THE ROLE OF SMALL LEUCINE-RICH REPEAT PROTEOGLYCAN ASPORIN IN EARLY EYE DEVELOPMENT

By

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DECLARATION

I, Kristin Anneliese Luehders, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

ABSTRACT

There has been a longstanding research interest in understanding the exact mechanisms underlying the correct development of the eye, with the goal of treating eye disease and blindness. While there have been enormous advances in the field of regenerative medicine, there is still some way to go before these advances can be translated into clinical applications. Very little is currently known about the regulatory mechanisms controlling the very early stages of eye development. While the genes, which regulate eye field development have been well characterised, their induction and mechanism of action, including downstream signalling targets and associated downstream signalling systems, still largely remain to be elucidated.

The small leucine-rich repeat proteoglycan (SLRP) family of proteins play important roles in a number of biological events, such as proliferation, growth and differentiation. Class I SLRP Asporin (ASPN) has so far been mainly associated with research relating to cartilage homeostasis, osteoarthritis susceptibility and more recently cancer. In this study, I introduce ASPN as a new important factor in *Xenopus laevis* early eye development.

During frog embryogenesis, ASPN is broadly expressed in the neuroectoderm of the embryo. The overexpression of ASPN causes the induction of ectopic eyes. In contrast, blocking ASPN function with morpholino-oligonucleotides inhibits eye formation, indicating that ASPN is an essential factor for eye development. Detailed molecular analyses revealed that ASPN interacts with insulin growth factor receptor (IGF1R) and is essential for activating the IGF-receptor mediated intracellular signalling pathway. Furthermore, ASPN perturbed the Wnt, BMP, and Activin signalling pathways, suggesting that ASPN thereby creates a favourable environment in which the IGF signal can dominate.

ASPN is thus a novel secreted molecule critical for eye induction through the coordination of multiple signalling pathways.

PUBLICATION

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ABBREVIATIONS

5'UTR	5' untranslated region
ARE-luc	Activin-Responsive Element
ASPN	Asporin
BMP	Bone morphogenic protein
BRE-luc	BMP responsive element
CB	Ciliary body
cDNA	Complementary DNA
Chd	Chordin
CMZ	Ciliary marginal zone
CNS	Central nervous system
CSPG	Chondroitin sulphate-containing proteoglycan
D	Aspartic acid
ddH_2O	Double distilled water
DiI	1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate
Dkk	Dickkopf
DMZ	Dorsal marginal zone
DN-IGF1R	Dominant negative form of IGF1R
dpc	Days post coitum
DSPG	Dermatan sulphate proteoglycan
ECM	Extracellular matrix
EFTF	Eye field transcription factor
EGF	Epidermal growth factor
Eif6	Eukaryotic initiation factor 6
ELS	Egg-Laying-Solution
ET	Eye T-box
FGF	Fibroblast growth factor
fz3	Frizzled 3
Gab-1	Grb2-associated binder-1
GAG	Glycosaminoglycan
GPI	Glycosylphosphatidylinositol
GSK3β	Glycogen synthase kinase 3β
HCG	Human Gonadotropin hormone
hDPSC	Human adult dental pulp stem cell
hESC	Human embryonic stem cells
Hh	Hedgehog
hpf	Hours post fertilisation
HSPG	Heparan sulphate proteoglycan
IGF	Insulin-like growth factor
IGF1R	IGF1-receptor
IGFBP	IGF binding protein
IL	Inflammatory cytokine like interleukin

IP	Immunoprecipitation
IRS	Insulin receptor substrate
L	Litre
LRR	Leucine-rich repeat
MAB	Maleic acid buffer
MAP	Mitogen-activated protein
MBS	Modified Barth's Solution
miRNA	Micro RNA
MO	Morpholino
mTOR	Mammalian target of rampamycin
Mya	Million years ago
N-terminal	Amino terminal
Otx2	Orthodenticle homeobox 2
PAMP	Pathogen-associated-molecular-pattern
Pax6	Paired box 6
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PG	Proteoglycan
PI3	Phosphoinositide-3
PMSG	Pregnant mare serum gonadotropin
RA	Retinoic acid
RISC	RNA-induced silencing complex
RPE	Retinal pigment epithelium
RT	Room temperature
RTK	Receptor tyrosine kinase
Rx1	Retinal homeobox 1
Shh	Sonic hedgehog
Six3	Six homeobox 3
Six6	Six homeobox 6
SLRP	Small leucine-rich repeat proteoglycan
SSC	Saline sodium citrate
SUMO-1	Small ubiquitin-like modifier protein-1
TAE	Tris-acetate-EDTA
TGF-β	Transforming growth factor beta
TNF	Tumour necrosis factor
TOPFLASH	TCF/LEF Optimal Promoter
VMZ	Ventral marginal zone
β-gal	β-Galactosidase

CHAPTER 1: GENERAL INTRODUCTION

1.1 Preface

A recent statistic from the eye research charity 'Fight for Sight' estimates that someone in the world goes blind every five seconds. Millions of adults and children struggle with the effects of their eye conditions. The eye forms part of the central nervous system and relays around one fifth of all sensory information, making it the most important sensory organ for humans to perceive their environment (Cunningham, 2001). Vision is certainly one of our most treasured senses and surveys consistently show that loss of sight ranks in third place on the list of most feared conditions – just behind cancer and cognitive impairment. Loss of vision has a severe impact on social as well as work-related aspects of life (e.g. use of computers, driving cars, etc.). A study by Langelaan (Langelaan et al., 2007) found that health related quality of life is considerably reduced in vision-impaired patients. Compared to other chronic conditions like Diabetes type II, coronary syndrome and hearing impairment, quality of life in patients with impaired sight received the lowest rating.

The eye is composed of a number of different specialised cell types, such as neurons and glia cells, which develop in a strictly controlled and precise manner (Kohwi and Doe, 2013, Reese, 2011). Unsurprisingly, there has been a longstanding research interest in understanding the exact mechanisms underlying the correct development of the eye, with the goal of mastering the growth of eye tissue in test tubes for clinical transplantation and other therapeutic purposes. Thanks to enormous research efforts and technical advances in the field of regenerative medicine, the future for treating blindness is looking promising. However, there is still some way to go before these advances can be translated into clinical applications.

Little is currently known about the regulatory mechanisms controlling the very early stages of eye development, i.e. eye induction and in particular the induction of eye determining genes. While the genes that regulate eye field development have been well characterised, their induction and mechanism of action, including downstream signalling targets and associated downstream signalling systems, still largely remain to be elucidated.

In this project I introduce the small leucine-rich repeat proteoglycan (SLRP) 'Asporin' (ASPN) as a new important factor in *Xenopus laevis* early eye development. I will explore its role and importance by means of gain-of-function, as well as loss-of-function studies and then delve in to explore some of the underlying molecular signalling pathways through which it transduces its effects, in particular the IGF signalling pathway.

In the following general introduction the vertebrate visual system and principles of eye development shall be introduced in more detail. Naturally, the proteoglycan of interest in this study, ASPN, and its SLRP family will also be covered. Finally, I will describe IGF signalling and its importance during early neural development, and in particular its role during eye development.

1.2 Introduction to the visual system

The ability to see is crucial for most species and offers a definite evolutionary advantage over organisms who do not possess vision (Fernald, 2000). While the eye as a sensory organ is widespread amongst metazoans, there is a huge variety and diversity in eye types in terms of their anatomical, developmental and organisational features. In the following paragraphs the structure and evolutionary origin of the eye as a sensory organ shall be discussed and then the advantages of the frog *Xenopus laevis* as a model organism to study eye will be introduced.

1.2.1 Structure and evolution of eyes

The basic principle of the eye as a sensory organ can be summarised as follows: Light is collected through a kind of aperture, then often focused with the help of a lens and thereby directed onto specialised photoreceptor cells containing opsins (photon capturing visual pigments), which convert the photons into a neural signal (Fernald, 2000).

The simplest type of eye found in nature, consists of a single photoreceptor cell in close proximity to another cell; expressing a dark shielding pigment. Darwin referred to this as the eye prototype in his work "The origin of species" (Jonasova and

Kozmik, 2008, Darwin, 1859). This protypic eye can be found in species such as the planarian *Polycelis auricularia*, as seen in Figure 1.1 by Gehring (2014).

The first image forming eyes are suggested to have appeared after the Cambrian explosion around 540 Million years ago (Mya). Eyes developed in different ways in different species, though the underlying genetic networks including Pax and Rx transcription factor family members were preserved. A vertebrate camera-type eye, very similar to the modern vertebrate eye, probably already existed by 500 Mya (Land, 2012, Martinez-Morales and Wittbrodt, 2009, Lamb et al., 2007).

Today, there are three main types of eye found amongst metazoans: the compound eye, as found in the fruit fly Drosophila; the mirror eye, as found in the scallops of the Pecten genus; and finally the camera or lens eye, as found in humans and other vertebrates (Gehring and Seimiya, 2010). These main eye types are further illustrated in Figure 1.2. The camera-type eye found in vertebrates is by far the most successful and high performing eye prototype, which yields the highest resolution compared to other eye types. Its success can be traced back to the convergent lens, which focuses the light onto a hemispheric retina, which contains a high density of photoreceptors. While chambered eyes also developed in invertebrate species, vertebrates are the only phylum to possess bilateral chambered eyes (Martinez-Morales and Wittbrodt, 2009).

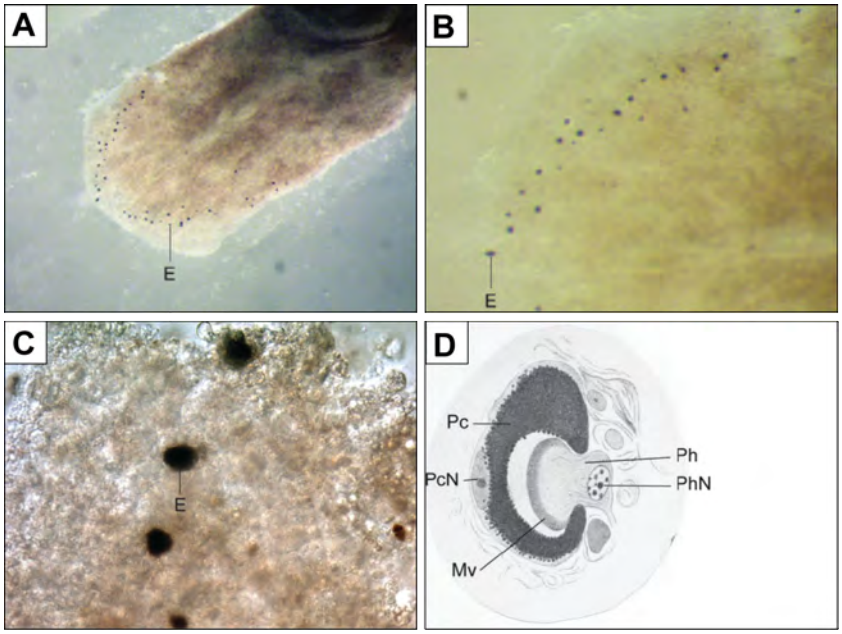


Figure 1.1: The protypic eyes found in planarian *Polycelis auricularia*. Protypic eyes are shown in the planarian *Polycelis auricularia* (A, B, C). Also a histological section of an eye of *Planaria torva* can be seen in (D). Pc, pigment cell; PcN, pigment cell nucleus; Mv, microvilli; Ph, photoreceptor cell; PhN, photoreceptor cell nucleus. Adapted from Gehring (2014).

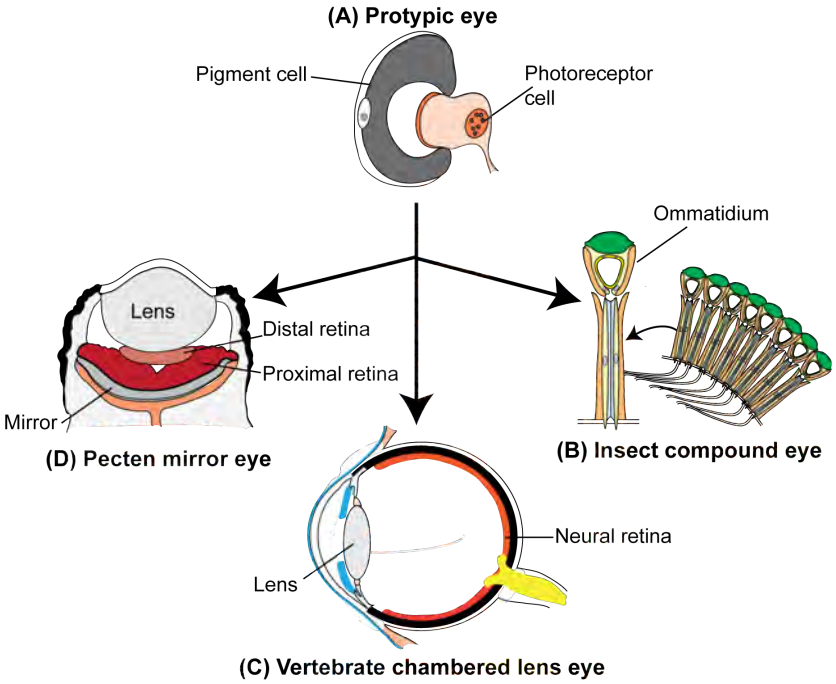


Figure 1.2: Schematic diagram of the evolution of different eye prototypes. Darwin's protypic eye, consisting only of two cells (pigment and photoreceptor cell) can be seen in (A). The main types of eyes found amongst metazoans today are the compound eye (B), which is found in insects; the mirror eye (D), which occurs in sea scallops; and finally the chambered lens eye (C), as found in vertebrates and humans. After Gehring (2014).

The evolutionary addition of a refractory lens and cornea greatly enhanced the function of the eye as a sensory organ and contributed to an image producing vision system. Until this point, organisms could only sense the intensity and direction of the light (Jonasova and Kozmik, 2008). The level of eye complexity is not directly correlated with the level of complexity of the rest of the organism's body plan, e.g. velvet worms (onychophorans) possess paired eyes with secreted cuticular corneas and acellular lenses formed of granular material. There is also a great diversity amongst mollusc species: squids have sophisticated camera eyes, while scallops have mirror containing eyes (Jonasova and Kozmik, 2008).

Depending on whether the animal is land or water based, as well as providing the eye with protection and nutrition, the cornea can function in an additional 'lens-like" fashion. In general, terrestrial animals use both lens and cornea to refract light, while aquatic species only need to use the lens. The correct refractive index can furthermore be achieved through the presence of different concentrations of lens proteins (Jonasova and Kozmik, 2008).

Animals today, express between two and 12 different types of opsins in their retinas, which distinguish themselves through the wavelengths they are able to absorb. The peak sensitivities of these opsins are often tailored to the animals' respective habitats, e.g. the African blind naked mole rat, which lives under ground, is still capable of detecting the wavelengths required for determining the circadian rhythms (Fernald, 2000). However, the opsins are remarkably homologous across species (Jonasova and Kozmik, 2008, Fernald, 2000), so while the anatomical features and morphology may differ, the molecular mechanisms underlying the capture of photons and conversion to a nerve signal are highly conserved amongst all organisms (Fernald, 2000).

Through evolution, two distinct broad categories of photoreceptor cells have developed: microvillar photoreceptors, which are most often found in invertebrate species, and ciliary photoreceptors, which are found in vertebrates such as humans (Fernald, 2000). The photoreceptor classes are also referred to as rhabdomeric (containing apical microvilli) and ciliary, which were initially thought to only occur in invertebrates and vertebrates, respectively. However, it is now known that they

can co-exist, such as in molluscs and anthropods (Lamb et al., 2007). The two types of receptor cells differ not only in morphology, but also in their method of signal transduction. The main difference is the type of G-protein used (Lamb et al., 2007), as well as the second messenger systems: vertebrates use cyclic GMP, while invertebrates use inositol triphosphate (Fernald, 2000).

Another significant difference is the tissue from which the eye originates during development, as this differs enormously between species and classes. The cephalopod eye – an advanced type of camera eye, not dissimilar to the human eye – stems from the epidermal placode-folding, while the vertebrate eye originates from the neural plate and overlying epidermis (i.e. the lens placode), which eventually forms the lens (Fernald, 2000).

The types of cells and proteins, which form the lens, again vary between species. The most important property of the lens is translucency and to provide the correct refractive index to focus the light onto the photon-transducing photoreceptor cells (Fernald, 2000). Often the proteins contained in lens tissue are not lens specific, and fulfil further activities (e.g. enzyme activities) in other tissues; this phenomenon is referred to as 'gene sharing' (Jonasova and Kozmik, 2008, Fernald, 2000). The crystalline proteins of the lens are not homologous across species, however the genetic factors, which govern eye and lens development, are remarkably similar amongst metazoans (Jonasova and Kozmik, 2008).

The question as to whether the eye as a sensory organ is of monophyletic (i.e. originating from one evolutionary event) or polyphyletic (i.e. eyes developed in several evolutionary events separately from each other) origin has been fiercely debated over the years (Jonasova and Kozmik, 2008, Fernald, 2000). More recent findings, which revealed a conserved genetic network of transcription factors involved in eye development point in the direction of monophyletic eye origin (Fernald, 2000). In particular, the families of Pax and Rx transcription factors exhibit a wide conservation across species, and shall be introduced in more detail later on (Jonasova and Kozmik, 2008).

1.2.2 *Xenopus laevis* as a model organism – a historical perspective

Xenopus laevis is a frog species, which is native to central and South Africa. These days, *Xenopus* populations can be found in research labs all over the word. The majority of developmental research is carried out using six main model organisms: *drosophila*, mouse, chick, *c.elegans*, zebrafish and *Xenopus laevis* (including its diploid relative *Xenopus tropicalis*, which has become a very attractive model for genomic studies). When asked, why *Xenopus laevis* is so popular amongst developmental biologists, many will refer to the ease with which they can be maintained, a relatively short life cycle, large number of eggs which are fertilised externally and large enough for microsurgery and lastly, a year round possibility for breeding and egg supply thanks to commercially available hormones (Gurdon and Hopwood, 2000).

While these are all very good reasons, there is also an interesting historical explanation for the popularity and widespread use of *Xenopus*. As part of imperialism, many European zoologists were travelling around the world documenting new and exotic species of flora and fauna. *Xenopus laevis* was firstly described at the beginning of the 19th century. In the 1880s leading experimental biologists in Germany, like Hans Spemann, used mainly local species of amphibia for their experiments, such as *rana* and newts. *Xenopus laevis* was already used in South Africa by local physiologists and also for biology teaching at schools. Lancelot Hogben, a British endocrinologist, worked in South Africa for several years, where he was introduced to working with *Xenopus*. He was very excited about the possibilities of *Xenopus laevis,* referring to it as a gift from God and even named his house after the clawed frog species. When he eventually returned to the UK, he brought a population of *Xenopus* with him and introduced this species into mainstream science. A lot of work was required to domesticate *Xenopus* and to find the best conditions to induce egg laying. The true rise to fame for *Xenopus* occurred after World War II, for at that time there was an increasing demand for pregnancy tests. While many scientists sought to develop tests with mice and rabbits, *Xenopus* turned out to be better suited (Figure 1.3). After subcutaneous injection of a pregnant woman's urine, *Xenopus laevis* would start laying eggs within the following four to 12 hours. Soon the *Xenopus* pregnancy test became available all around Europe and North America. Little additional work was required by developmental biologists to

adopt them for their own studies: techniques which had been perfected in other amphibians, worked well in *Xenopus* and - thanks to commercially available hormones - eggs could be harvested all year round. The latter reason in particular made it the model organism of choice for a lot of initial biochemical and cellular biology work. Consequently, most of the work on early animal development was carried out in *Xenopus laevis* (Gurdon and Hopwood, 2000).

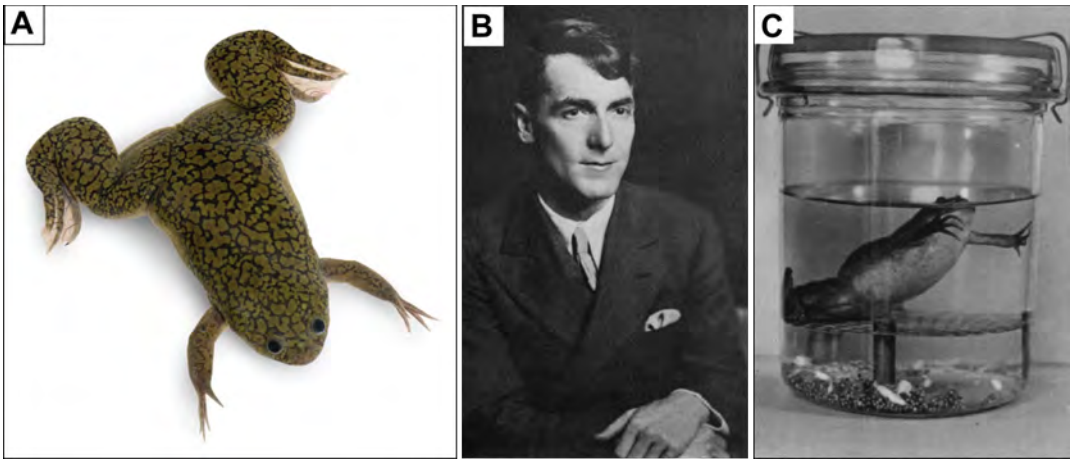


Figure 1.3: Images of the African clawed frog, Lancelot Hogben and the Xenopus pregnancy test. (A) A Xenopus laevis female is shown (image source: https://www.enasco.com/product/LM00715MX; accessed 20/02/2016). (B) British Endocrinologist Lancelot Hogben, who introduced Xenopus laevis as a model organism into mainstream science. Image adapted from Wells (1978). (C) The 'Xenopus pregnancy test', whereby urine of a possible pregnant woman was injected subcutaneously into the frog. If pregnant, the frogs would start ovulating four to 12 hours post injection. Image adapted from Elkan (1938).

1.2.3 *Xenopus laevis* is a great model organism to study eye development

As previously discussed, developmental biologists have been using amphibians for their studies for over a century, prominent examples being Hans Spemann and Hilde Mangold, who performed their important early work in newts (Spemann and Mangold, 1924).

Xenopus laevis is generally easy to maintain and culturing of the embryos is also not difficult. The female frogs can be induced to lay eggs, which gives the researcher the power to control ovulation and is therefore no longer reliant on seasonal breeding

behaviours. The embryos themselves are relatively large (around 1.2 mm) and are cultured and fertilised externally. The embryos are also surprisingly resistant to various manipulations, including transplantations and excising of tissues. Wound healing is also very fast in *X. laevis* embryos. Furthermore, *Xenopus* embryos exhibit a fast and very regular cleavage pattern, which made it possible to obtain consistent cell lineage/fate maps (Henry et al., 2008). Thanks to these fate mapping studies, it is now known which areas of the embryo later contribute to the eye. This can now be used to study eye development in more detail.

A variety of tools are available to *Xenopus* researchers, particularly when it comes to analysing gene function. Gain-of-function experiments work really well in *Xenopus,* due to easy access and the comparative robustness of the embryos. For gain-of-function experiments, synthetic RNA or DNA expression constructs can be microinjected into the embryo (often, capped synthetic RNAs are used) and the resulting phenotype subsequently analysed (Henry et al., 2008). Loss-of-function assays can also be carried out in *X.laevis* embryos, by injecting antisense RNA, morpholinos or dominant negative constructs. Morpholinos are synthetic oligonucleotides, which are designed to target the 5'UTR and/or the translational start site to prevent translation of the mRNA into protein (Gene Tools, 2016).

A potential concern with *X. laevis* is its pseudotetraploidy, which is thought to be the result of a whole-genome-duplication, following the hypothesised hybridisation of two diploid species (Uno et al., 2013, Harland and Grainger, 2011). While some of the genes may still be present in a diploid state, other genes are preserved as duplicated "allogenes", which can show different levels of divergence (usually less than 10%). Allogenes can even have distinct expression patterns (Pollet and Mazabraud, 2006). So the duplicated genes in the *X. laevis* genome may have slightly different sequences, but a conserved function. To overcome this in loss-of-function experiments, it is sometimes necessary to co-inject two different morpholinos, which target the different allogenes (Henry et al., 2008).

Eye and lens development have been studied extensively in *Xenopus*. Early on its particular ability to regenerate parts of its eye (shared with many other amphibians) made it of interest to researchers. While not as proficient as other amphibians, *X.*

laevis is capable of regenerating some body parts including eye tissues, especially while still in larval stages (Lee et al., 2013, Vergara and Del Rio-Tsonis, 2009). Pieter Nieuwkoop and Job Faber were the first to publish a detailed description of *X. laevis* development in their 'Normal table of Xenopus laevis' (Nieuwkoop and Faber, 1967), which is still used by scientists today (Henry et al., 2008).

Much of what we know today about vertebrate eye development is based on studies carried out in *X. laevis*. This includes crucial work on neural induction and the discovery of various BMP-inhibiting factors such as Noggin, Chordin, Follistatin and Cerberus (Harland, 2000, Henry et al., 2008). Another example is Zuber and colleagues' study (2003) regarding the network of eye field transcription factors (EFTFs) in *Xenopus*: using double in *situ* hybridisation techniques they demonstrated the EFTFs unique overlapping expression patterns. With overexpression experiments they were able to construct the relationships between these factors. *Xenopus* work also contributed to a better understanding of later stages of eye development, like the signalling pathways and transcription factors involved in the differentiation of neural cell types, as well as axon guidance from eye to brain (Mann et al., 2004, Perron et al., 1998).

Overall, *Xenopus* is a good model for studying eye development. Due to some technical advantages over other model organisms, it offers a great model to study the mechanisms underlying eye development, as well as the processes of development and regeneration.

1.3 Early embryonic development

During gastrulation, the simple one layered embryo, which at this stage is a spherical ball of cells, develops into a multi-layered organism. To achieve this transformation, large cell migrations take place throughout the embryo to form the three primary germ layers endoderm, mesoderm and ectoderm, which later give rise to the various differentiated parts of the embryo. Also during gastrulation, the so-called 'primary embryonic induction' takes place, which leads to cell fate determination and axis formation within the developing embryo. Neural induction, i.e. the specification of

cells to a fate of neural, brain (and also in part eye tissue) is part of these inductive events and shall be introduced in a bit more detail in the following paragraphs.

1.3.1 Primary embryonic induction and neural induction

The dorsal mesoderm is known as the "organizer" and is a well-known source of neural inducing agents. It was Hans Spemann and Hilde Mangold, who first discovered the organizer and neural induction, nearly a century ago. Early transplantation experiments in newts, carried out by Spemann and Mangold, showed that the dorsal lip of the blastopore has its fate autonomously determined (Harland, 2000, Spemann and Mangold, 1924). When transplanted to a ventral site on the embryo, this particular tissue still retained its 'dorsal lip' character and was even able to induce gastrulation and embryongenesis in the developing embryo, eventually leading to a secondary axis (Gilbert, 2014, Recanzone and Harris, 1985). Spemann termed these dorsal lip cells and its derivatives (i.e. the notochord and head endomesoderm) the "organizer"; in his opinion an appropriate name since it has the ability to organize embryonic tissue with clear dorso-ventral and antero-posterior axes and can induce ventral tissue to form a neural tube and dorsal mesoderm (Gilbert, 2014, Spemann, 1938).

Since those initial experiments, it is now known that the interaction between the organizer and the ectoderm is not enough to organize the whole embryo. But these first interactions initiate a series of inductive events, which are required for embryonic development. Since this first induction forms the basis for all the others that follow, it is traditionally referred to as primary embryonic induction (Gilbert, 2014).

1.3.2 Induction of the organizer

The major characteristics of the organizer are the abilities to self differentiate, - to dorsalise the surrounding mesoderm and ectoderm (including neural tube induction), and to induce the gastrulation cell movements. But how exactly does the organizer itself form? The organizer cells are positioned directly opposite the sperm entry point, which is the exact location where two signalling pathways converge, namely a dorsalising and a mesodermal signal. Together, they seem to be key for the organizer tissue specification (Gilbert, 2014).

In more detail, the organizer is induced by the so-called "Neuwkoop center" cells (Gerhart et al., 1989, Nakamura and Takasaki, 1970, Nieuwkoop, 1973, Nieuwkoop, 1977). These are the most dorsal vegetal cells (i.e. prospective endoderm), which lie beneath the future organizer tissue. The Nieuwkoop center induces the overlying animal cells to become mesodermal tissue. The mesoderm then arises at the border (= equator) between the animal and vegetal pole of the embryo (Gilbert, 2014). The Nieuwkoop-center forming molecule is β -catenin, which acts as the required dorsalising signal. Through several mechanisms, β-catenin accumulates at the dorsal side of the embryo (Larabell et al., 1997, Schneider et al., 1996) and activates the expression of the two genes *twin* and *siamois*, which are involved in organizer induction. These in turn induce other proteins to be expressed, which are important for organizer function, such as Goosecoid, Xlim1 (dorsal mesoderm specification), Noggin, Chordin (BMP inhibitors), Frzb and Cerberus (Bae et al., 2011, Engleka and Kessler, 2001, Gilbert, 2014).

Phosphorylated Smad2 seems to be the other important mesodermal signal, which is required for the induction of the organizer cells. The vegetal cells of the Nieuwkoop center secrete nodal-related paracrine factors (high concentrations in dorsal areas with decreasing amounts towards ventral regions), which phosphorylate Smad2 in the overlying presumptive mesoderm (Brannon and Kimelman, 1996, Engleka and Kessler, 2001). Activated Smad2 then induces the expression of the *hhex* gene, which, together with *twin* and *siamois*, specifies the organizer cells and induces anterior brain development (Rankin et al., 2011, Smithers and Jones, 2002). Since there is a Smad2 gradient along the dorso-ventral axis, slightly lower levels of Smad2 seem to activate *goosecoid* expression in cells destined to become the prechordal mesoderm and notochord (Cho, 2012, Germain et al., 2000, Gilbert, 2014).

The cells of the Nieuwkoop center remain endodermal, while the organizer becomes dorsal mesoderm and starts its migration underneath the dorsal ectoderm. The organizer cells are thought to become part of the following tissues: pharyngeal endoderm, head mesoderm (prechordal plate), dorsal mesoderm (mainly notochord) and dorsal blastopore lip (Gont et al., 1993, Keller, 1976). The pharyngeal endoderm and prechordal plate lead the organizer tissue's migration and induce the forebrain

and midbrain, while dorsal mesoderm induces the hindbrain and trunk. The blastopore lip eventually becomes the chordaneural hinge and induces the tip of the tail in Xenopus (Gilbert, 2014).

In summary, dorsal mesoderm and organizer induction takes place through the actions of firstly the Wnt/β-catenin pathway, which activates genes coding for Siamois and Twin, and secondly, a vegetal pathway, which activates the secretion of Nodal-related paracrine factors, which activate Smad2 in mesodermal cells. The joint action of Smad2, Siamois and Twin define the dorsal mesoderm and organizer cells (Gilbert, 2014).

1.3.3 Neural induction and the role of BMP inhibitors

Neural induction can be defined as the process of multipotent embryonic cells differentiating into neural cell types, which eventually form the neural tube/plate. To explain the mechanisms of neural induction the 'default model' was proposed in the 1990s. It states that ectodermal cells will automatically adopt a neural cell fate unless other signals are given. The ectoderm can be induced to form epidermis through the actions of bone morphogenic proteins (BMPs). It was thought that BMPs are active throughout the entire ectoderm. With the start of gastrulation, the oragnizer acts by secreting BMP-inhibitors in the dorsal anterior region to 'protect' the ectoderm from the epidermal induction and to secure its neural fate (Hemmati-Brivanlou and Melton, 1997, Hemmati-Brivanlou and Melton, 1994), thereby creating a dorsal-ventral BMP gradient (Andoniadou and Martinez-Barbera, 2013, Harland, 2000, Stern, 2006). The major BMP inhibitors are Noggin, Chordin and Follistatin, which are downstream of the previously mentioned organizer proteins Twin and Siamois (Fan and Sokol, 1997, Kessler, 1997), and Norrin, TSK, Xnr1 and Cerberus (Gilbert, 2014). The neural inducers' inhibitory effects seem to extend to other members of the TGF-β family (Harland, 2000).

The major epidermal inducers are the BMPs, especially BMP4 and its close relations BMP2 and BMP7. At late blastula stage, BMP4 expression is restricted to the ventrolateral marginal zone, due to the presence of Goosecoid, which represses *bmp4* and *wnt8* transcription (Glavic et al., 2001, Hemmati-Brivanlou and Thomsen, 1995). As previously mentioned, there is a concentration gradient in the mesodermal cells,

whereby a lack of BMP4 results in dorsal mesoderm formation, low levels of BMP4 in intermediate mesoderm, and high concentrations of BMP4 in lateral mesoderm induction (Gilbert, 2014, Hemmati-Brivanlou and Thomsen, 1995).

Noggin was first identified by Smith and Harland (1992). It is a secreted protein, which induces the ectoderm to form neural tissue and also dorsalises mesodermal cells. It is first expressed in the dorsal lip and later in the notochord. Noggin binds BMP4 and BMP2 and thereby stops them from binding to their receptor (Gilbert, 2014, Zimmerman et al., 1996). Out of all the organizer-secreted proteins, Chordin (Sasai et al., 1994) is most acutely activated by β-catenin. Like Noggin, it binds BMP4 and BMP2 and prevents receptor binding (Gilbert, 2014, Piccolo et al., 1996). Follistatin, first described by Hemmati-Brivanlou and Melton (1994, 1992), inhibits both the BMPs and Activin. Norrin is an exception, in that it is already present in the animal part of the embryo and not secreted by the organizer. It therefore blocks BMP activity in a cell-autonomous fashion (Gilbert, 2014, Kuroda et al., 2004, Savage and Phillips, 1989, Xu et al., 2012).

Recent studies, however, suggest that BMP signal inhibition alone is not sufficient for neural induction initiation. It has become clear that the process of neural induction is much more complex and the default model therefore too simplistic. Other work indicates, that neural induction is not a single, but rather a specific sequence of signalling events, whereby BMP inhibition might act as a late stage maintenance event (Stern, 2006).

While surely contributing to neural induction, ablation experiments and knockout studies have shown that the organizer is not essential for neural plate formation (Harland, 2000). Since then, some researchers proposed the existence of additional 'head', 'trunk' and 'tail' organisers – an idea that is still heavily debated. More recent studies suggest that neural induction in the anterior part of the embryo is governed by BMP inhibition, while trunk and tail end neural induction is linked to FGF signalling (Stern, 2006). The organizer's properties differ from species to species and reflect different modes in early development. However, organizers of all classes of animals have the ability to recruit the surrounding ectoderm into a patterned neural tube (Harland, 2000).

1.3.4 Regional specificity of neural induction and axes formation

An important feature of neural induction is the regional specificity with which the different neural regions are induced. There is obviously a great need for neural structures to be organised in a proper anterior to posterior, as well as dorso-ventral, fashion. The organizer cells do not simply induce; they also specify the different regions of the neural tube. Otto Mangold already demonstrated this in 1933. A few of the underlying principles and the molecules involved in this regional specification process will be introduced in the following paragraphs (Gilbert, 2014).

BMPs have been shown to be very important for correct dorsal-ventral fate specification. Follistatin-like-product (fstl2) and Noggin1 act redundantly with chordin to facilitate dorsal-ventral axis (Stern, 2006). The Wnt family is involved in many aspects of neural development such as neural induction, axis formation and axon guidance. The role of Wnt signalling during neural induction is somewhat controversial, but differing results are most likely due to differences in timing during the experiments (Stern, 2006). During the early development of ectoderm to neural plate and then to neural tube, Wnt signalling seems to be involved in every step of the process, including anterior-posterior axis specification in the neural plate, regulation of morphology of the neural tube, neural stem cell proliferation and differentiation and neural migration (Mulligan and Cheyette, 2012). Wnt signalling, alongside retinoic acid and FGF, acts as a caudalising agent. Results from recent studies suggest that activated FGF and Wnt signalling pathways are needed to inhibit Smad1 activity, which leads to BMP inhibition (Andoniadou and Martinez-Barbera, 2013).

The role and importance of Wnt signalling in antero-posterior axis formation, seems to be preserved across all vertebrate species studied, including many invertebrates such as echinoderms, cnidarians and flatworms. In a majority of organisms, Wnts are produced in the posterior tissues, while Wnt antagonists block their actions in the anterior head regions (Petersen and Reddien, 2009). In the forebrain, the notochord and prechordal mesoderm not only secrete BMP, but also Wnt inhibitors (Gilbert, 2014, Petersen and Reddien, 2009).

Cerberus (Bouwmeester et al., 1996) is another important factor, which is secreted by the organizer (the pharyngeal endomesoderm part, to be more specific). It was named after the three-headed mythical dog, which guards the entrance to Hades in Greek mythology. It promotes the development of the cement gland, eyes and olfactory placodes in *Xenopus* embryos. When overexpressed in a vegetal ventral blastomere of the 32-cell *Xenopus* embryo, Cerberus induces ectopic heads. Cerberus can bind BMPs, Nodal-related proteins and Wnt8. Blocking of Cerberus action, results in greatly increased levels of BMPs, Wnts and Nodals, and a severely inhibited head induction (Gilbert, 2014, Silva et al., 2003).

Frzb is a small soluble form of Frizzled (the Wnt receptor) and it is capable of binding Wnt proteins in solution. Forced overexpression leads to embryos, which lack all posterior body structures due to the systematic Wnt inhibition. Dickkopf (Dkk) also directly interacts with the Wnt-receptor and thereby blocks Wnt signal transduction. Inhibition of Dkk results in head malformations or a lack of forebrain development. Tiki – which has been more recently discovered – forms a complex with Wnt proteins and prevents them from binding to their respective receptor. It cleaves the proteins and thereby renders them non-functional. Knockdown of Tiki confirmed its crucial role in head development (Gilbert, 2014, Zhang et al., 2012).

Pera et al. (2001) first showed the importance of IGFs (insulin-like growth factors) for normal anterior neural tube development. IGFs accumulate at the dorsal midline of the embryo, particularly in the anterior neural tube. Their overexpression in the ventral mesoderm leads to the formation of ectopic heads, while a block leads to no head formation. IGFs seem to work through receptor tyrosine kinase (RTK) signalling cascades and are capable of blocking BMP and Wnt signalling (Gilbert, 2014). The importance of IGFs will be discussed in more detail in chapter 1.6.

While research into neural induction mainly focuses on signalling molecules secreted by the organiser, a full understanding can only be gained when the associated regulatory processes of gene expression are known. Neural and other cell fate specific gene activation or suppression, along with transcription factors and changes in chromatin structure, histone methylation/acetylation, need to be elucidated (Stern, 2006).

1.4 Development of the eye and visual system

The head's sensory organs, such as the eye, develop mostly through interactions between the neural tube and the various cranial placodes. These include the olfactory placode, which gives rise to the nasal epithelium; the otic placode, which forms the inner ear; and the lens placode, which goes on to form the lens of the eye. Also crucial to the correct formation of the eye, is a reciprocal interaction between the lens placode and the presumptive optic vesicle, which originates from the diencephalon of the forebrain (Gilbert, 2014). In this chapter, the general mechanisms of vertebrate eye development shall be introduced, with an emphasis on the genetic network and molecules known to regulate the development of the visual system. Following on from general principles underlying vertebrate eye formation, *Xenopus* eye development will be explained in more detail.

1.4.1 Vertebrate eye development

1.4.1.1 Eye field specification

The bilateral protrusions of the optic vesicles from early anterior forebrain, are the earliest visible anatomical signs of vertebrate eye development. For the last 80 years it has been known, however, that the eye field exists long before the optic vesicles become visible (Lopashov and Stroeva, 1964).

The eye field forms as a result of progressive inductive events in the anterior neural plate. As previously described, during neural induction, embryonic ectoderm is exposed to various neural inducers such as Noggin and Chordin, which then induce the formation of the neural plate (Harland, 2000, Mulligan and Cheyette, 2012, Stern, 2006). Increased expression of factors like Otx2 (orthodenticle homeobox 2), facilitate forebrain specification in the embryo. However, the eye-field specification progress itself is orchestrated by the so-called 'eye field transcription factors' (EFTFs). While numerous inductive and patterning events take place in the anterior neural plate to 'prepare' for the generation of the vertebrate eye, co-ordinated expression of the EFTFs is crucial to eye field specification itself (Zuber et al., 2003). Correct positional information is therefore important for the specification of the eye field.

The specification of the eye field starts around the time of neural tube specification. In the anterior part of the neural tube, BMP- and Wnt-pathways are inhibited through the actions of the different neural inducers. Noggin plays an important role for eye field induction as it allows Otx2 to be expressed in the anterior neural tube. It also inhibits ET (eye T-box) – an important eye field transcription factor - from being expressed too early. Only when enough Otx2 has accumulated in the ventral head region, is the Noggin driven inhibition of ET expression lifted. Once ET is expressed, the EFTF induction-signalling cascade is kick started (Gilbert, 2014). ET activates the next important eye field marker called Rx1 (retinal homeobox), which is crucial for the specification of the retina. Rx1 seems to activate Pax6 (paired box 6), but at the same time represses the expression of Otx2. Pax6 is a major eye field-forming gene, which seems to be conserved across all phyla as a photoreceptor-cell specifying gene in both vertebrates and invertebrates (Halder et al., 1995b, Zuber, 2010, Zuber et al., 2003).

Pax6 then activates a network of other EFTFs such as Six3, Six6 and Sox2. Together, their actions result in one single eye field, located centrally in the ventral forebrain region of the embryo (Fuhrmann, 2010, Tetreault et al., 2009). Since vertebrates possess a pair of eyes, the eye field needs to split in two. This is where sonic hedgehog (Shh) signalling comes into play. Secreted from the prechordal plate, Shh inhibits Pax6 expression in the central part of the eye field and splits it down the middle. If not enough Shh is expressed, the eye fields fail to separate, which results in an embryo with a single eye - a condition referred to as cyclopia (Chiang et al., 1996, Kelley et al., 1996, Roessler and Muenke, 2001). The opposite case has also been observed, whereby too much Shh has been released, which leads to a complete inhibition of eye formation, as is the case with the blind cave fish *Astyanax mexicanus* (Yamamoto et al., 2009).

1.4.1.2 Neural retina, lens and optic cup development

Following eye field specification, the two optic vesicles of the forebrain bulge out and extend to the overlying surface ectoderm in the head region. The head ectoderm has already received 'lens forming competency' during gastrulation from the underlying foregut endoderm and the heart-forming mesoderm (Jacobson, 1966, Zygar et al., 1998). This is probably achieved through the supply of BMP and Wnt

antagonists, which in turn create an environment where Pax6 can be induced (Ogino et al., 2012). Pax6 was shown to be critical for the conferral of the lens forming competence to the head ectoderm, thereby enabling it to respond to optic cup inducers (Gilbert, 2014).

The Rx1 protein, which is expressed in the two – now separated - eye fields, activates the *Nlcam* gene, whose cell-surface product is required for the evagination of the optic vesicles from the forebrain (Brown et al., 2010). They extend towards the surface ectoderm and upon contact flatten out against it.

The optic vesicles then release paracrine factors such as BMP4, FGF8 and Notch, which induce the overlying head ectoderm to form the lens placode (Ogino et al., 2012). BMP4 activates Sox2 transcription factors in the lens placode (Furuta & Hogan, 1998), while FGF8 induces the L-Maf transcription factor. Pax6, Sox2 and L-Maf expression in the ectoderm is needed to activate lens specific genes, such as the crystalline proteins (Ogino and Yasuda, 1998, Vogel-Hopker et al., 2000). In return, the newly induced lens placode also starts to secret paracrine factors such as FGFs, which activate the *Vsx2* gene in the optic vesicles, which is needed for neural retina development (Gilbert, 2014). The dermal mesenchyme surrounds the optic vesicles and induces the expression of the *Mitf* gene in the outer layers, which instructs them to produce the pigment melanin (Burmeister et al., 1996, Nguyen and Arnheiter, 2000). The optic vesicles' cells touching the surface ectoderm, will therefore develop to form the neural retina, while the adjacent cells of the optic vesicles will form the pigmented retina (Fuhrmann, 2010, Gilbert, 2014).

In summary, during gastrulation, the foregut and prechordal plate interact with the head ectoderm to confer a lens forming bias, for which transcription factor Pax6 is important. The activation of the lens forming potential occurs through paracrine factor secretion by the two optic vesicles, which extend from the diencephalon of the forebrain to be right next to the future lens placode (Gilbert, 2014). The thereby stimulated head ectoderm lengthens upon receiving the vesicles' paracrine signals, and forms the lens placode. Receiving signals itself from the lens placode, the optic vesicles bend inwards to form the two-layered eyecup. The lens placode is also drawn inwards into the eyecup invagination. The two eyecup layers start to

differentiate, whereby the outer layer becomes the pigmented retina and the inner layer the neural retina. Within the neural retina, the retinal ganglion cells extend, meet at the optic disc at the base of the eyecup and travel along the optic stalk, which will later be the optic nerve (Chauhan et al., 2009, Gilbert, 2014).

1.4.1.3 Vertebrate lens, cornea and retina differentiation

To perform its function the lens needs to be transparent. The transparency of the lens tissue is achieved through lens specific proteins called crystallins. When lens cells start to grow and differentiate, they accumulate lots of crystalline proteins until they fill up the entire cell. For the correct lens curvature, which is crucial for its function, microfilaments contract and extend accordingly along the apical-basal axis, regulated by the Rho-Rac signalling pathway (Chauhan et al., 2011, Gilbert, 2014).

Relatively little is known about cornea development. Shortly after the lens vesicle detaches from the surface ectoderm, the lens vesicle induces the overlying ectoderm to secrete layers of collagen. Neural crest cells migrate into these layers to form a new cell layer and secrete corneal specific extra cellular matrix (Kanakubo et al., 2006). As the cornea matures, this cell population condenses and forms a flat cell layer, which turns into cornea precursor cells (Cvekl and Tamm, 2004). As part of the maturation process, the cells dehydrate and form very tight junctions, which unite them with the surface ectoderm (Gage et al., 2005). For the correct curvature of the cornea, intra ocular fluid pressure is required (Gilbert, 2014).

The retinal neural precursor cells have the competency to turn into any of the seven retinal cell types: retinal ganglion cells, bipolar cells, amacrine cells, horizontal cells, rod and cone photoreceptor cells and Müller glia cells (Yang, 2004). In amphibians, timing of gene translation and not location of gene transcription was found to determine which type of neuron the retinal stem cells turn into. This time dependent translational regulation is orchestrated by specific microRNAs (Decembrini et al., 2006). Of course not all cells comprising the optic cup mature into neural cells; the tips of the optic cup adjacent to the lens form the pigmented muscular ring, known as iris. Also, at the junction of the neural retina and the iris lies the ciliary body, which secrets the aqueous humour (Gilbert, 2014). A schematic diagram of the vertebrate eye and retina can be seen in Figure 1.4.

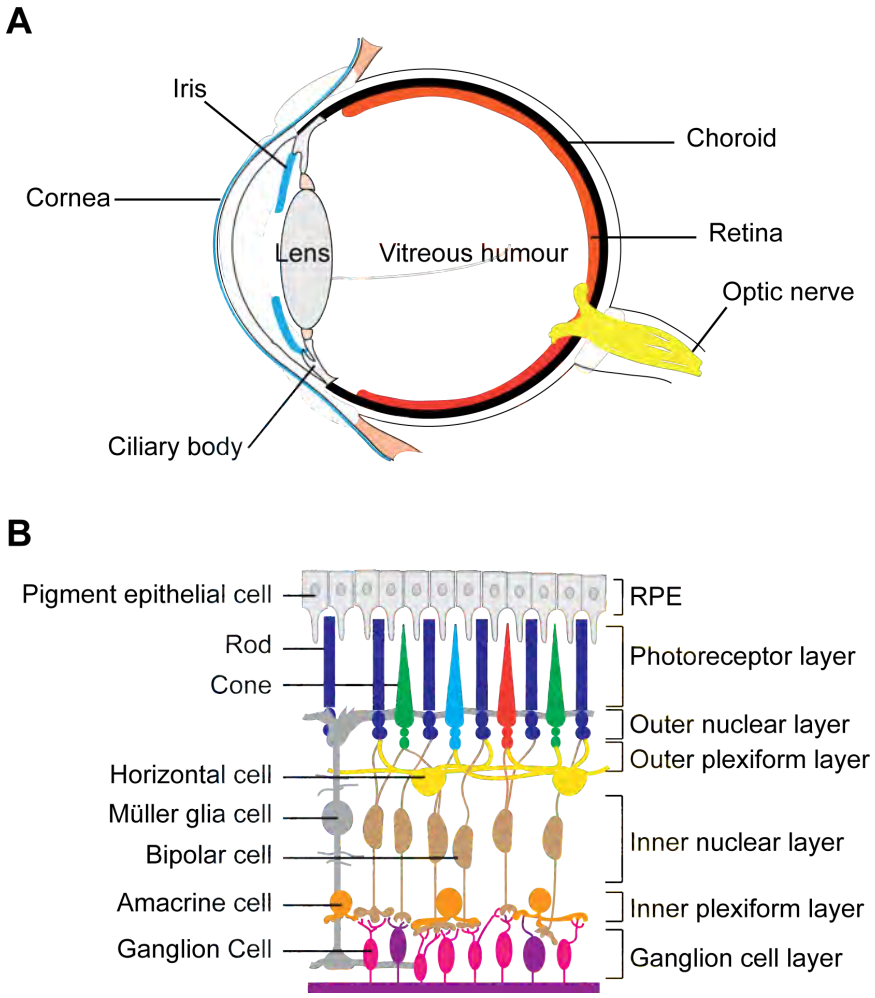


Figure 1.4: The vertebrate eye and neural retina. (A) A schematic diagram of the anatomical structure of vertebrate eye is shown. (B) The neural retina consists of seven retinal cell types (retinal ganglion cells, bipolar cells, amacrine cells, horizontal cells, rod and cone photoreceptor cells and Müller glia cells), which are arranged in a layered fashion. After Wilkinson-Berka (2004).

1.4.2 *Xenopus* eye development

The eyes of *Xenopus* are very similar to those of other vertebrates such as chick, mouse and human, and stem from the anterior part of the neural plate. *Xenopus* eyes also originate from one single eye field located in the central forebrain (diencephalon to be more precise). As described above, during neurulation the eye field separates due to midline signals (Henry et al., 2008, Li et al., 1997, Patten and Placzek, 2000, Roessler and Muenke, 2001). Due to the availability of detailed fate maps of the *Xenopus* embryo, which are based on tracer studies, it is now known that the cells

contributing to the eye mostly arise from blastomere D1.1 and D1.2 cells, with some minor contributions from the V1.2 pool of cells (see Figure 1.5). For the lens, cells are mainly derived from the D1.2 and V1.2 blastomeres (Moody, 1987a, Moody, 1987b).

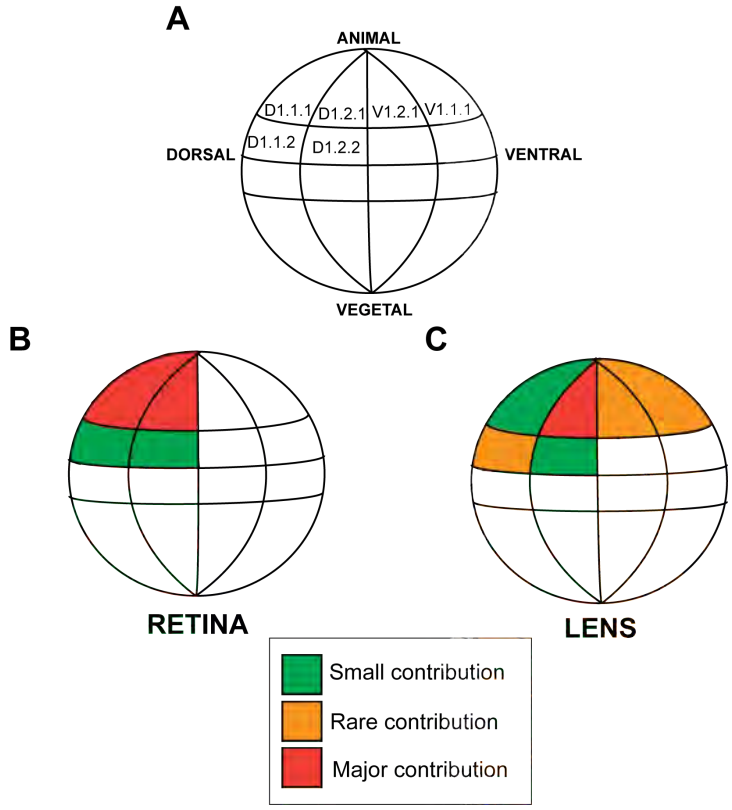


Figure 1.5: Blastomeres of the 32-cell *Xenopus* embryo, which contribute to lens and retina. Lineage tracing studies revealed that (B) tissues of the D1.1.1, D1.2.1 blastomeres make a major contribution (red) to the prospective retina, while blastomeres D1.1.2 and D1.2.2 make small contributions (green). (C) For lens, blastomere D1.2.1 mainly contributes (red), with small contributions from D1.1.1 and D1.2.2 (green). D1.1.2, V1.2.1 and V1.1.1 rarely also contribute (orange) to prospective lens. Orientation and naming of blastomeres is shown in (A). After Moody (1987b) and Xenbase (2016).

At early neural plate stage of *Xenopus* development, the initial retinal rudiment is found in the anterior neural plate at around stage 13/14. Prior to optic vesicle formation (around stage 15-19), the retinal rudiment extends to the region of the anterolateral folds (Brun, 1981). At stage 18/19 the optic vesicles start protruding from the sides of the neural tube. By stage 19-21 the optic vesicles come into contact with the overlying head ectoderm, which will eventually give rise to the lens and

cornea. The bulging optic vesicles are externally noticeable on the embryos by stage 21 (Henry et al., 2008).

Much like in other vertebrates, in *Xenopus* there is a reciprocal inductive relationship between the optic vesicles and the lens placode, which drives the development of lens and the optic cups. Uniquely in *Xenopus*, the embryonic ectoderm consists of a pigmented outer layer (whose final fate is not well known) and a non-pigmented sensorial inner layer. Also, in *Xenopus* the lens does not form through invagination of the surface ectoderm, as is the case for chick, mouse and human. A thickened lens placode forms at stage 26/27 in *Xenopus*. This lens rudiment then enlarges and finally separates from the sensorial ectoderm by stages 33/34. A cavity appears and the lens placode transforms to lens vesicle by stages 35/36. At the same time, cells facing the developing eye cup give rise to elongated primary lens fibres, which start synthesising lens crystallin proteins. The cells facing away from the eyecup, go on to form the lens epithelium, which is mitotically active (Henry et al., 2008). By stage 41, the lens cavity disappears as the lens epithelium and lens fibres draw together and touch. The lens goes on to mature further.

In *Xenopus* so called "free-lenses" have been observed and carefully described. These are lenses that have developed in the absence of an optic vesicle. The part of the optic vesicle that touches the lens placode, goes on to form the neural retina, while the more proximal region develops into RPE. First pigmentation in the RPE can be seen at stage 32, while it appears entirely black by stage 35/36. At stage 35/36 the neural retina starts to differentiate and by stages 37/38 the three distinct layers can be seen consisting of outer-, inner-nuclear layer and ganglion cell layer. By stage 42, the photoreceptor cells (rod and cones) are distinguishable. Before metamorphosis, a lot of cell proliferation takes place in the ciliary marginal zones (CMZ). It seems that much of the adult frog retina stems from the ventral CMZ cells (Henry et al., 2008). Without the presence of the lens placode, the retina does not form normally (Lupo et al., 2005, Mann et al., 2004, Perron et al., 1998).

1.4.3 Eye field transcription factors in Xenopus

It is known that the eye field becomes established after neurula stages. From lineage tracing/fate mapping experiments it is also known, that already at 32-cell stage, nine

of the blastomeres are competent – if not yet specified – to contribute to retinal development. It is at the start of the neural plate formation, that specific molecular events take place, which commit a population of neuroectodermal cells to a retinal fate. This process is also referred to as 'eye field specification' (Gallagher et al., 1991, Huang and Moody, 1993, Moody, 1987a, Zuber, 2010).

During gastrulation/neural induction, cells involute and travel along the inside of the blastocoel and finally induce the overlying cells to form neural ectoderm tissue. The process of neural induction is, as previously mentioned, regulated by BMP inhibitors, FGFs and Wnt signalling pathways. At mid-gastrula stages, all areas of the presumptive neural plate have the competency to form eyes. It is only at later stages, that the eye formation ability becomes restricted to the very anterior regions of the embryo (Li et al., 1997, Saha and Grainger, 1992, Zuber, 2010).

Both the process of eye field specification and forebrain patterning of the neural plate, are governed by BMPs, FGFs, Wnts, Nodals, hedgehogs and retinoic acid (RA) proteins, as well as their inhibitors. A fine tuned control of these signalling gradients exists across the antero-posterior and dorso-ventral axes of the embryo, thereby regulating the patterning events. Malfunctioning of any of these signalling networks will not only affect the forebrain patterning process, but may also have a knock-on effect on eye field specification and thus eye development (Zuber, 2010).

The frog retina is remarkably similar to the human retina in terms of its structure, function and (despite different time scales) development. The *Xenopus* retina contains all seven retinal cells types, which are also arranged in a three-layered retina. Importantly, a homologous network of genes seems to control eye development. These are referred to as the "eye field transcription factors" (EFTFs), which shall be introduced in more detail in the following paragraphs. Much of the initial work was carried out in Drosophila, where the homologs to the vertebrate EFTFs are known as the 'retinal determining genes in Drosophila' (Erclik et al., 2009, Gehring, 2004, Zuber, 2010).

1.4.3.1 Otx2 (orthodenticle homeobox 2)

The *Xenopus* Otx2 is a homolog of the Drosophila 'orthodenticle' gene. Prior to gastrulation Otx2 is detected in the dorsal marginal zone of the embryo (Gammill and Sive, 1997, Pannese et al., 1995). During gastrulation, it is expressed in the involuting mesoderm and later also in the overlying ectoderm, which will eventually form the rostral brain and eye field. Just before eye field specification starts (at stage 12), Otx2 expression stretches from the very anterior presumptive cement gland to the region of the midbrain. But this expression pattern changes quickly as eye field specification progresses, and soon an expression gap appears in the forebrain. By stages 12-13, Otx2 can no longer be detected in the eye field, while other EFTFs start to be expressed (Pannese et al., 1995, Zuber et al., 2003). It has been shown that the knockdown of Otx2 results in abnormal anterior structures and eye (Carron et al., 2005). It was also shown that in order for the eye to develop normally, it needs to be suppressed in the eye field as described, as continued expression of Otx2 in the eye fields results in a 'no eye' phenotype. Otx2 seems to supress the expression of other EFTFs at eye field specification stages. The forced overexpression of Otx2 was also shown to induce ectopic cement gland (Pannese et al., 1995, Zuber, 2010).

1.4.3.2 Six3 (six homeobox 3)

Xenopus Six3 is expressed in the developing neural plate at all stages, but it can be first detected by *in situ* hybridisation at stage 12 alongside other early phase EFTFs (Ghanbari et al., 2001, Zhou et al., 2000, Zuber et al., 2003). The miss-expression of Six3 seems to have a dose-dependent effect: low doses of Six3 increase the eye field and eventually eye size, while high doses affect the entire head region and result in abnormal structures (Bernier et al., 2000). It seems that excessive amounts of Six3 convert midbrain cells to retinal progenitors. Considering the rather broad expression domain of Six3, together with the range of phenotypes observed following overexpression, suggests it plays an important role for both eye field specification and rostral brain development (Zuber, 2010).

1.4.3.3 Rx1 (retinal homeobox 1)

In *Xenopus*, two highly homologous Rx genes have been identified (referred to commonly as Rx1a and Rx1b), which are more than 95% identical and exhibit the same expression pattern (Casarosa et al., 1997, Mathers et al., 1997a, Wu et al.,

2009). This may be a result of a partial duplication of the *Xenopus* laevis genome during evolution. For the purposes of this study, I will refer to both with 'Rx1'. Rx1 is first detected at stage 12 with a limited expression domain in the presumptive forebrain, which includes regions of telencephalon, hypothalamus, eyes and diencephalon, but not cement gland (Casarosa et al., 1997, Mathers et al., 1997a). The overexpression of Rx1 has been shown to result in hyperproliferation of the retina (sometimes with duplicated retinas) and ectopic RPE or retinal tissue in the region between eye and brain. Overexpression of Rx1 had no effect on Pax6 and Six3 expression domains at early stages (e.g. stage 13), while an expansion of both could be observed at later tailbud stages (stage 23) (Andreazzoli et al., 1999, Mathers et al., 1997a). The knockdown of Rx1 results in small or absent eyes. It has been found that Rx1 supresses Otx2 expression, and it seems to control proliferation and neurogenesis in the anterior neural plate. Overall, Rx1 is an important factor for eye specification and forebrain development (Andreazzoli et al., 1999, Zuber, 2010, Zuber et al., 2003).

1.4.3.4 Pax6 (paired box 6)

By *in situ* RNA hybridisation, Pax6 is first detectable at stage 12 in the anterior neural plate, where it is expressed in a band across the embryonic midline, as well as in two broad stripes along the presumptive neural tube (Hirsch and Harris, 1996, Li et al., 1997). At neural plate stages, the anterior expression domain includes regions for presumptive eye, telencephalon, diencephalon, olfactory bulbs and hindbrain, as well as the presumptive lens ectoderm (Zuber, 2010).

The forced overexpression of Pax6 leads to lens induction in both the whole embryo and animal cap. Considering the crucial role Pax6 plays in lens induction (mentioned previously), this finding comes as no big surprise (Altmann et al., 1997). What was however surprising is that early miss-expression studies found no evidence of Pax6 being able to induce ectopic eyes. For these studies, researchers injected the Pax6 mRNA at the 4-cell stage of the frog embryo (Altmann et al., 1997, Halder et al., 1995a, Hirsch and Harris, 1996, Zuber et al., 1999). However later experiments, where injections were carried out at 16- or 32-cell stage showed that Pax6 could induce ectopic eye-like structures. Both the concentration of Pax6 mRNA injected, as well as location of injection, proved to be critical for this. Pax6 overexpression

induced Otx2, Six3 and Rx1 expression. The knockdown of Pax6 reduced or even blocked eye formation in *Xenopus* (Chow et al., 1999b). Based on the Pax6 expression domain, its ability to induce other EFTFs and ectopic eyes, it can be concluded that Pax6 represents a crucial factor for eye field specification (Zuber, 2010).

1.4.3.5 ET (eye T-box)

ET can be detected by *in situ* hybridisation at stage 12 in two expression domains within the presumptive eye field and in the presumptive cement gland. Amongst the EFTFs, ET exhibits one of the most restricted expression domains within the anterior neural plate (Li et al., 1997, Zuber et al., 2003). The miss-expression of ET at the 2-cell stage of the embryo leads to abnormal eye morphology and development, and if overexpressed medially, cyclopia (i.e. fused retinas). This can be explained by the fact that ET regulates both part of the Shh signalling pathway and the expression of ventral retinal markers (and vice versa - Shh regulates ET expression) (Takabatake et al., 2002, Wong et al., 2002). ET can act as a transcriptional repressor (He et al., 1999), however very little is known about its role in early eye field specification. The miss-expression of ET has also been shown to down regulate Otx2 – whether this is a direct effect or an indirect effect via Rx1 remains to be seen (Takabatake et al., 2002, Zuber et al., 2003). There was no induction of ectopic retina or expanded eye tissue, when ET was overexpressed. The knockdown of ET produces headless tadpoles. In $ET^{-/-}$ knockout mice, no eye phenotype could be found (Davenport, 2003, Rana et al., 2006, Ribeiro et al., 2007). While ET is a crucial component of the EFTF cocktail that regulates eye development, it may not be crucial for eye field specification itself – its role may be to regulate the expression of other EFTFs or other factors such as Shh (Zuber, 2010).

1.4.3.6 Six6 (six homeobox 6)

Six6 is the last of the EFTFs to be expressed in the *Xenopus* eye field. It has the smallest and most eye field centric expression pattern (Zuber et al., 2003). Overexpression of Six6 has been shown to dramatically increase eye field and consequently eye size (Zuber et al., 1999). It expands the Pax6, Rx1 and ET expression domains. The eye enlarging effects of Six6 can be reversed with the mitotic inhibitor hydroxyurea, which suggests that this effect is based on excessive

cell proliferation. It has been proposed, that Six6 (like Six3) transforms midbrain cells to retina (shown to down regulate midbrain markers, while up-regulating EFTFs) (Bernier et al., 2000). Both Six6 and Six3 seem to regulate the proliferation of retinal precursor cells, possibly through similar mechanisms. Due to Six6 being expressed only in the late stage of eye field specification, it must be assumed that it contributes to eye development only after initial specification processes have occurred (Zuber, 2010).

1.4.4 Mechanisms underlying the EFTFs regulation of eye development

A lot of the initial work regarding eye field specifying factors was carried out in *Drosophila*. Results showed that there is a tightly co-ordinated hierarchical network of transcription factors and feedback loops, that controls eye development in *Drosophila*. The *Xenopus* EFTFs Otx2, Six3/6, Rx1, Pax6, Lhx2, ET and Nr2e1 are in fact homologs of the *Drosphila* eye regulating genes *orthodenticle* (*otd*), *sineoculis/optix* (*so/optix*), *Drosophila Rx* (*drx*), *eyeless/twin of eyeless* (*ey/toy*), *apterous* (*ap*), *optomotor-blind* (*omb*) and *tailless* (*tll*), respectively (Acampora et al., 2001, Daniel et al., 1999, Davis et al., 2003, Lunardi and Vignali, 2006). Zuber et al. (2003) found that the same network of regulating factors can be found in vertebrates (specifically *Xenopus*) (see Figure 1.6). They found that the miss-expression of 'cocktails' of EFTFs is sufficient to induce ectopic eye field and ectopic eye-like structures in *Xenopus*. 90% of injected tadpoles had developed ectopic retinal pigment epithelium (RPE) on the injected side, while 20% exhibited large ectopic eyes of either similar or sometimes larger size than the endogenous eye. These ectopic structures had a cup-like structure with a tri-layered retina and expressed different retinal markers, such as for lens, RPE, photoreceptor and retinal ganglion cells. These ectopic eyes could be observed both inside and outside of the nervous system (Zuber, 2010).

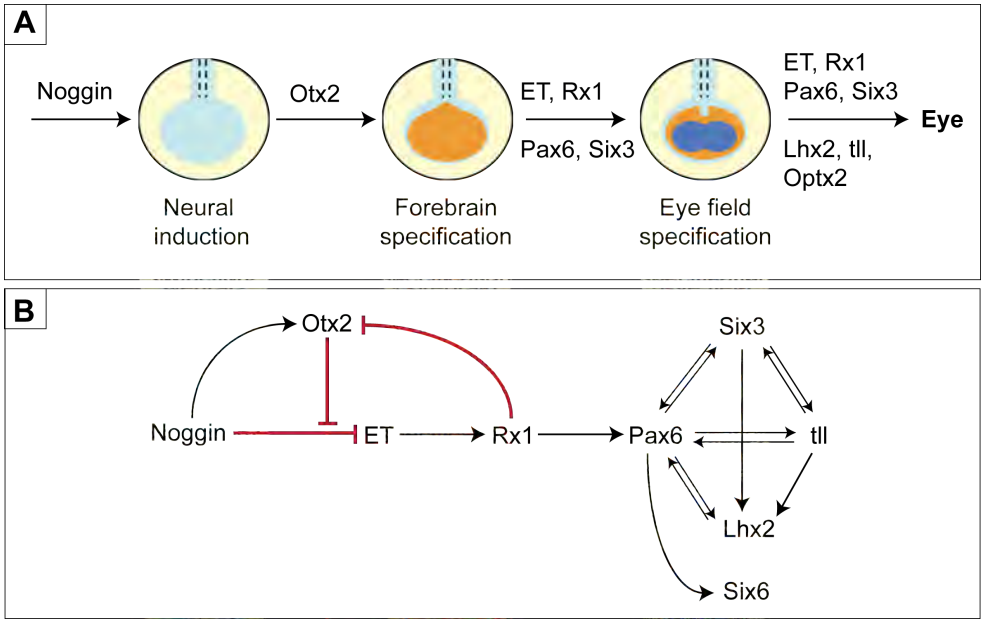


Figure 1.6: The formation of the eye field in the anterior neural plate. (A) A schematic diagram to illustrate the dynamic formation of the eye field. Neural inducer Noggin is widely expressed in the neural plate (light blue) during neural induction and paves the way for Otx2 expression (orange), as part of forebrain specification. This is followed by the orchestrated expression of EFTFs such as ET, Rx1, Pax6 and Six6 (dark blue) to specify the eye field, which eventually leads to eye formation. (B) Before stage 10, Noggin inhibits ET expression, but promotes Otx2 expression. Once Otx2 accumulates sufficiently, it lifts the noggin-induced inhibition and ET is finally expressed. ET then induces Rx1, which inhibits Otx2 expression, but promotes Pax6, which in turn activates a network of other eye field transcription factors to facilitate eye field specification (after Zuber et al. (2003) and Gilbert (2014).

The EFTFs have been shown to regulate each other's expression (see Figure 1.6): Rx1 is required for Six6 expression in *Xenopus* (Terada et al., 2006), while in the mouse Pax6 and Lhx2 are required for *Six6* expression (Tetreault et al., 2009). Rx1 may be transcriptionally regulated by Otx2 (Danno et al., 2008). Pax6 and Six6 can also work in synergy to coordinate eye size together (Zuber, 2010, Zuber et al., 1999).

It seems that the expression of EFTFs in pluripotent cells determines them to a retinal cell fate (Viczian et al., 2009). Otx2 and the EFTFs are required, and in some conditions even sufficient, for proper eye development in *Xenopus*. Following these

experiments, the question still remained through which mechanisms exactly the EFTFs regulate the eye field specification and development of the eye (Zuber, 2010).

Some of the mechanisms have since been identified, such as controlling neural patterning signals. As already described in Chapter 1.3.3 about neural induction, the neural patterning events in the *Xenopus* embryo are regulated by BMPs, FGFs, Wnts, Nodals and RA. These proteins and their signalling systems have been shown to influence the expression domains of EFTFs and in some cases, vice versa (Zuber, 2010). In order for neural induction to take place, BMP signals need to be inhibited in the dorsal anterior part of the embryo. But even after the forebrain patterning events have taken place, the BMP inhibition needs to be maintained, so that the eye field can develop. So when BMP4 coated beads were implanted in the forebrain region of the developing *Xenopus* embryo, Otx2, Rx1 and Pax6 expression was inhibited, which completely hindered eye development (Gestri et al., 2005, Hartley et al., 2001). It was shown that Six3 directly suppresses BMP4 and thereby preserves the eye field. In summary, the signalling factors that pattern the neural plate have a continual influence on the eye field and the EFTFs do in part 'protect' the eye field from these invading factors (Zuber, 2010). There needs to be a balance of these factors for the patterning/development process to occur properly.

EFTFs have also been shown to take part in the regulation of cell migration in the neural plate. During gastrulation and neurulation, large numbers of cells migrate within the embryo. It has been found that EFTFs play a role in regulating the migration and mobility of retinal precursors. Cells expressing Otx2, Rx1 and Pax6 migrate into the anterior neural plate and eye field. The BMP inhibitor and neural inducer Noggin was shown to have a similar effect (Kenyon et al., 2001, Zuber, 2010). It is known that FGF, ephrin and Wnt signalling pathways are all important for the proper positioning of the retinal progenitor cells within the eye field (Lee et al., 2006, Lee et al., 2009, Moore et al., 2004). While there is no direct link yet to show that EFTFs are directly involved in this process, it is known that EFTFs are regulated by and can themselves regulate parts of these pathways (Zuber, 2010).

Some EFTFs have been shown to maintain the proliferative state of the eye field cell population. Neural differentiation begins soon after gastrulation in the posterior

regions of the neuroectoderm. However, cells of the eye field continue to proliferate to achieve the large eye size. Therefore, proneural genes, which facilitate neural differentiation, such as Xngnr-1 and Xdeta-1, are not expressed in the eye field. Rx1 seems to be able to repress the expression of these pro-neural genes (Andreazzoli et al., 2003). Six3/Six6, as previously mentioned, regulate retinal progenitor proliferation. Not only do they inhibit BMP4, but they also promote the expression of anti-neurogenic genes such as Zic-2, hairy2, CyclinD1 and p27Xic1. So EFTFs use different mechanisms to maintain eye field cells in a proliferative state (Gestri et al., 2005, Zuber, 2010).

The EFTFs are highly conserved across species through evolution and their malfunctioning leads to abnormal or no eye development. While there are certainly species differences, most of the mechanisms mentioned have been discovered in other common model organisms. The EFTFs seem to be individually important, as they have varied roles, such as regulation of neural patterning, control of cell migration and proliferation, as well as controlling other EFTFs expression. In *Xenopus* at least, the EFTFs collectively seem to be sufficient to form normal eye (Zuber, 2010).

1.5 Extracellular matrix and role of SLRPs

The extracellular matrix (ECM) is a three dimensional, non-cellular structure, which can be found in the space in between cells. It is composed of a network of insoluble macromolecules, such as glycoproteins, proteoglycans (PGs) and collagens, which are secreted by the surrounding cells. It not only provides mechanical support and stability, but also creates appropriate microenvironments, controlling levels of growth factors, hydration, pH and electrochemical-gradients (Chen and Birk, 2013).

The ECM is actively involved in the development and maintenance of differentiated tissues and it also regulates tissue homeostasis, i.e. the constant remodelling, breakdown and synthesis of tissue (Bonnans et al., 2014, Hynes, 2009). It is crucial for cell adhesion, as well as cell migration, as it can act as scaffolding for cells to attach to or migrate along, but it can also provide direction cues and signals (Gilbert, 2014). The ECM is essential for survival, which is exemplified by loss-of-function

studies of various ECM components (Bateman et al., 2009, Bonnans et al., 2014, Jarvelainen et al., 2009). Components of the ECM continuously interact with epithelial cells, e.g. as ligands for cell surface receptors. The ECM signals are involved in all areas of a cell's life, such as adhesion, migration, proliferation, survival, differentiation and apoptosis. Also, the ECM can locally secrete growth factors, such as EGF, FGF, Wnts and TGF-βs (Bonnans et al., 2014, Hynes, 2009). Cleaved parts of the ECM can regulate ECM architecture and also influence cell behaviour. Cells are constantly rebuilding and remodelling the surrounding ECM (Bonnans et al., 2014).

The architecture of the ECM is highly organised and our understanding of its key components, structure and detailed function is continuously increasing (Mecham, 2001, Rozario and DeSimone, 2010). In mammals there are around 300 proteins that make up the ECM – these proteins are referred to as the 'core matrisome'. The main groups are collagens, PGs and glycoproteins. There are two main types of ECM, which differ in location and composition: firstly, the interstitial connective tissue matrix, which surrounds the cells and offers structural support, and secondly the basement membrane, which is a specialised ECM to separate the epithelium from the surrounding stroma (Bonnans et al., 2014).

The macromolecules that make up the ECM are specialised and their relative composition is tissue specific (Gilbert, 2014, Mecham, 2001, Mouw et al., 2014). The ECM macromolecules often aggregate to form supramolecular structures. The ECM mainly consists of fibrous proteins such as collagens and elastin, and glycoproteins; such as proteoglycans, Fibronectin and Laminin. The collagens represent the bulk of the ECM proteins (Mecham, 2001, Mouw et al., 2014).

There are 36 types of proteoglycans, which will be introduced in more detail in the following section, with particular emphasis on the family of small leucine-rich repeat proteoglycans to which Asporin belongs. Briefly, the PGs can be found in the interstitial space between the collagens fibres. They generally consist of a core protein with covalently bound glycosaminoglycan (GAG) chains. These GAG components readily bind water, so that the presence of PGs serves the hydration of the ECM and also contributes to higher resistance to compression forces. PGs are

very abundant in the ECM of cartilage and neural tissues (Bandtlow and Zimmermann, 2000, Knudson and Knudson, 2001, Mouw et al., 2014). PGs can be subdivided according to the type of GAG chains bound, as well as their distribution and density along the core protein (Cui et al., 2013, Schwartz and Domowicz, 2004).

There are around 200 complex glycoproteins, with diverse functions, such as laminins, fibronectins and elastin. They are involved in ECM assembly, but also interact with cell surface receptors (Bonnans et al., 2014). Laminin and Fibronectin are examples of so called 'connector proteins', as they function as bridges between the different ECM proteins, thereby reinforcing the overall ECM structure, and also connecting it to the cells. These connector glycoproteins have multiple binding domains and can thereby attach to a multitude of molecules (Mecham, 2001). There are also many ECM-associated proteins, which are not counted part of the matrisome, but are none the less important for its functions (e.g. galectins, semaphorins, plexins) (Bonnans et al., 2014, Hynes and Naba, 2012).

1.5.1 Proteoglycans

Proteoglycans are biological molecules, which posses a protein core and covalently linked glycosaminoglycan (GAG) chains. The GAG chains themselves are linear, negatively charged polysaccharides, which belong to either of two classes (sulphated or non-sulphated). They consist of disaccharide repeat regions, containing acetylated amino sugar moieties and uronic acid. After synthesis, proteoglycans are mostly secreted into the extracellular space. There, they not only fulfil structural and supportive roles, but are also involved in cell signalling activities and other regulatory functions. Due to their extracellular location, proteoglycans can affect and regulate upstream elements of cell signalling cascades, such as intracellular phosphorylation (Schaefer and Schaefer, 2010).

The proteoglycan family consists of 43 distinct genes in mammals, although due to alternative splicing the actual number of PGs is much higher. For 20 years a classification has been used which does not really encompass all the different proteoglycans. Iozzo and Schaefer (2015) therefore suggested a new and more encompassing proteoglycan classification, which is based on three criteria: cellular/subcellular location, overall gene/protein homology and the presence of

specific protein modules within the PG protein core. Nearly all mammalian PGs can be divided into four main classes: intracellular PGs, cell surface PGs, pericellular and basement membrane zone PGs and lastly extracellular PGs (Iozzo and Schaefer, 2015). So far there has been only one intracellular PG identified – Serglycin (Douaiher et al., 2014). Heparan sulfate proteoglycans (HSPGs) are mainly associated with the cell surface or pericellular matrix and are usually closely linked to the cells (either directly or indirectly). Moving away from the cells, chondroitin sulfate-containing proteoglycans (CSPGs) and dermatan sulfate proteoglycans (DSPGs) are the next types of PGs to dominate. They are usually components of complex matrices such as found in cartilage, brain and cornea, and provide viscoelastic properties to the tissues. The largest class of PGs form the small leucine-rich repeat proteoglycans, which are most abundant in terms of gene number. As will be explained in more detail later - this class of PGs has both structural and signalling functions. Particularly in tissue remodelling environments, such as during cancer, diabetes, inflammation and atherosclerosis, SLRPs are known to play roles. SLRPs interact with several different receptor tyrosine kinases (RTK) and toll-like receptors (Iozzo and Schaefer, 2015).

1.5.1.1 Intracellular proteoglycans

So far, the only known intracellular proteoglycan is Serglycin. It is substituted with heparin and found in mast cells where it regulates the packaging of proteases, which are released during inflammation (Douaiher et al., 2014).

1.5.1.2 Cell surface proteoglycans

There are 13 genes in this class, seven of which are transmembrane PGs, while the other six are glycosylphosphatidylinositol–anchored (GPI-anchored) PGs. With the exception of two PGs (NG2 and Phosphocan), all PGs in this subgroup are substituted with heparin sulphate side chains (Iozzo and Schaefer, 2015). To the transmembrane spanning PGs belong the Syndecans; this family includes four distinct genes for single-pass transmembrane protein cores (Couchman, 2010). Syndecans are involved in a huge variety of functions. In development, syndecans have been shown to bind growth factors and thereby influence morphogen gradients (Christianson and Belting, 2014). In a newly emerging role, syndecan-1 has been shown to transfer to the nucleus (Chen and Sanderson, 2009). Other PGs belonging

to this group of transmembrane PGs are CSPG4/NG2 (CSPG which has been shown to promote tumour vascularization), betglycan/TGFb type III receptor (which acts as co-receptor for members of the TGF-β family of Cys knot growth factors, such as activins, inhibins and BMP) and Phosphocan (a CSPG which interacts with neurons and neural cell adhesion molecules/N-CAMs). The glypicans are GPI-anchored HSPGs and are known to bind and modulate Hedgehog (Hh) as well as the canonical Wnt pathway via Frizzled (Iozzo and Schaefer, 2015).

1.5.1.3 Pericellular and basement membrane zone proteoglycans

In this class are four PGs, which are all closely associated with the cell's surface and they are mostly HSPGs. Perlecan is a modular HSPG, with a large gene and complex promoter. It has various biological functions and a wide distribution. It interacts with various ligands and receptor tyrosine kinases and is known to be a complex regulator of vascular and tumour angiogenesis (Iozzo and San Antonio, 2001, Iozzo and Schaefer, 2015). Another member of this class is Agrin. It is also an HSPG and found to be responsible for acetylcholine receptor clustering. It is highly expressed in axons and dendrites (Marneros and Olsen, 2005).

1.5.1.4 Extracellular proteoglycans

This is the largest class amongst the proteoglycans with a total of 25 distinct genes. Four of these are the hyalectans, which are key structural components of cartilage, blood vessels and the nervous system. Three genes code for the SPOCK family of calcium-binding HSPGs, which are still poorly studied. The largest family form the SLRPs to which Asporin belongs. 18 genes have been identified so far (Iozzo and Schaefer, 2015).

1.5.2 Small leucine-rich proteoglycans (SLRPs)

1.5.2.1 Structure and Function

The SLRP-family consists of 17 members of secreted proteins. Based on genomic and protein homologies, they can be divided into five subfamilies: traditionally defined classes I to III and the non-canonical classes IV and V (see Figure 1.7). SLRPs consist of two main structural components, one being the protein core and the other a varying number of GAG chains (e.g. chondroitin, keratin, dermatan and heparin-sulphate). SLRPs exhibit a variable number of tandem 'leucine-rich-repeats'

(LRRs) in their central protein domain, which follow a characteristic motif: LXX-LXLXXNXL, whereby X = any amino acid, L = leucine/isoleucine/valine and N = asparagine/cysteine/threonine. The LRRs are considered particularly important for protein-protein interactions. The N-termini contain four cysteines combined with class conserved spacing, which has been found to vary depending on SLRP function, while the C-termini contain cysteine-rich capping motifs. Canonical SLRP classes I, II and III also contain the so-called 'ear-repeat'. This refers to the penultimate and longest LRR, which extends outwards of the molecule and thereby maintains protein conformation and its ability to bind ligands. A mutation in the ear repeat has been found in human congenital stromal corneal dystrophy patients. There is great molecular diversity between SLRP members, which is a result of different numbers and combinations of GAGs with which the protein core can be substituted (Schaefer and Schaefer, 2010). SLRPs have been shown to be important regulators of biological processes. While GAG chains certainly contribute to the specific functions of the SLRPs, it has not been investigated in detail (Chen and Birk, 2011, Chen and Birk, 2013, Dellett et al., 2012, McEwan et al., 2006, Schaefer and Iozzo, 2008, Schaefer and Schaefer, 2010). The GAG side chains are processed in different ways depending on the tissue and developmental stage, thereby resulting in varying GAG chain lengths, number, sulphation and epimerization. SLRPs are also subject to modifications via N-glycosylation, which affects their conformation, stability and secretion (Chen and Birk, 2013). This variability in glycosylation and differential processing, provide SLRPs with multiple binding abilities (Krishnan et al., 1999, Seo et al., 2005).

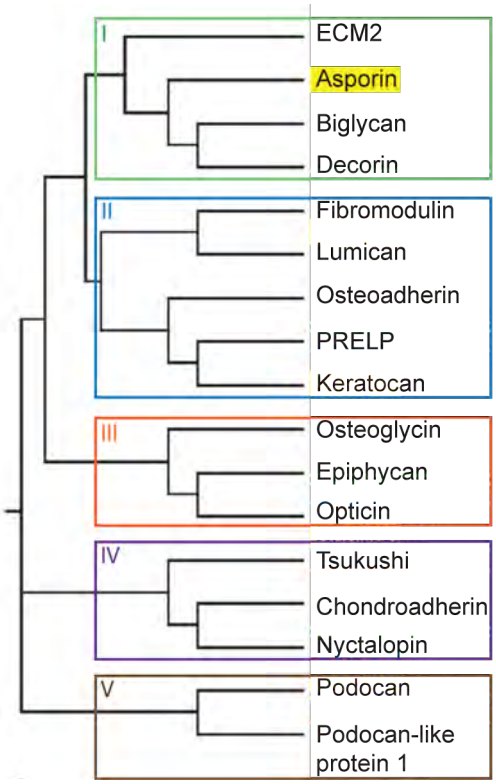


Figure 1.7: The SLRP family of proteins. Shown is a phylogenic tree of the SLRP family members, which are divided into traditionally defined classes I to III, and the non-canonical classes IV and V. Asporin – the SLRP of interest in this study – has been highlighted in yellow and belongs to class I, alongside Biglycan, Decorin and ECM2.

The division of SLRPs into families based solely on sequence might not be appropriate and should possibly be based on function instead. A major property shared by most members of SLRP classes I, II and IV, is the ability to bind collagen via their LRR domains with high affinity (K_d in nM range) (Lorenzo et al., 2001).

Due to their proteoglycan structure, SLRPs tend to accumulate in the ECM, where they are mainly associated with collagens in the interstitial connective tissues, like cornea, bone and tendon. SLRPs were initially thought to be exclusively involved with collagen fibril assembly, organisation and degradation. During early development, however, there is also collagen independent secretion in tissues. It is now known that SLRPs directly regulate ligand induced signalling pathways, such as TGF-β family (including BMP) signalling. SLRPs coordinate several signalling pathways which regulate cell processes such as proliferation, growth, differentiation,

survival, adhesion, migration, tumour growth and metastasis formation (Chen and Birk, 2013, Dellett et al., 2012, Wilda et al., 2000).

SLRPs are dynamically synthesised, secreted, deposited and degraded *in vivo*. Their binding ability, the speed of secretion and presence of other modulating molecules all affect the form and function of SLRPs. Differential splicing, variable polyadenylation and the use of several promoters guarantee the tissue specific regulation at different developmental stages. Specific surveillance chaperone molecules and post-transcriptional modifications take place in the endoplasmic reticulum as part of the secretory pathway (Chen and Birk, 2013, Tasheva et al., 2004). Once in the extracellular matrix, SLRPs act as substrates for various proteases such as matrix metalloproteinases, aggrecanases, BMP-1 and Granzyme B. Some SLRP members are resistant to degradation from selected metalloproteinases. When bound they can thereby protect collagen fibrils from cleavage through collagenase (Boivin et al., 2012, Geng et al., 2006, Melching et al., 2006). There are structural similarities between SRLPs and also overlaps in properties. The spatial distribution of SLRPs is tissue specific and dynamically changes during development. Thereby SLRPs have both instructive and structural roles (Chen and Birk, 2013).

1.5.2.2 Role of SLRP in matrix assembly

Collagen fibres are the key component of extracellular matrices. Pro-collagen is synthesised within the cell, folded in the endoplasmic reticulum (ER), packaged in the Golgi apparatus and then finally transported and secreted via specialised elongated intracellular compartments at the cell surface. Once in the extracellular space, pro-collagen is converted to collagen, which in turn is then assembled to protofibrils. The subsequent end-to-end assembly of protofibrils, results in mature collagen fibrils. SLRPs play an important supportive and stabilizing role during the collagen assembly process through a continuum of interactions, which is both tissue and developmental stage dependent (Birk et al., 1995, Canty and Kadler, 2002, Chen and Birk, 2013).

SLRPs of different classes share several binding sites, but they also contain specific non-shared sites. Although shared binding sites exist, their individual affinities towards ligands vary. This suggests some functional redundancy between SLRPs and

possibly a scenario where several SLRPs participate in a fine tuned regulatory process. SLRPs bind collagen via their central domain to regulate fibril growth (Svensson et al., 1995), e.g. class I SLRPs bind collagen via "d" and "e" bands (Pringle and Dodd, 1990). SLRPs belonging to the same class bind collagen through the same sites and therefore compete for collagen binding, as is the case with Asporin and Decorin. However, there is no competition for binding between the different SLRP classes. The GAG chains of SLRPs are thought to play a role in the regulation of fibrillogenesis and the interfibril spacing, as well as ECM organisation (Chen and Birk, 2013). SLRPs may also play a role in cell to matrix interactions, by interfering with cell surface receptors and pericellular matrix molecules (Chen and Birk, 2013, Kadler et al., 2008).

1.5.2.3 Diseases linked to SLRPs

There are numerous human diseases, which have been linked to SLRP mutations. Interestingly, a lot of inherited SLRP-linked diseases seem to be ocular abnormalities. In general, functional compensation seems to take place between SLRP members. For example, in muscle, diseased kidney and bone cells an inherited lack of Biglycan is compensated by an increased expression of Decorin. This emphasises the point that SLRPs work in a context dependent and tissue specific manner (Schaefer and Iozzo, 2008).

As previously mentioned, SLRPs have been shown to regulate cell receptor mediated signalling. They can interact with some LRR receptors and their adapter molecules, which are involved in pathogen recognition. SLRPs are part of the innate immune response, by either acting as pathogen-associated-molecular-pattern (PAMP) analogues, or being involved in presenting PAMPs to receptor complexes. SLRPs may also direct neutrophil infiltration into inflamed tissue by establishing immobilized chemokine gradients (Schaefer and Schaefer, 2010). SLRPs also have the ability to bind certain pathogens and toxins, such as borrelia burgdorferi (the cause of Lyme disease), which is bound by Decorin in the ECM. Also, Decorin and Biglycan bind low-density lipoproteins and apolipoproteins to collagen, thereby causing their accumulation in atherosclerosis. Decorin has also been shown to have a crucial role in the formation of amyloid plaques in Alzheimer's disease (Brown et al., 2001, Chen and Birk, 2013, Snow et al., 1992).

1.5.2.4 Signalling pathways modulated by SLRPs

Multiple cellular signalling pathways are modulated by SLRPs including: receptor tyrosine kinases, toll-like receptors and, especially, BMP/TGF- β signalling pathways. Many SLRP members, including ASPN (along with Decorin and Biglycan), are able to bind to and block BMP/TGF- β signalling. SLRPs regulate several signalling networks and are involved in processes like tissue morphogenesis, cancer growth and native immunity. Specific spatial distribution of SLRPs and the resulting abundance in certain locations may favour certain pathways, while absence of SLRPs could allow other types of signals. If this knowledge could be translated into a protein-based therapy, it might be possible to target diseases like fibrosis, cancer and inflammatory disorders (Schaefer and Iozzo, 2008).

SLRPs have been shown to execute substrate specific functions in a tissue specific manner. Decorin, Biglycan and Fibromodulin can bind all three isoforms of TGF-β, however with different binding affinities. There have been several modes of interactions suggested between Decorin and TGF-β, amongst them: direct binding of the two molecules and inactivation as a result; sequestration of the Decorin/TGF-β complex into the ECM; and Decorin mediated inhibition of TGF-β signalling via Smad2 (Schaefer and Schaefer, 2010).

1.5.2.5 The role of SLRPs during development

Most members of the SLRP family are expressed during development, and also in adult neural tissues – particularly ocular tissue (Le Goff and Bishop, 2007, Ohta et al., 2006). SLRP expression is increased in ECM rich tissues such as connecting tissues; e.g. cornea and vitreous, which consist of ECM proteins such as collagens. The reported molecular activities of SLRPs and their expression patterns, suggest an important role for SLRPs in neural development and maintenance. Many SLRP family members are highly expressed in the eye and mutations have been shown to lead to severe eye defects in humans, such as high myopia (Lin et al., 2010, Majava et al., 2007) and congenital dystrophy of the cornea (Bredrup et al., 2005), but also more severe diseases, such as gastric cancer (Wang et al., 2011), brain tumours (Castells et al., 2010), atherosclerosis and Progeria (Lewis, 2003, Singla et al., 2011).

The SLRP family is involved in nearly all signalling pathways of development, such as TFG-β and BMP signalling pathways (Moreno et al., 2005b, Morris et al., 2007, Yamaguchi et al., 1990). Until recently SLRPs have been studied only in respect to one certain signalling pathway. But a much more complex picture is emerging, whereby SLRP members each regulate several pathways (Brandan et al., 2006, Dellett et al., 2012, Desnoyers et al., 2001, Goldoni et al., 2009, Inkson et al., 2009, Iozzo, 1999, Kresse and Schonherr, 2001, Schaefer and Iozzo, 2008).

In *Xenopus*, Tsukushi and Biglycan regulate the dorsal-ventral axis and secondary axis formation by modulating the anti-BMP4 activity of Chordin. Biglycan, for example, promotes the binding of BMP4 and Chordin and thereby contributes to the BMP signal inhibition (Moreno et al., 2005b, Morris et al., 2007, Ohta et al., 2006). Another SLRP – Decorin – also interacts with ECM components such as collagen and Fibronectin. It binds and sequesters TGF-β and thus inhibits the TGF-β signalling pathway (Yamaguchi et al., 1990). Developing embryos where Decorin was inhibited with an antibody showed disturbed anterior-posterior axes, which suggests a role for Decorin in convergent extension cell movements (Zagris et al., 2011). The SLRP Tsukushi, which controls BMP and Delta activity, is thought to play an important role in neural crest formation (Kuriyama et al., 2006). SLRPs Keratocan, Lumican and Mimecan are all involved in the formation of collagen fibrils, the hydration of the corneal stroma and the regulation of corneal transparency (Ali et al., 2011, Chen et al., 2010, Tanihara et al., 2002).

In addition to their role in embryonic neural development, the SLRP family also maintain the nervous system by regulating adult stem cell and CNS injury response. Neural stem cell niches are areas where embryonic stem cells reside after the completion of the embryonic development. In amphibians and fish, neural stem cell niches have been located in the retina. This peripheral region is referred to as the 'ciliary marginal zone' (CMZ) and is known to produce neurons and glia cells continuously throughout the animal's life. In chick and mammals, a similar structure termed the 'ciliary body' (CB) has been identified, even though there is much controversy regarding the mouse CB (Bilitou and Ohnuma, 2010, Dellett et al., 2012). Tsukushi is selectively expressed at the CMZ and CB and has been shown to regulate proliferation through the Wnt signalling pathway (Ohta et al., 2011, Ohta et

al., 2008). Canonical Wnt signalling has been found to regulate retinal stem cell proliferation by Kubo and Nakagawa (2008). Decorin is thought to be involved in the recovery processes after CNS injury, as it was found to be up-regulated widely around lesions in injured rat brain (Stichel et al., 1995). Decorin could also be contributing to the healing of damaged neural retina. It has been shown to supress scar formation and promotes axon growth after CNS injury (Davies et al., 2004, Logan et al., 1999, Minor et al., 2008).

1.5.3 Asporin – a novel class 1 SLRP

1.5.3.1 Identification and characterisation of Asporin

The Asporin protein was first purified from human articular cartilage and meniscus. The name 'Asporin' is derived from the presence of poly-**asp**artate residues and its overall similarity to dec**orin**. Lorenzo et al. (2001) initially classed ASPN as a non-proteoglycan when compared to decorin and biglycan, as it lacks the typical Ser-Gly dipeptide and flanking amino acids needed for glycanation. However, it contains a stretch of aspartate residues and an acidic domain, located either near the N- or C-terminal. Polymorphisms have been identified, whereby the number of consecutive aspartate residues varied from 11-15 (Lorenzo et al., 2001, Schaefer and Iozzo, 2008).

ASPN is expressed in several adult tissues but in varying amounts. High expression levels have been found in the liver, heart, aorta and uterus. Low levels of ASPN are found in lung, bone marrow and trachea. No ASPN could be found in the CNS, spleen and thymus. ASPN is a very acidic peptide and is closely related to its fellow class I SLRPs Decorin and Biglycan. ASPN's four amino terminal cysteines show the typical class I pattern: C-X_3-C-X-C-X_6-C. Like Decorin and Biglycan, ASPN contains a putative pro-peptide with a conserved cleavage site for BMP-1. The *ASPN* gene (human) is divided into eight exons, whereby the introns are inserted into the coding sequence at exactly the same positions as found in Decorin and Biglycan. The human ASPN gene spans 26 kilobases and is located on chromosome $9_{q31.1\text{-}32}$. It is not clear, whether ASPN contains an additional alternatively spliced exon 1, as is the case with Decorin. Like with Biglycan, there is no TATA box found in the 5' flanking region of exon 1. Several transcription factor recognition sites have been detected (Lorenzo et al., 2001).

As previously mentioned, the ASPN N-terminal is unusual as it contains an extended stretch of aspartate residues. mRNA similar to ASPN has been found in other vertebrate species, such as zebrafish, which also contains stretches of aspartate residues. In mouse and fish, poly-aspartate stretches are interrupted by other amino acids, but still exhibit a conserved number of aspartates. This suggests that the aspartates are important to the function of ASPN. It has no consensus sequence for GAG attachment between the N-terminal cysteine motif and the propeptide, but it does contain a conserved consensus sequence for asparagine-linked-glycosyl. This single linked oligo-saccharide shows variability in structure. The highest concentrations of ASPN mRNA have been found in articular cartilage, as well as aorta and uterus. Intermediate levels of mRNA were also found in other tissues, containing smooth muscle cells (Lorenzo et al., 2001).

1.5.3.2 ASPN – known signalling properties and disease implications

In the same way as Decorin and Biglycan, ASPN can bind type I and type II collagen (Kou et al., 2010) - probably via its LRR10-12. ASPN competes with Decorin, but not Biglycan, for collagen binding (Kalamajski et al., 2009).

ASPN has been shown to inhibit TGF-β function, through a direct interaction of ASPN with TGF-β *in vitro* (Kizawa et al., 2005). ASPN inhibits the TGF-β/Smad signal upstream of TGF-β type I receptor activation by co-localising and directly binding to TGF-β1 via ASPN's LRRs, and thereby stopping it from interacting with the TGF-β type II receptor (Ikegawa, 2008, Nakajima et al., 2007). The LRR motif is well conserved amongst species and is known to bind metal ions, DNA and proteins, and it exerts a variety of functions (Tomoeda et al., 2008). On the other hand, TGF-β was shown to increase ASPN expression in articular cartilage *in vitro* (Nakajima et al., 2007). In fact, all three isoforms of TGF-β (-1, -2 and -3) similarly induce ASPN mRNA expression in chondrogenic cells in human. However, TGF-β/Smad-3 mediated ASPN induction is indirect, as it requires *de novo* protein synthesis (Kou et al., 2007). Mouse ASPN seems to bind TGF-β via LRR4-5, in contrast to fellow class I SLRP Decorin, which binds via LRR4-8. Upon binding of TGF-β, the protein conformation of ASPN is expected to be similar to that of Decorin. ASPN, Decorin, Biglycan and Fibromodulin are all thought to compete for TGF-β binding. The

ability to bind TGF-β and collagens is a trait shared amongst many SLRP family members (Kou et al., 2010).

Irreversible destruction of cartilage, tendon and bone are hallmark signs of both rheumatoid and osteoarthritis. While osteoarthritis is due to chronic overuse and/or injury, rheumatoid arthritis is a systemic autoimmune disease. In both diseases inflammatory cytokine like interleukin (IL) 1β and tumour necrosis factor (TNF)-α stimulate matrix metalloproteinase enzymes (Torres et al., 2007). Kizawa et al. (2005) investigated the potential role of ASPN in osteoarthritis and found a significant association between (knee) osteoarthritis and the aspartic acid (D) repeat polymorphism in the N-terminal of the ASPN protein. The ASPN allele D14 (= 14 D repeats) seems to be overrepresented in patients compared to the common D13 allele. Furthermore, the frequency of the D14 allele was found to increase with disease severity. D14 is also overexpressed in patients suffering from hip osteoarthritis. Asporin suppresses TGF-β mediated expression of Aggrecan 1 and collagen type II α 1 genes and also decreased proteoglycans levels in an *in vitro* model of chondrogenesis (Torres et al., 2007). Kizawa et al. (2005) suggest that ASPN plays an important role in cartilage homeostasis by regulating cell and extracellular material regeneration via the inhibition of TGF-β. Allele variant D14 exhibits the strongest inhibitory effects. The stronger the TGF-β inhibition, the faster the disease should theoretically progress. Therefore, the multi D-repeat polymorphism has no direct role in the susceptibility for rheumatoid arthritis, but it influences the outcome of the disease (Torres et al., 2007). The ASPN D-repeat polymorphism seems to play a role in disc degenerative disease (Eskola et al., 2012, Tian et al., 2013) and there also seems to be an association with hand osteoarthritis (Bijsterbosch et al., 2013). Duval and colleagues (2011) examined the induction of ASPN in chondrocytes via both the cytokines interleukin (IL)-1β and tumour necrosis factor (TNF)-α. Both cytokines promote matrix degradations, while TGF-β promotes matrix and tissue repair. It was found that the pro-inflammatory cytokines decreased ASPN levels, while TGF-β increased ASPN levels in human chondrocytes (Duval et al., 2011).

ASPN seems to have a positive effect on the mineralization of human adult dental pulp stem cells' (hDPSCs) predentin and dentin (Park et al., 2009). The knockdown

of ASPN in hDPSCs suppressed mineralisation (Lee et al., 2011). In the periodontal ligament, ASPN decreased mineralization by inhibiting BMP-2 activity (Yamada et al., 2007). ASPN binds directly to BMP-2, most likely via LRR5, and thus prevents it from attaching to its BMP receptor 1B, as found in PDL cells *in vitro*. ASPN was also shown to inhibit BMP dependent Smad proteins *in vitro* (Tomoeda et al., 2008). Kalamajski and colleagues (Kalamajski et al., 2009) showed that ASPN can in fact bind calcium via its poly aspartate region and promote osteoblast collagen mineralization. Osterix, an osteoblast specific transcription factor, was later found to regulate ASPN and OMD expression in human osteoblasts (Zhu et al., 2012).

ASPN forms a feedback loop with both TGF-β and BMP2: ASPN down-regulates TGF-β and BMP2, and in turn both of these proteins increase ASPN levels (Ikegawa, 2008). FGF2, on the other hand, was shown to decrease ASPN levels (Yamada et al., 2007).

More recently, ASPN has become of interest in cancer research. Increased ASPN levels have been detected in the following tumour tissues: pancreatic (Turtoi et al., 2011), breast (Dumont et al., 2012), prostate (Orr et al., 2012) and scirrhous gastric cancer (Satoyoshi et al., 2015). ASPN seems to play opposing roles in different types of cancer, for example it acts as a tumour suppressant in breast cancer (Maris et al., 2015), but has a pro-invasive effect in scirrhous gastric cancers (Satoyoshi et al., 2015). SLRPs offer a great potential therapeutic target as they are more accessible and they form the first line of physical interaction of the cancerous cells with its surrounding (Turtoi et al., 2011). Until now there have been no studies regarding ASPN's role in early development.

1.6 The role of insulin-like growth factor (IGF) signalling in development

Research carried out over the past 20 years, shows that IGF signalling plays a crucial role in the normal development and growth of the central nervous system (CNS). The IGF signalling system includes growth factors IGF1 and IGF2, their receptors IGF1R and IGF2R, and the IGF binding proteins (IGFBPs). Generally, IGF signalling

promotes cell proliferation, maturation, survival and growth of neural cells. Most of its effects are mediated through the type 1 IGF receptor (IGF1R). The nature of IGF's effects seems to depend on cell type, tissue microenvironment and the developmental stage. Even though the time course differs between species, IGF1 and IGF1R seem to be involved at every stage of CNS development - neurulation, neurogenesis, differentiation into neurons and glia, neuronal migration, dendritic and axon outgrowth, natural cell death, synaptogenesis and myelination (O'Kusky and Ye, 2012). It is now also known that IGF signals work together with other neural signalling systems to direct neural stem cells towards specific fates during early development (O'Kusky and Ye, 2012). In the following section, the IGF signalling system shall be introduced, with particular emphasis on its role in eye development.

1.6.1 Overview of the IGF signalling system

1.6.1.1 IGF1 and IGF2

Growth factors IGF1 and IGF2 are anabolic peptides of 70 and 67 amino acids, respectively. Both share homology with pro-insulin and are produced by a single large gene. IGF expression starts early during development, but the exact regulatory mechanisms of *igf1* and *igf2* gene expression, are not well understood. IGF1 is expressed in all regions of the CNS, while peak expression often coincides with localised active spurts of proliferation, development and growth of neural cells. IGF1 production seems to take place predominantly in neurons and less so in glial cells (O'Kusky and Ye, 2012). In post-natal development, the pituitary growth hormone regulates IGF1 outside of the brain and, to an extent, also within the brain. Variant forms of IGF1 exist in the brain, which is due to post-translational N-Terminal cleavage (Ballard et al., 1987, O'Kusky and Ye, 2012). IGF1 has been shown to promote neuron progenitor proliferation and differentiation (Arsenijevic and Weiss, 1998, Arsenijevic et al., 2001, DiCicco-Bloom and Black, 1988, Zackenfels et al., 1995), and has an anti-apoptotic effect, which promotes survival (Yamada et al., 2001). IGF1 is also thought to regulate neurite outgrowth and synaptogenesis (Torres-Aleman et al., 1990).

IGF2 is highly expressed in mesenchymal tissues. In the brain, peak levels occur prenatally (Ayer-le Lievre et al., 1991). As development progresses, the IGF2 expression levels continuously decrease and eventually become restricted to the

meninges and choroid plexus in adults (Zhang et al., 2007). Genetic knockout studies showed that IGF2 is important for growth in early development (Baker et al., 1993, O'Kusky and Ye, 2012). *In vitro*, IGF2 has similar effects to IGF1 on cell growth and development. Postnatal overexpression of IGF2 has no effect on brain growth in mice (van Buul-Offers et al., 1995). An increased amount of IGF2 at this stage may have no further growth enhancing effects, as IGF1 signalling through the IGF1R is already occurring at maximum levels. IGF2 most likely modulates important non-growth related neuronal functions, e.g. when injected into rodent brains IGF2 was shown to improve memory (Chen et al., 2011). At high concentrations both IGF1 and IGF2 can also bind to the insulin receptor (InR), which can mediate IGF actions (Louvi et al., 1997, Moreno et al., 2005b, O'Kusky and Ye, 2012).

1.6.1.2 IGF binding proteins

IGF1 and IGF2 are mostly bound to IGFBPs in the extracellular space and in circulation. Ten IGFBPs have been identified so far, including six high affinity members termed IGFBP1-IGFBP6 (Jones and Clemmons, 1995), which all share structural homology. They specifically bind IGF1/2, with no affinity for insulin. IGFBP2, 3, 4, and 5 are the most abundant in the brain, while IGFBP6 can only be detected in low concentrations. IGFBP1 is not expressed in the brain at all. Each of the IGFBPs exhibits a particular temporal and spatial expression pattern within the CNS, but their roles still need to be elucidated in more detail (O'Kusky and Ye, 2012). By transporting bound IGFs in plasma, IGFBPs are thought to determine receptor binding, tissue and cell specific localization of IGFs and to prolong IGFs half-life in circulation (Jones and Clemmons, 1995). When bound to an IGFBP, IGFs cannot readily leave the circulation, which prolongs their half-life 70-90 fold. The resulting pool of circulating IGFs is thought to be a reservoir for times of stress (Guler et al., 1989, Hodgkinson et al., 1989).

1.6.1.3 IGF1R and IGF2R

The IGF1R is a heterotetrameric glycoprotein. It is made up of paired, di-sulfide linked α and β subunits. The α-subunits are located in the extracellular space and bind to the IGFs, while the β-subunits have a long intra-cytoplasmic domain, which contains intrinsic tyrosine kinase activity, as well as critical sites for tyrosine and serine phosphorylation (O'Kusky and Ye, 2012). The IGF1R shares around 46%

homology with the Insulin Receptor (InR). Both receptors can form heterodimeric hybrid receptors. Although their exact physiological function is not fully understood, the hybrid receptors may play a role in certain forms of cancer (Belfiore et al., 2009, Kim et al., 2012). IGF1 binds with high affinity to IGF1R. IGF2 and insulin also bind, but with a 10-fold and 100-fold lower affinity, respectively (O'Kusky and Ye, 2012). The IGF1R seems to be expressed in neural stem cells and all other neural cells evaluated (Baron-Van Evercooren et al., 1991). Once IGFs bind to the α-subunit of the IGF1R, a conformational change takes place, which results in auto-phosphorylation of the β-subunit. This activates a series of intracellular substrate proteins, which will be explained in more detail later (O'Kusky and Ye, 2012).

The largest abundance of the InR and IGF1R is found in Hensen's node, neural fold, neural tube and the developing eyes (Girbau et al., 1989). IGF1R is ubiquitously expressed in all neural cell types. High abundance of this receptor seems to coincide with high rates of cell proliferation and growth (Popken et al., 2005). Furthermore, the IGFs and IGF1R often seem to be expressed in close proximity of one another, which may suggest that IGFs act locally in an autocrine or paracrine fashion. However, IGF1 can cross the blood brain barrier, suggesting that circulating IGF1 must also be able to influence signalling in the brain (Aberg et al., 2007). IGF1 and IGF1R are expressed during early development, before the establishment of neural tissues (Ayaso et al., 2002, O'Kusky and Ye, 2012). IGF1 seems to be more abundantly expressed in anterior/head regions during late neurulation and organogenesis. In the developing eyes, IGF1 was detected in the epithelial cells (de Pablo et al., 1993).

The IGF2R is a single chain transmembrane protein, which is identical to the cation-independent mannose-6-phosphate receptor. It translocates IGF2 and proteins containing mannose-6-phosphate units to lysosomes for degradation. The global ablation of IGF2R has been shown to lead to overgrowth, due to the subsequent accumulation of IGF2 and signalling through the IGF1R (Efstratiadis, 1998, Eggenschwiler et al., 1997, O'Kusky and Ye, 2012).

1.6.1.4 IGF signalling through the IGF1R

Most of the IGF signals affecting growth are mediated via the IGF1R. Binding of IGF1 or IGF2 to the IGF1R leads to the activation of the tyrosine kinase in the receptor's β-subunit. The subsequent auto-phosphorylation of the tyrosine residues recruits docking proteins (LeRoith et al., 1995), such as insulin receptor substrates IRS-1, IRS-2, IRS-3, IRS-4, Grb2-associated binder-1 (Gab-1), Ras and Src homology containing proteins. The group of IRS molecules are involved in insulin signalling and are widely expressed throughout the CNS. IRS-1, -2, -4 and Gab-1 exhibit spatial/temporal expression patterns (Fantin et al., 1999, Folli et al., 1994, Numan and Russell, 1999, Sciacchitano and Taylor, 1997, Ye et al., 2002b), while only low levels of IRS-3 can be detected in the brain. Knockout studies in mice, suggest that IRS-1 is not essential in IGF neural signalling. These results may be due to other IRS members (IRS-2 and -4) compensating for the lack of IRS-1 (Ye et al., 2002b). IRS-2 seems to be more important for the IGF and insulin mediated signalling in the CNS (Schubert et al., 2003). The phosphorylated docking proteins then recruit down-stream signalling molecules to transduce the IGF signal. The Ras-Raf-MAP (mitogen-activated protein) kinase and Phosphoinositide-3 (PI3)-AKT kinase pathways are known to play crucial roles in IGF signalling (O'Kusky and Ye, 2012).

Furthermore, *in vitro* studies in non-neural cells have shown that the IGF1R can be modified by SUMO-1 (small ubiquitin-like modifier protein-1) and translocate into the cell nucleus, where it acts as a transcription factor to regulate its own expression (Sehat et al., 2010). Since neural cells are able to internalise the IGF1R, it is possible that IGF1R can act as a transcription factor in neural tissues too (Romanelli et al., 2007). There is some evidence that G-protein mediates some of IGF's neural actions (Kuemmerle and Murthy, 2001).

IGF treatment of cells leads to an increase in AKT phosphorylation and activation, which is sustained for a minimum of 24 hours (unlike the transient activation, that takes place with the Raf-ERK pathway) (O'Kusky and Ye, 2012, Romanelli et al., 2007). The PI3K-Akt pathway plays a key role in the survival of neural cells and it is also important for neural and IGF stimulated proliferation (Johnson-Farley et al., 2007). Its critical downstream effectors are glycogen synthase kinase 3β (GSK3β)

and β-catenin, which is a common mediator of IGF-I and Wnt signalling to promote neural cell proliferation and survival. Another downstream effector is mammalian target of rampamycin (mTOR), which is involved in signals relating to cell maturation and function (Guardiola-Diaz et al., 2012).

IGF signalling through the MAP pathway (Ras-Raf MAP kinase pathway) has been well studied. MAP kinase signalling is organised in three tiers of signalling cascades (Ye et al., 2010). Based on *in vitro* studies with pharmacological inhibitors, it is known that the Raf-Erk pathway seems to be key to IGF stimulated cell proliferation (D'Ercole et al., 1996). In neural IGF signalling, the MAP kinase pathway seems to be mainly involved in cell maturation and survival (O'Kusky and Ye, 2012).

1.6.1.5 IGF signalling through the IGF2R

There is little evidence to suggest that the IGF2R mediates the growth actions of both IGF1 and IGF2. Studies suggest that it may mediate some IGF actions, such as acetylcholine release from cultured neurons (Hawkes et al., 2006), and facilitate memory improvement in rodents (Chen et al., 2011). No enzymatic activity has been observed at IGF2R's intracellular domain and potential associated intracellular pathways are still unclear. G-protein has been identified as a key molecule for IGF2-IGF2R actions in neuronal cells (Hawkes et al., 2006). Other molecules such as protein kinase C (Hawkes et al., 2006), MAP kinase (McKinnon et al., 2001) and GSK3 (Chen et al., 2011) are also likely to participate.

1.6.2 IGF signalling in eye development

Pera et al. (2001) were the first to present evidence that IGF signalling is involved in the anterior neural development in *X.laevis*. They found that IGF1, IGF2, IGF3 (particular to *X.laevis*) and IGFBP-5 promote anterior development, and when overexpressed, expand the head region at the expense of trunk tissue in the frog embryos.

The overexpression of both IGF1 and IGF2 (in the 4 to 8 cell embryo) resulted in expanded head structures and ectopic eyes (IGF1 induced ectopic eyes: 66%; IGF2 induced ectopic eyes: 29%). In the animal cap, forced overexpression of IGFs increased the expression of anterior neural and some eye specific markers (Pera et

al., 2001). A dominant negative form of IGF1R (DN-IGF1R) had the opposite effect, whereby head tissue was drastically supressed and eye development was inhibited. The presence of DN-IGF1R inhibited Chordin mediated neural induction. Forced overexpression of IGF2 in the ventral marginal zone, resulted in embryos with ectopic heads and cement glands reminiscent of the Cerberus overexpression phenotype (Pera et al., 2001).

Overall, Pera and colleagues found that IGF mRNA injection in the neural plate region caused embryos to develop ectopic eyes, while injection in the prospective ventral mesoderm resulted in ectopic head formation. IGF signalling may regulate how much tissue is allocated to both head and trunk portions of the embryo. Not only is an inhibition of BMP, Wnt and Nodal necessary for head induction, but it seems also an active IGF signal (Pera et al., 2001).

The study by Pera and colleagues (2001) was closely followed by Richard-Parpaillon et al. (2002), who also confirmed a crucial role for IGF signalling in head formation in *Xenopus laevis*. Their study focussed more on the effects of IGF1. Much like Pera et al., they found that IGF1 overexpression expanded head, eye and cement gland tissue, while depletion of the IGF1R lead to drastic reduction in the aforementioned tissues. They found that IGF1 elicits its effect through inhibiting Wnt signalling in the early embryo at β-catenin level (Richard-Parpaillon et al., 2002).

In *Xenopus*, IGF1 is expressed maternally, and after midblastula transition both IGF1 and IGF2 are expressed in the embryo. By *in situ* hybridisation, the IGF1R can be detected after the end of gastrulation and in higher amounts in anterior and dorsal regions (Richard-Parpaillon et al., 2002). IGF1 did not cause generalised hyperplasia, but did cause different phenotypes depending on the site of mRNA injection. Dorsal injections caused enlarged head structures, whereby mid and hindbrain was regularly expanded, while the forebrain and neural tube structure remained unchanged. IGF1 was also shown to expand the expression of Otx2, NCAM and Pax6, with a strong induction of cement gland marker XAG. A morpholino targeted at the IGF1R caused microcephaly, as well as small or no eyes (Richard-Parpaillon et al., 2002).

Richard-Parpaillon et al. (2002), also found that IGF1 overexpression affects convergent extension movements in the developing embryos. Dorsal injections often resulted in gastrulation defects with delayed mesoderm involution and open blastopores. The IGF1 overexpression phenotype (with the dorsally curved embryos and a shortened anterior-posterior axis) seemed overall very similar to that of Wnt inhibitors such as Cerberus and Dickkopf. This prompted Richard-Parpaillon et al. (2002) to examine if IGF1 acts by inhibiting Wnt signalling. They found that IGF1 inhibits Wnt target genes *siamois*, *xnr-1*, *wnt-8*, *DN-GSKb* and *β-catenin*. The hypothesized IGF1 induced eye induction via Wnt inhibition is also in keeping with a study in an eyeless phenotype in Zebrafish, which showed that suppression of Wnt is required for both eyes and the telencephalon to develop properly (Richard-Parpaillon et al., 2002, van de Water et al., 2001).

1.6.3 Factors thought to play a role in IGF mediated eye development

Since the original studies by Pera et al. (2001) and Richard-Parpaillon et al. (2002), more work has been carried out which further confirms the important role of IGF signalling in eye development and introduced other key players in more detail. With IGF being quite broadly expressed throughout the embryo during development, the question remains - how can it propagate eye development in a specific spatial and temporal fashion?

1.6.3.1 Kermit2/XGIPC is important for eye development

Kermit2 (also known as XGIPC) is an IGF receptor interacting protein, which acts downstream of the IGF1R. It is required for the maintenance of IGF induced AKT activation. There is a long list of identified binding partners for Kermit2, including several membrane proteins, but their importance and biological functions are not well understood. Kermit2 binds to the intracellular domain of the IGR1R in *Xenopus* (Wu et al., 2006) and the human equivalent 'GIPC' has also been shown to bind the human IGF1R (Ligensa et al., 2001). Furthermore, the expression of Kermit2 is very similar to that of the IGF1R (Wu et al., 2006).

Kermit2 has been shown to be required for IGF mediated eye development. A knockout of Kermit2 results in embryos with inhibited development of the anterior structures, particularly the eyes. Kermit2 is apparently needed to maintain the IGF

induced AKT activation, which plays an important role in eye development (Wu et al., 2006).

Previously, it had been shown that the inhibition of IGF signalling leads to severe disruption of all anterior development in the *Xenopus* embryos. However, Kermit2 loss-of-function experiments only affected the eyes. A possible explanation is that Kermit2 acts only through the AKT signalling pathway, which has been shown to be particularly important for eye development. Another explanation might be that eye development requires specific temporal IGF signals. The induction of anterior structures may only require an early IGF signal, whereas proper eye development calls for a maintained IGF signal. As Kermit2 is required for prolonged maintenance of the IGF-AKT signal, its knockdown therefore only affects eye development (Wu et al., 2006). Kermit2 might also regulate the subcellular location of IGF1R. Mammalian GIPC is known to be involved in the regulation of endocytic trafficking, which raises the possibility that the *Xenopus* orthologue has similar abilities (Wu et al., 2006).

A study by La Torre et al. (2015) investigated whether mammalian GIPC has the same role in eye development, as Kermit2 has in frog. They found that GIPC binds and interacts with the IGF1R and activates Akt1. A DN-GIPC1 causes an inhibition of eye field cells with a down regulation of endogenous GIPC1. Pharmacological inhibition of Akt1 phosphorylation mimicked the DN-GIPC1 phenotype. Their results indicate that GIPC1 is important to separate eye field fate from telencephalic fate and it is likely to act through the IGF1R via the Akt1 pathway (La Torre et al., 2015).

1.6.3.2 The translational initiation factor eif6 affects eye development in *Xenopus*

Eif6 (eukaryotic initiation factor 6) is a highly conserved translational initiation factor, which regulates ribosome assembly and mediates both selective mRNA translation and apoptosis. It is thought to act as part of the 'RNA-induced silencing complex' (RISC) and many of its functions have been traced back to the phosphorylation of its serine 235 by protein kinase C (De Marco et al., 2011). In the developing embryo, Eif6 is expressed in abundance in the dorsal mesoderm,

presumptive eye field and the mid-hindbrain border. When overexpressed in *X. laevis* embryos, a disrupted eye development was observed. This phenotype was shown to be only temporary however and, by stage 42, eye development had recovered. Other anterior structures remained unaffected by eif6 overexpression. While morpholino application had no effect on the embryo, already small amounts of eif6 mRNA caused transient eye defects. It can be concluded, that for normal eye development, no eif6 must be present (De Marco et al., 2011).

Eif6 is regulated by extracellular signals such as IGF (Gandin et al., 2008, Tussellino et al., 2012). Eif6 has been shown to interact with the IGF1R and Kermit2 in the kidney, where eif6 seems to down regulate Kermit2 levels. It is also plausible that in eye development eif6 interacts with Kermit2. Looking at expression patterns in development - eif6 and Kermit co-localise. More work is required to establish whether eif6 and Kermit2 are required for the eye inducing IGF signal. It may be possible that eif6 regulates Kermit2 levels, which then influences IGF mediated downstream AKT signalling (Tussellino et al., 2012).

1.6.3.3 IRS-1 is important for eye development

IRS-1 belongs to the family of insulin receptor substrates (IRS). IRS-1 mediated receptor phosphorylation can activate two branches of the so-called canonical IRS pathway. The first one is the Ras/MAPK pathway, which is important for cell growth, division and differentiation. The second is the PI3/AKT (Ras independent) pathway, which is involved in mitogenesis, cell motility, metabolism, cytoskeleton organization, cell survival and differentiation. It has also been directly linked with IGF mediated eye induction and development (Bugner et al., 2011). IRS-1 is a major substrate for both the IGF1R and the InR and links both receptors to common downstream signalling pathways, such as the PI3K and MAPK pathways. IRS-1 seems to preferentially activate the PI3K/AKT kinase pathway (Bugner et al., 2011).

IRS-1 is already present in the oocyte and is further expressed throughout all of embryogenesis with increased expression levels in the developing eye and brain, branchial arches, otic vesicles and pronephrons (Bugner et al., 2011). The IRS-1 expression levels coincide with expression for IGF-1, which is mainly observed in anterior and dorsal tissues of the developing embryo. The expression pattern of IRS-

1 suggests that it plays a role in neural induction and eye development. Since the IGF1R is present at higher levels during *X. laevis* embryo development than the insulin receptor (InR), it is likely that IRS-1 mainly mediates IGF1R signalling (Bugner et al., 2011). An IRS-1 morpholino strongly decreased the expression of EFTFs Rx1, Pax6 and Otx2. Since blocking of IGF1R signal transduction leads to a reduction of anterior/head structures, one might expect the same phenotype with the IRS-1 loss-of-function experiment. This was however not the case. There is potentially a redundancy between different members of the IRS family (e.g. between IRS-1 and IRS-2). Furthermore, overexpression of IRS-1 did again not induce the expected expansion of head, eye and cement gland (Bugner et al., 2011).

1.7 Thesis Aims

How eyes are formed has been a longstanding research interest from both the scientific and clinical points of view. Recent outstanding technological progress has made molecular-based therapies a realistic prospect. In contrast, the whole molecular picture from naïve neuroectoderm to retinal precursor cells, still remains to be fully elucidated. Compared to the extensively characterised eye-field specific transcription factors, there are still a number of questions left unanswered with regards to the inductive signals that initiate the expression of these transcription factors, and thus specify the eye field.

The SLRP family of proteins is involved in a large number of biological events, and Prof. Ohnuma's group is particularly interested in their functions during neural development. During a systematic functional screening in *Xenopus* embryos, the SLRP ASPN showed a strong eye-inducing activity when overexpressed and I became interested in the molecular function of this protein.

The work presented in this thesis aims to investigate the role of ASPN in early embryonic eye development, in the *Xenopus laevis* embryo. To this end, ASPN's temporal and spatial expression patterns during frog development will be investigated and the induced overexpression eye phenotype analysed in more detail (Chapter 3). Chapter 4 employs a morpholino based loss-of-function approach to

verify that ASPN is indeed essential for eye development. Chapter 4 also explores whether ASPN is unique amongst SRLPs in affecting eye development and what effect ASPN has in the developing zebrafish embryo. Finally, the molecular mechanisms underlying the actions of ASPN will be investigated in Chapter 5.

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

2.1.1 Standard solutions

Table 2.1: Composition of standard solution used in this project

Solution	Composition
100 x Denhardt's solution	10 g Ficoll 400 (GE Healthcare), 10 g Polyvinylpyrrolidone (Sigma), 10 g Bovine Serum Albumin. Dissolved in 500 ml double distilled water (ddH_2O), filter sterilised and stored at -20°C.
6 x DNA loading dye	10 mM Tris-HCl (pH 7.6), 0.15 % Orange G, 60 % Glycerol, 60 mM EDTA. Made up in ddH_2O.
8 x ELS (egg laying solution)	For 4 liters (L): 205.71 g NaCl, 4.77 g KCl, 2.72 g $Na_2HPO_4.2H_2O$, 5.815 g Tris Base, 5.38 g NaH CO_3, 16.149 g $MgSO_4x7H_2O$ dissolved in 4 litres of ddH_2O and pH adjusted to 7.6 with glacial acetic acid.
4 % Ficoll	20 g Ficoll (GE Healthcare) dissolved in 500 ml of 0.2 x MBS; 500 µl Gentamicin. Solution filter sterilised and stored at 4°C
10 x MEM	209.2 g MOPS (1 M), 7.6 g EGTA (20 mM), 2.47 g $MgSO_4.7H_20$ (10 mM) in 1 L ddH_2O and adjust pH to 7.4. Filter sterilise and store protected from light.
10 x PBS	For 1 L: 80 g NaCl, 2 g KCl, 18.1 g $Na_2HPO_4.2H_2O$, 2.4 g KH_2PO_4; pH adjusted to 7.4 with NaOH or HCl and autoclaved.
4 % PFA	32 % PFA stock (Electron Microscopy Sciences) was diluted in 1 x PBS to a 4 % solution.
20 x SSC	175.3 g NaCl, 88.2 g Sodium citrate. Made up to 1 L ddH_2O and pH adjusted to 7.0 with 1 M HCl, autoclaved and stored at RT.
50 x TAE	242 g Tris base, 57.1 ml Glacial acetic acid, 100 ml 0.5 M EDTA. Make up to 1 L with ddH_2O.
1 x Transfer buffer	1 g SDS, 14.4 g Glycin, 3 g Tris was dissolved in 800 ml ddH_20 and 200 ml MeOH
5 x Tris-glycine running buffer	15.1 g Tris base, 94 g Glycine, 5 ml 20 % SDS. Dissolved and made up to 1 L of ddH_2O.
Alkaline Phosphatase (AP) - buffer	15 ml 1 M Tris-HCl pH 9.5, 3 ml 5 M NaCl, 7.5 ml 1 M $MgCl_2$, 1.5 ml 10 % Tween 20. Dissolved in 150 ml ddH_2O.

Bleaching solution	42.5 ml ddH_2O, 2.5 ml Formamide, 2.5 µl 20 x SSC, 5 ml H_2O_2 (30 % solution). Made fresh before use and kept on ice.
Blocking buffer (immunohistochemistry)	10 % Goat serum (Sigma), 0.3 % Triton-X 100 (Sigma), 0.1 % Sodium azide (Sigma) in 1 x PBS, filter sterilized and stored at 4°C.
Blocking buffer (Western Blot)	5 % non-fat milk, 0.1 % Triton-X 100 in 1 x PBS.
Blocking solution (whole mount *in situ*)	MAB containing 2 % blocking agent (Roche) was heated in the microwave until fully dissolved with milky appearance. Once solution had cooled on ice, 20 % heat treated lamb serum (Invitrogen) was added.
2 % cysteine	4 g cysteine (Sigma) in 200 ml ddH_2O water and pH adjusted to 7.8 with NaOH.
Hybridisation buffer	500 ml Formamide, 250 ml 20 x SSC, 10 ml 100 x Denhardt's solution, 10 ml 10 % Tween-20, 1 g CHAPS (Sigma), 2 ml 0.5 M EDTA, 1 g Torula RNA (Sigma), 100 mg Heparin sodium salt (Sigma). Made up to 1 L with ddH_2O and stored at -20°C in a glass bottle.
Luria-Bertani (LB) agar	40 g LB-agar powder (Fisher Scientific) in 1 L ddH_2O, autoclaved.
LB-Broth	25 g LB Broth powder (Fisher Scientific) in 1 L ddH_2O, autoclaved.
Lysis buffer	200 µl 1 M TrisHCl pH 8.0, 300 µl 5 M NaCl, 40 µl 500 mM EDTA, 100 µl NP40. Made up 10 ml with ddH_2O.
MAB	Dissolve 11.61 g Maleic acid, 8.77 g NaCl, 7.8 g NaOH in 1 L ddH_2O and adjust to pH 7.5 with 10 N NaOH.
10 x MBS	102.6 g NaCl, 1.5 g KCl, 4 g $NaHCO_3$, 47.6 g Hepes, 4 g $MgSO_4$ $7H_2O$, 1.56 g $Ca(NO_3)_2$ $4H_2O$, 1.18 g $CaCl_2$ $2H_2O$ in 2 L of ddH_2O and pH adjusted to 7.5. Autoclaved and stored at 4°C.
10 x MEMFA	For 50 ml: 5 ml 10 x MEM solution, 5 ml 37 % formaldehyde, 40 ml ddH_2O
10 x MOPS	83.7 g MOPS, 13.6 g Sodium Acetate, 3.7 g EDTA, dissolved in 1 L of nuclease free distilled water, pH adjusted to 7 with NaOH.

10 x MOPS-EDTA buffer	41.86 g MOPS, 4.102 g Sodium Acetate anhydrous, 20 ml 0.5 M EDTA (pH 8). Fill to 800 ml with DEPC.H_2O and pH to 7.0 using 10 M NaOH. Finally fill to 1000 ml with DEPC.H_2O and add 200 µl DEPC. Solution was mixed, left to stand over night prior to autoclaving. The buffer was then stored at 4 °C in the dark.
RNA Gel Loading Buffer	750 µl deionised Formamide, 250 µl 37 % Formaldehyde, 150 µ1 10 x MOPS-EDTA buffer, 360 µl 6 x DNA loading dye, 6 µl Ethidium Bromide (10 mg/ml).
Steinberg's Solution	For 1 L: 3.4 g NaCl, 0.05 g KCl, 0.08 g $Ca(NO_3)_2$•$4H_2O$, 0.205 g MgSO4•$7H_2O$, 0.56 g Tris. Add ddH_2O up to 1 L and adjust pH to 7.4 with HCl.
TN-buffer	150 mM NaCl, 5 mM KCl, 0.5 % NP-40 detergent, 10 mM Tris-HCl (pH 7.8)
TNEB-buffer	150 mM NaCl, 5 mM $MgCl_2$, 0.5 % NP-40 detergent, 10 mM Tris-HCl (pH 7.8)
Wash buffer (Western Blot)	1 L PBS with 0.1 % Triton-X 100.
X-gal (stock solution)	Dissolve 0.1 g of X-gal (Melford, UK) in 5 ml dimethylformamide-DMF (Sigma, UK), store in the dark, -20°C
X-gal staining solution	0.15 g $K_3Fe(CN)_6$, 0.2 g $K_4Fe(CN)_6$·$3H_2O$, 0.024 g $MgCl_2$.$6H_2O$, 0.01 g Sodium deoxycholate, 20 µl NP40 (IGEPAL), 100 ml 1 x PBS, store protected from light at RT.

2.2 Methods

2.2.1 *Xenopus laevis* embryo handling and techniques

2.2.1.1 *Xenopus* housing

Xenopus laevis pigmented frogs were purchased from Nasco (US) and housed in the UCL Institute of Ophthalmology biological resource unit according to UK Home office regulations.

Xenopus frogs were housed in opaque tanks at a density of 10 females or males per 27 L (same sex tanks), supplied with tank enrichments including 20 cm long dark coloured plastic tubes. Frog water was filtered and re-circulated through the

Tecniplast (Italy) system. Housing conditions were carefully controlled and closely monitored. The temperature was kept between 17°C and 19°C degrees, and frog water at pH 8. *Xenopus* frogs were kept on a 12/12-hour light-dark cycle and fed twice a week with *Xenopus* Diet Pellets (Scientific Animal Food and Engineering, France). Each frog was identified by the unique pigmentation pattern on its back, which was photographed and made into an identity card. All injections were carefully recorded on the identity card and in log-books for regulated procedures, as required by the Home Office.

2.2.1.2 Induction of superovulation in *Xenopus* females

The minimum age of female *X.laevis* used for superovulation is two years old. By law, females can be used no more than four times a year, which equates to a resting period of at least 12 weeks between induced superovulation.

The veterinary grade hormones used to induce egg laying were PMSG-Intervet® (PMSG = Pregnant Mare Serum Gonadotropin) and CHORULON® (HCG = Human Gonadotropin hormone; both purchased from Intervet, UK). The 5000 IU vial of PMSG powder was reconstituted in the 25 ml of provided solvent (final concentration 200 IU/ml), subsequently aliquotted and stored at -20°C. Aliquots were only thawed once and unused hormone was discarded. Female *X.laevis* were injected with 50 IU of PMSG (i.e. 250 µl of reconstituted hormone) into the dorsal lymph sac, at least three days (usually a week) prior to HCG injections.

HCG hormone was freshly prepared on the day, just before use. Chorulon vials containing 1500 IU powder were resuspended in 1.5 ml of the solvent provided (remaining solvent was discarded if not used within 24 hours) giving a final concentration of 1000 IU/ml. Around 12-14 hours before the planned egg-laying, females were injected with between 200 - 400 µl (200-400 IU) subcutaneously into the lymph sac, depending on size of the animal. After the HCG injection, the female frogs were transferred to tanks containing egg filters overnight.

2.2.1.3 Testis isolation

Sexually mature *X.laevis* males were injected subcutaneously with the anaesthetic MS-222 (also known as ethyl 3-aminobenzoate methanesulfonate or Tricaine

methanesulfonate; Sigma, UK). After ensuring the animal had lost consciousness (i.e. with toe-pinch and testing for gag-reflex), the abdomen was opened, the testis isolated and then the heart and major blood vessels were cut. After removing all excess blood, testis were kept in a vial with 1 x Modified Barth's Solution (MBS; see Table 2.1) at 4°C and used within 7 days.

2.2.1.4 Egg collection and *in vitro* fertilisation

The next morning, females were transferred to the lab and kept at 18 °C room temperature in individual tanks with 1 x Egg-Laying-Solution (ELS; see Table 2.1). With its high salt content, the ELS preserves oocyte quality until fertilisation.

Eggs were collected and washed three times in 1 x MBS. A small piece of testis was cut and crushed in a 1.5 ml microfuge tube containing 1 x MBS. As much liquid as possible was removed from the dish containing the eggs, before applying the crushed testis solution. The egg and testis solution were gently mixed with a plastic stirrer and left covered for five minutes before being flushed with 0.1 x MBS. Lowering the salt concentration from 1 x MBS to 0.1 x MBS induces sperm motility and aids fertilisation. After circa 20 minutes, successfully fertilised eggs undergo cortical rotation, which is visible by the dark animal poles facing upwards. Female frogs were returned to the animal house and kept overnight in egg-filter containing tanks, before being returned to their holding tanks the following day.

2.2.1.5 De-jellying of embryos

Approximately one hour post-fertilisation (or when the first signs of cell division became visible), eggs were de-jellied with 2% cysteine solution (see Table 2.1). For this, 0.1 x MBS was removed from the embryos and replaced with cysteine solution. The dish was then gently swirled until embryos started to detach from the dish and floated freely. The cysteine solution was left for no longer than four minutes, before embryos were thoroughly and repeatedly washed in 0.1 x MBS (ca. seven to 10 washes).

2.2.1.6 Rearing and staging embryos

Xenopus embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1994). *Xenopus* embryos were maintained in 0.1 x MBS solution, which was

regularly replaced with fresh solution, while the animals grew to the desired stage. Before developmental stage 13, embryos were kept below 16°C to minimise spontaneous gastrulation defects. After stage 13, the embryos were kept at temperatures between 14°C and 20°C, according to the requirement of the experiment - the higher the incubation temperature, the faster the embryos' development progressed.

2.2.1.7 Microinjection of *Xenopus* embryos

Microinjection is a powerful technique to reliably deliver an accurate amount of substance, such as messenger RNA, DNA or morpholinos, into early stage *Xenopus* embryos. Microinjection was carried out using a PLI-100 Pico-Injector (Harvard Apparatus, US). Microinjection needles were pulled from borosilicate capillary glass (Harvard Apparatus) using a P-97 Flaming/Brown micropipette puller (Sutter Instrument, US). The injection dishes were prepared using 49-well silicone moulds, which were placed on molten 1% agarose solution in small petri dishes and left to set at room temperature (RT). 4% Ficoll solution was prepared (see Table 2.1).

On the injection day, the workstation and tools were wiped down with RNase Zap™ (RNase removing agent, Sigma) and the needle calibrated using sharp forceps to give an injection volume of 10 nl. Embryos were de-jellied at the first sign of cleavage and then transferred into the injection dishes containing 4% Ficoll solution. Injection solution (e.g. mRNA, morpholinos) was prepared and kept on ice. The needle was then filled and embryos injected at the desired site and stage. Injected embryos were kept in the injection moulds with 4% Ficoll solution overnight and then transferred into petri dishes containing 0.1 x MBS the following day.

2.2.1.8 Animal Cap assay

Following fertilisation, embryos were kept at 14 °C over night in 0.1 x MBS until they reached stage 8 to 9 (usually the morning following fertilization). The embryos were then transferred to 1% agarose (in water) injection dishes, filled with Steinberg's Solution (see Table 2.1). Covering the dissecting dishes with 1 % agarose is necessary to protect the dissected and rather sticky animal caps from attaching to the plastic dish. Using sharp forceps, the vitelline membrane of the embryos was carefully removed, without damaging the animal hemisphere and the

embryo carefully pushed back into shape. Using two very sharp forceps the animal cap was cut out, taking extra care not to excise any marginal zone cells (white fluffy appearance), and placed with the inner side facing up. After 30 minutes to one hour the animal caps formed spheres and were then cultured at 20 °C alongside control whole embryos to the desired stage for further analysis.

2.2.1.9 Fixing *Xenopus* embryos

Once *Xenopus* embryos reached the required stage, they were anaesthetised in a 0.1 x MBS solution containing 0.2 mg/ml MS-222. After being anaesthetised, embryos were transferred to 4 ml borosilicate glass vials (Fisher Scientific, UK) and fixed in either 4% paraformaldehyde (PFA; Table 2.1) or MEMFA (Table 2.1) solution for one hour at RT on a rocking platform or at 4°C overnight. Injected MEMFA-fixed embryos were either processed for β-Galactosidase (β-gal) staining or else washed in 1x phosphate buffered saline (PBS; Table 2.1) three times and then stored in methanol at -20°C, until further use.

2.2.1.10 β-gal staining

MEMFA fixed embryos were washed three times with 1x PBS and then incubated in 1x PBS with 2 mM $MgCl_2$ for 15 minutes at RT. Then PBS was replaced with 500 µl X-Gal mixer with 25 µl of 20 mg/ml X-gal solution (X-gal purchased from Melford, for both solutions' composition see Table 2.1) in each vial, and all samples were incubated at 37°C, protected from light, until staining was satisfactory. Embryos were then washed in PBS three times and stored in methanol at -20°C.

2.2.1.11 DiI Staining of neural retina and optic nerve

DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; Sigma) is a lipophilic non-toxic fluorescent dye, which inserts into cell membranes. It can be used in both live or aldehyde fixed tissue to label neuronal processes and somata. Fixed tissue labels through dye diffusion along the cell membranes while staining in live cells seems to be mainly localised to intracellular vesicles. In this experiment, DiI was used to stain potential nerve projections from the ectopic eye structures towards the brain or spinal cord. The precise administration of the dye is very important to limit its spread. Post-injection incubation conditions and time frames are also important as too little incubation may result in incomplete labelling, while too

lengthy incubation tends to increase non-specific labelling.

Tadpoles were anaesthetized in 0.2 mg/ml MS-222 and then fixed in 4% PFA for one to two hours at RT or at 4°C overnight. Glutaraldehyde fixed tissue is not suitable for analysis in whole-mounts, because it produces intense background fluorescence. 4% PFA fixed embryos were then immobilized in a dish with 1 x PBS on plasticine with micro-pins. Using sharp forceps, the skin overlying the eye was carefully removed and a small cut made into the sclera. The lens and vitreous were removed without damaging the rest of the eye and retina. Solution was removed from the dish, so that the eye was buffer-free but still moist. Using a micro-pin probe, DiI crystals were pushed into the eyecup. The labeled tadpoles were then put back into fixative and cultured in the dark at RT, until optimal fluorescent labeling of the tissue was achieved. Depending on age and size of the tadpole, incubation time ranged between three days and two weeks.

2.2.1.12 Luciferase assay in *Xenopus* embryos

The reporter constructs of ARE-luc (Activin-Responsive Element; (Chen et al., 1997)), TOPFLASH (the TCF/LEF Optimal Promoter monitoring the WNT activity; Upstate) and BRE-luc (BMP responsive element; (Tozer et al., 2013)) were used. pRL-CMV (Promega, Madison, USA) was used as a normalization control, and luciferase assays were performed by a dual-luciferase assay system (Promega). Embryos were harvested in triplicates at stage 10.5 by freezing on dry ice. Luciferase assays were performed using Dual-Luciferase® Reporter Assay System (Promega) according to the manufacturer's protocol. The luminescence levels were measured using a FLUOstar OPTIMA plate reader (BMG LABTECH, Ortenberg, Germany). The Relative Luciferase Unit (RLU) was calculated as the ratio of firefly luciferase against Renilla luciferase.

2.2.2 *Xenopus* tissue processing and sectioning

2.2.2.1 Embedding and cryo-sectioning of *Xenopus* embryos

Embryos were fixed in 4% PFA for one to two hours at RT (or at 4°C overnight). Embryos were then placed in 30% sucrose (in 1 x PBS) in embedding moulds (Sigma) for one to two hours on a rocker at room temperature or until embryos were saturated and sank to the bottom. All sucrose solution was then removed from the

mould and the embryos covered in liquid Tissue-Tek® O.C.T. compound (Sakura Finetek, USA) and properly orientated using forceps. The moulds containing embryos in O.C.T. compound were then placed onto a pre-chilled metal plate, sitting on dry ice. Once the O.C.T. compound was completely set, the blocks were stored in a tissue paper padded box at -80 degrees. Cryosections were cut at 10 microns thickness using a cryostat-microtome (Leica, Wetzlar, Germany) and collected on Superfrost™ Plus microscope slides (Fisher Scientific). Before sectioning, embryo containing O.C.T. blocks were stored inside cryostat chamber at -20 °C for 10 minutes. Cut sections were left at RT until O.C.T. compound had melted and dried. All slides were then stored at -80 °C until further processing.

2.2.2.2 Embedding and paraffin sectioning of *Xenopus* embryos

Embryos were fixed in 4% PFA as described in previous section 2.1.8. The embryos were then placed in labelled cassettes. Using a tissue processor in the Institute of Ophthalmology Pathology department, the cassettes were then taken through a series of graded ethanol washes to dehydrate the tissue and were finally bathed in Xylene and hot paraffin wax. After tissue processing, the embryos were embedded in paraffin wax and once hardened sectioned on a manual rotary microtome (Leica RM 2235). Tissue sections were cut at 5-7 micron thickness and collected on Superfrost™ Plus slides (Fisher Scientific) and stored at RT. For de-waxing, slides were washed three times with xylene for five minutes each, and then rehydrated by passing through decreasing gradients of ethanol: 100%, 95%, 75%, 40% for five minutes each, followed by double distilled water (ddH_2O).

2.2.2.3 Hematoxylin and Eosin (H&E) staining of paraffin sections

H&E staining was performed in an automated system (Leica ASP300S) in the Pathology department. Every wash lasted around 10 seconds. Briefly, paraffin sections were de-waxed in xylene and passed through two washes of absolute alcohol, two washes in 90% alcohol, one wash in distilled water and were then stained in Harris hematoxylin for five washes. Then, sections were washed in running tap water, differentiated in 1% acid alcohol for 10 seconds and washed once again in tap water. Afterwards, they were passed through two washes of 90% alcohol, counterstained with eosin for three washes, dehydrated in 95% alcohol for

two washed and finally cleared with xylene, before being mounted using DPX mounting medium (Fisher Scientific).

2.2.3 DNA Techniques

2.2.3.1 Transformation of plasmids into bacterial cells

The *E.coli* bacterial strain used in this study was XL1-Blue competent cells (Stratagene, US). Prior to transformation, 100 µl XL1-Blue aliquots were thawed on ice. Then 50 ng of DNA was added to each aliquot, mixed gently and incubated on ice for 30 minutes. Then the tubes were placed in a 42°C water bath for exactly 45 seconds, followed by immediate incubation on ice for 2 minutes. The brief heat-shock treatment facilitates the entry of the DNA plasmids into the bacterial cells. 900 µl pre-warmed S.O.C. medium (Invitrogen, US) was added to each tube and the bacterial samples were further incubated at 37°C for one hour with shaking at 225-250 rpm for the antibiotic resistant gene to be expressed. 100 µl of each transformation mixture was plated onto a pre-warmed LB agar plate (see Table 2.1), containing 50 µg/ml ampicillin, using aseptic techniques. The plates were inverted and incubated at 37°C overnight for the bacterial colonies to form.

2.2.3.2 Preparation of liquid bacterial cultures for midi prep

Individual bacterial colonies were picked up from the LB plates using sterile toothpicks and placed into snap-cap (BD-Falcon) tubes, containing 3 ml of LB broth (see Table 2.1) with 100 µg/ml ampicillin (or if required other selection antibiotics). The cultures were incubated for eight hours at 37°C shaking at 225 rpm. 250 µl of the bacterial culture was then added to 100 ml of LB broth (+ ampicillin) in an autoclaved beaker and incubated overnight at 37°C shaking at 225 rpm.

2.2.3.3 Midi prep – harvesting DNA plasmids from bacterial cultures

For the plasmid harvest, the Qiagen Plasmid Midi Prep kit (Qiagen, Germany) was used and all procedures carried out according to manufacturer's protocol. For the final elution step, DNA plasmids were eluted in 50-100 µl ddH_2O instead of the buffer supplied by Qiagen, to avoid adverse cross reactions in subsequent analysis.

2.2.3.4 Restriction enzyme digest

Enzymes were chosen according to known restriction sites on the plasmids used and were purchased from New England Biolabs UK (NEB, UK). Reaction buffers were supplied alongside the enzymes. Usually 10 µg of the circular plasmid was digested with 3 µl of the enzyme in a total volume of 100 µl. Samples were incubated at 37°C for three hours. Once successful digestion was confirmed by running a small amount of sample on a 1% argarose gel, the rest of the sample was cleaned using the GeneJET PCR purification kit (Fermentas, US) and the clean linearized plasmid re-suspended in 30 µl RNase free water.

2.2.3.5 Agarose gel electrophoresis

To analyse quality, size and linearization, samples were run at 100 Volts on an agarose gel. The gel percentage was chosen according to sample molecule sizes. To make the gel, agarose powder was added to an appropriate amount of 1x Tris-acetate-EDTA (TAE, see Table 2.1) buffer and the mixture heated in the microwave to boil. Once all the agarose was dissolved, SYBR® Safe DNA Gel stain (Invitrogen) was added at a ratio of 1 in 10,000 and the liquid poured into a gel cast and left to set. The set gel was then transferred into a running tank and submerged in TAE buffer. DNA samples and 1 Kb Plus DNA ladder (Invitrogen) was mixed with 6x loading dye and loaded into gel wells. The tank was closed and connected to a power source and left to run at 80 – 100V. Gels were visualised using an ultraviolet trans-illuminator (Gene Flash, Syngene Bio imaging).

2.2.4 RNA techniques

2.2.4.1 *In vitro* synthesis of capped mRNA

The mMessage mMachine kit (Ambion, UK) was used for the transcription reaction (either SP6, T3 or T7 RNA polymerase depending on the plasmid and promoter used, see Table 2.1). For the transcription reaction 1 µl to 1.5 µl of DNA/linearized plasmid, 2 µl of 10 x transcription buffer, 10 µl 2 x ribonucleotide mix, 2 µl 10 x enzyme mix was made up to a total volume of 20 µl with ddH_2O. Samples were then incubated for three hours at 37°C. Finally 1 µl of Turbo DNase (included in the kit) was added to each sample and incubated for a further 15 minutes at 37°C. To stop the reaction 115 µl nuclease free water and 15 µl ammonium acetate were added.

Table 2.2: Plasmids used for mRNA production

pCS2+ β-galactosidase	pCS2+ Epiphycan
pCS2+ Asporin	pCS2+ Chondroadherin
pCS2+ Asporin-myc	pCS2+ Chordin
pCS2+ DN-IGF1R	pCS2+ Wnt8
pCS2+ Lumican	pCS2+ IGF2
pCS2+ Lumican-myc	pCS2+ Xnr1
pCS2+ Decorin	pCS2+ BMP4
pCS2+ Decorin-myc	

2.2.4.2 Extraction and cleaning of capped mRNA

Following the mRNA *in vitro* synthesis, equal volumes of phenol (150 µl) were added to each mRNA sample, mixed well and centrifuged at maximum speed of 13,300 rpm (Heraeus Fresco 17 centrifuge, Thermo Fisher) for two minutes at 4°C. The aqueous top layer was then transferred into a separate microfuge tube. The phenol extraction step was repeated once more. In the fumehood, an equal amount of chloroform was added to the collected aqueous solution, mixed by inverting and tapping the microfuge tube and centrifuged for two minutes at full speed (13,300 rpm). Again as much of the aqueous layer as possible was collected in a fresh microfuge tube, while not touching the walls of the tubes to avoid contamination. An equal volume of ice-cold iso-propanol (stored at -20 °C) was added to the collected solution, gently mixed by tapping the microfuge tube and stored at -20 °C over night.

The next day, the samples were centrifuged for 30 minutes at 4°C at 13,300 rpm. The supernatant was carefully discarded without disturbing the mRNA pellet and then re-suspended in 200 µl of ice-cold 75% ethanol. The sample was centrifuged for a further 20 minutes at 13,300 rpm and the supernatant again very carefully removed. The pellet is not sticky at this point and can easily be lost. The microfuge tube containing the pellet was air dried in the fume hood for ca. 5-10 minutes on ice, before re-suspending in 10 µl of RNase free water (Qiagen) and determining the concentration using a NanoDrop™ 2000 (Thermo Scientific). Samples were diluted in RNase free water to a concentration of 1 µg/µl and then store at -80°C.

2.2.4.3 Preparation of RNA-gel

To assess the quality of RNA, samples were run at 80 - 100 V on a RNA gel. The following steps were carried out in a laminar fume hood due to the use of formaldehyde. For a 30 ml solution, 0.3 g of agarose was added to 21.6 ml of ddH_2O water and boiled in the microwave. In the fume hood, 3 ml of 10 x MOPS (see Table 2.1) and 5.4 ml of 12.3 M formaldehyde were added to the agarose mix. 2 µl of RNA constructs, including RNA ladder, were mixed with 8 µl of RNA Gel loading buffer (see Table 2.1) and the samples incubated at 65-70 °C for 10 minutes, placed on ice for two minutes and then loaded into the gel pockets. The gel was run in a 1 x MOPS solution for 10 minutes and then checked in a UV transilluminator for distinct bands representing the RNA samples.

2.2.4.4 Synthesis of DIG-labelled riboprobes

The plasmids used for riboprobe synthesis are shown in Table 2.3. Pax6- and Rx1-plasmids were kindly provided by Dr. Michael Zuber. 1 µg of linearised template plasmid was added to a reaction mixture containing 2 µl transcription buffer (Roche), 2 µl RNA/DIG labelling mix (Roche; 10 mM ATP, 10 mM CTP, 10 mM GTP, 6.5 mM UTP, 3.5 mM DIG-11-UTP), 2 µl of the appropriate enzyme (Roche; e.g. SP6) and nuclease-free water to a total volume of 20 µl. The solution was incubated at 37°C for two hours. 2 µl of DNase1 (equals 2 units) was then added to remove template DNA and incubated at 37°C for a further 15 minutes. To stop the reaction 0.8 µl of 0.5 M EDTA was added and the probe precipitated with 1.25 µl of 8 M LiCl and 75 µl of 100% EtOH. Contents were mixed well and left overnight at -20 °C.

The following day, the mixture was centrifuged at 13,000 rpm for 30 minutes at 4 °C. The supernatant was carefully removed and the pellet re-suspended in 200 µl of 75% ice-cold ethanol. The mixture was centrifuged for another 20 minutes and again the supernatant was removed with out disturbing the pellet. After air-drying for 5-10 minutes, the pellet was re-suspended in 10 µl of RNase free water and then added to 5 ml of Hybridisation buffer (see Table 2.1) to be stored at -20 °C. The probes could be reused for at least a year, without significant reduction in signal strength.

Table 2.3: Plasmids used for riboprobe synthesis

Plasmid name	Restriction enzyme	RNA polymerase
Krox20	EcoRI	T3
Otx2	NotI	T7
En2	XbaI	T3
ET	NotI	T7
Rx1	BamHI	T7
Pax6	XbaI	T7
Asporin	NotI	SP6
Six3	EcoRI	T7
Six6	EcoRI	T7
FoxG1	EcoRI	T7

2.2.4.5 Whole mount *in situ* hybridization of *Xenopus* embryos

Xenopus embryos which had been fixed and stored in methanol at -20°C were first allowed to warm to room temperature, and then rehydrated by washing for 10 minutes each in 75%, 50% and 25% methanol, followed by three washes of 5 minutes each in PBST (0.1% Tween-20 in 1x PBS). The embryos were then treated with ice-cold proteinase K solution (Roche, use at 2 µg/ml in PBST) for two minutes, followed by one wash in 0.1 M triethanolamine for five minutes and one wash in 0.1 M triethanolamine with 0.5% acetic anhydride for 10 minutes. Subsequently, two five-minute washes in PBST were performed, followed by a 20-minute fixation in 4% formaldehyde in PBST and six five-minute washes in PBST again. Then the embryos were washed once in hybridization buffer that was pre-warmed to 60°C and incubated in fresh hybridization buffer for two hours at 60°C. This pre-hybridisation step, helps to decrease non-specific binding. For hybridization, embryos were incubated with DIG-labeled riboprobes at 60°C overnight.

On the second day, the embryos were removed from the riboprobe solutions and washed in 2 x saline sodium citrate (SSC) four times, 20 minutes each time at 60°C, followed by two 20-minute washes in 0.2 x SSC at 60 °C and two 5-minute washes in maleic acid buffer (MAB) at RT (Table 2.1). In the meanwhile, blocking solution (Table 2.1) was prepared and the mixture was allowed to cool on ice before use. Blocking was performed for one hour at RT and blocking solution was replaced with anti-dig alkaline phosphatase (AP) Fab fragments antibody (Roche), diluted 1 in 5000 in fresh blocking solution. The embryos were incubated with antibody at 4° C

overnight on a rocking platform.

On the third day, embryos were washed once in alkaline phosphatase (AP) buffer (see Table 2.1) first and then twice for 5 minutes each time in AP buffer containing 2 mM levamisole (Sigma), which helps inhibit endogenous alkaline phosphatase activity and hence lowers the background signal. The colour reaction was developed using the chromogenic AP substrate BM Purple (Roche) with 2 mM levamisole protected from light. When the colouration was complete, embryos were washed in 100% methanol for 30 minutes to stop the reaction and fixed in MEMFA overnight at RT.

2.2.4.6 Depigmentation of *Xenopus* embryos

After two five-minute washes in 1 x PBS, embryos were incubated in bleaching solution (see Table 2.1) on a light box in a laminar flow hood. When bleaching was complete, embryos were again washed in 1 x PBS and stored at RT in MEMFA.

2.2.4.7 RNA extraction and reverse transcription

RNA used for PCR (polymerase chain reaction) was extracted using the RNeasy® RNA extraction kit (Qiagen). The complementary DNAs (cDNAs) were synthesised with the reverse transcription enzyme SupersScript™ II and Random Hexamers (both Life Technologies), which are random sequences of short oligodeoxyribonucleotides. 11 µl of the extracted RNA and 1 µl of random hexamers was incubated for 10 minutes at 70°C and then immediately put on ice. Then, 4 µl of 5 x First Strand Buffer (250 mM Tris-HCL, pH 8.3 at RT, 375 mM KCL, 15 mM $MgCl_2$), 2 µl 0.1 M DTT, 1 µl 10 mM dNTP and 1 µl of Superscript II enzyme (Thermo Fisher) was added and incubated for one hour at 42°C. After the incubation, 20 µl of ddH_2O was added and samples stored at -20°C.

2.2.4.8 Semi-quantitative RT-PCR

For semi-quantitative reverse transcription (RT) PCR, Platinum® Pfx DNA polymerase (Life Technologies) was used. The primer sequences were taken from previous reports (Mizuseki et al., 1998; Shimizu et al., 2013) and the De Robertis group laboratory web page and can be seen in Table 2.4 (website address: http://www.hhmi.ucla.edu/derobertis/protocol_page/Pdfs/Frog%20protocols/Primers

%20for%20RT-PCR.pdf). For the PCR reaction 0.5 µl of Platinum Pfx enzyme, 5 µl 10x Pfx Amp Buffer, 1.5 µl dNTP (10mM), 0.5 µl of each forward and reverse primer (100 µM), 1 µl cDNA and 40.5 µl ddH_2O was mixed per sample. As a standard protocol the following cycle parameters were used in the PCR machine: 94°C for 5 minutes, and then 35 cycles of 30 seconds at 94°C, 30 seconds at 55°C, 30 seconds at 72°C, followed by 5 minutes at 72°C.

2.2.4.9 Quantitative RT-PCR

Quantitative RT-PCR (qRT-PCR) was performed using the 7900 HT Fast Real-Time PCR machine (Applied Biosystems) with the SYBR Green detection system (Applied Biosystems). Each gene expression level was normalised to that of ODC (ornithine decarboxylase). In detail, 10 µl of 2 x Q-PCR enzyme mix SYBR green (ABI), 9 µl of ddH_2O, 1 µl cDNA and 0.2 µl of primers (100 µM each) was mixed for each sample. A relative quantification of the target genes was achieved by comparing it to a reference gene transcript, such as ODC (or Histone 4). For this the Ct data was first linearised, normalised to ODC. The means of each set of technical duplicates were calculated and then the means and standard deviations of each set of biological triplicates determined. The data analysis followed the Pfaffl method (Pfaffl, 2001), whereby the relative expression ratio is calculated from the real-time PCR efficiencies and the crossing point deviation of an unknown sample versus a control. The primers used for qRT-PCR are shown in Table 2.4.

Table 2.4: Primers used in semi-quantitative PCR and qRT-PCR

Gene	Semi-quantitative PCR primers		Reference	qRT-PCR primers	
	forward primer	reverse primer		forward primer	reverse primer
ASPN	5'-CCTGGAGCATTTGATGGACT-3'	5'-TTTGTTGTGGTCAAGGTGGA-3'	this study	5'-TTTTCTGGCTTTGTGCTACG-3'	5'-CATCATTGTCATCGTCGTCA-3'
c-Actin	5'-GCTGACAGAATGCAGAAG-3'	5'-TTGCTTGGAGGAGTGTGT-3'	De Robertis's lab webpage		
En2	5'-ATGAGCAGAATAACAGGGAAGTGGA-3'	5'-CCTCGGGGACATTGACTCGGTGGTG-3'	De Robertis's lab webpage	5'-ACATCTCCCTACTGGCTGCT-3'	5'-GTTTCACTTCAGCCAAGCAA-3'
Histone 4	5'-CGGGATAACATTCAGGGTA-3'	5'-TCCATGGCGGTAACTGTC-3'	De Robertis's lab webpage		
Krox20	5'-CCGGCCCATCCTCAGACCCAGAAA-3'	5'-CGCCACGCCGCTGTTGCCGAGTTC-3'	De Robertis's lab webpage	5'-GATTCAGATGAGCGGAGTGA-3'	5'-CAAGGGGTAGTTGGACGAGT-3'
Mix.2				5'-AGTCACCAAGATCCCCAGAG-3'	5'-GCTGAGATGCTGAAACCAAA-3'
NCAM	5'-GCGGGTACCTTCTAATAGTCAC-3'	5'-GGCTTGGCTGTGGTTCTGAAGG-3'	De Robertis's lab webpage		
ODC	5'-CAGCTAGCTGTGGTGTGG-3'	5'-CAACATGGAAACTCACACC-3'	De Robertis's lab webpage	5'-ACAAAGAAACCCAAACCAGA-3'	5'-CAAACAACATCCAGTCTCCAA-3'
Otx2	5'-GGATGGATTTGTTACATCCGTC-3'	5'-CACTCTCCGAGCTCACTTCCC-3'	Zuber et al., 2003	5'-TTTCACCAGAGCTCAACTGG-3'	5'-ACCAAACCTGGACTCTGGAC-3'
Pax6	5'-GCAACCTGGCGAGCGATAAGC-3'	5'-CCTGCCGTCTCTGGTTCCGTAGTT-3'	Zuber et al., 2003	5'-GTGTTTGCCAGGGAAAGATT-3'	5'-CTCCCTTCTCCACTTTGCTC-3'
Rx1	5'-CCCCAACAGGAGCATTTAGAAGAC-3'	5'-AGGGCACTCATGGCAGAAGGTT-3'	Zuber et al., 2003		
Rx2a				5'-CCAAGAAATGCCAAAGAGGT-3'	5'-GGATCTCCATAGCCACCAGT-3'
Slug	5'-TCCCGCACTGAAAATGCCACGATC-3'	5'-CCGTCCTAAAGATGAAGGGTATCCTG-3'	Mizuseki et al., 1998		
Sox2	5'-GAGGATGGACACTTATGCCCAC-3'	5'-GGACATGCTGTAGGTAGGCGA-3'	De Robertis's lab webpage		
XAG1	5'-CTGACTGTCCGATCAGAC-3'	5'-GAGTTGCTTCTCTGGCAT-3'	De Robertis's lab webpage		
XBF1/FoxG1	5'-TCAACAGCCTAATGCCTGAAGC-3'	5'-GCCGTCCACTTTCTTATCGTCG-3'	Shimizu et al., 2013		
Xnr3				5'-AATCCACTTGTGCAGTTCCA-3'	5'-GGACACAGGAGGGACACTCT-3'

2.2.5 Protein techniques

2.2.5.1 Immunohistochemistry on sectioned frog tissue

Cryo-sections were removed from - 80 °C, defrosted and then re-fixed with 4% PFA for 10-15 minutes, before commencing the immunohistochemistry protocol. The antibodies used in this study are shown in Table 2.5.

After three 10-minute washes in 1 x PBS, sections were blocked in blocking buffer (see Table 2.1) for 30 minutes at RT. In the meanwhile, primary antibodies were diluted in blocking buffer to the appropriate concentrations (Table 2.5). Sections were incubated with the primary antibody in a humidity chamber at 4°C overnight.

On the second day, sections were washed three times in 1 x PBS for 15-minutes each time and then incubated with secondary antibody diluted in blocking buffer (Table 2.5) at RT for 60 minutes, protected from light. Three 15 minute washes in 1 x PBS were performed to remove the unbound antibodies and sections were mounted using ProLong® Gold anti-fade mountant with DAPI (Life Technologies). Slides were stored at 4°C in darkness until images were captured using a fluorescent microscope (Carl Zeiss Axioskop 2 Plus) with a QICAM 12-bit Color Fast 1394 (Qimaging) and the Openlab software. Images were processed using Adobe Photoshop CS4 (Adobe Systems, US).

Table 2.5: Antibodies used for cytochemistry

Antibody name	Dilution used	Company
Calbindin D-28K	1 : 100	Sigma; C-2724
β-Crystallin	1 : 1000	Abcam; ab90379
Glutamine Synthetase	1 : 500	Millipore; MAB302
Hu-C/Hu-D	1 : 400	Life Technologies; A21271
Alexa Fluor 488 anti-mouse	1 : 400	Abcam; A-11001
Alexa Fluor 488 anti-rabbit	1 : 400	Abcam; A-11008

2.2.5.2 Western Blot

2.2.5.2.1 Protein extraction from *Xenopus* embryos

Lysis buffer (Table 2.1) was prepared, protease inhibitor cocktail (Roche) was added according to manufacturer's protocol and the solution kept at 4°C. *Xenopus* embryos

or animal caps were collected in centrifuge tubes and any medium carefully removed. 5 µl of lysis buffer per whole embryo or 3 µl per animal cap, was added and the animal tissues lysed on ice by pipetting up and down. The homogenised samples were centrifuged at 13,000 rpm for five minutes at 4°C. The clear supernatant was collected and either used immediately or stored at -80°C.

2.2.5.2.2 Whole cell extracts of HEK293

Media was removed and cells washed once in 1 x PBS. 900 µl of 1 x PBS was added to each 30 mm dish of cells and, using a scraper, cells were detached and then collected in a centrifuge tube and briefly centrifuged for 1-2 minutes at a very low speed (500-1000 rpm). Excess PBS was removed and cells then briefly snap frozen in liquid nitrogen and defrosted on ice. Next, 500 µl of RIPA (50mM Tris-HCl, pH 8.0, 150mM NaCl, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS, Sigma, UK), supplemented with protease inhibitor cocktail (Roche) and phosphatase inhibitors (Roche), was added to the samples. Cells were lysed, by pipetting the solution up and down. The lysed cell solution was then centrifuged at a high speed of 13,300 rpm for five minutes and the extract collected for further use in SDS-PAGE gel analysis.

2.2.5.2.3 SDS-PAGE

The amount of protein in each sample was determined using a BCA assay kit (Thermo scientific). 2 x Laemmli sample buffer (62.5 mM Tris, pH 6.8, 20% glycerol, 2% SDS, 0.01% bromophenol blue, Biorad, UK), supplemented with 1% 2-mercaptoethanol, was added in a 1:1 buffer to sample ratio, and then heated to 95 ºC for five minutes in a heat block for the purpose of protein denaturation. For the SDS-PAGE analysis, 4-15 % Mini PROTEAN® TGX™ gels were purchased (Biorad). 30 µg of each protein sample was run out on the gel, alongside the Precision Plus Protein™ All Blue protein standard (Bio-Rad, UK). Electrophoresis was performed with the Mini-Protean III system (Bio-Rad), at 70 - 100V, in 1 x running buffer (Table 2.1).

2.2.5.2.4 Western blot transfer, immunoblotting and development

Proteins were transferred onto PVDF nitrocellulose membranes (GE healthcare), which had been activated in methanol for 1 minute. The Bio-Rad wet transfer

chamber system was used, which was performed at 60 V for two hours.

Protein integrity was checked using Ponceau stain (Sigma, UK). Membranes were then blocked in blocking buffer (see Table 2.1) for 30 minutes at RT, and incubated overnight with the primary antibody (diluted in blocking buffer) according to the manufacturer's instructions (Table 2.6). The following day, membranes were washed in PBS containing 0.1% Triton-X, for three 10-minute washes, and incubated with appropriately diluted secondary HRP-conjugated antibody (Table 2.6) blocking buffer, for one hour at RT.

Membranes were washed thrice in PBS containing 0.1% Triton-X, for 10 minutes per wash. ECL reagents (GE Healthcare) were applied according to manufacturer's protocol for 5 minutes and membranes then wrapped in cling film and exposed to autoradiography film (Kodak, UK). When required, membranes were stripped in stripping buffer (Bio-Rad, UK) for 15 minutes, and were subsequently washed thrice in PBS with 0.1% Triton-X, for 10 minutes per wash, before staining with a different antibody. This was particularly useful to detect levels of the protein of interest in relation to the loading control. The antibodies used in the western blots are summarised in the Table 2.6, below.

Table 2.6: Antibodies used for Western blotting

Antibody name	Dilution used	Company
ERK	1 : 1000	Cell Signaling Technology; 9102
phosphorylated ERK	1 : 2000	Cell Signaling Technology; 9101
AKT	1 : 1000	Cell Signaling Technology; 4691
phosphorylated AKT	1 : 2000	Cell Signaling Technology; 4060
myc	1 : 1000	Cell Signaling Technology; 2276
IGF1R	1 : 1000	Cell Signaling Technology; 9750
FLAG	1 : 1000	Sigma; F3165
HA	1 : 1000	Sigma; 6908
Anti-mouse IgG, HRP-linked	1 : 5000	GE Healthcare; NA9310
Anti-rabbit IgG, HRP-linked	1 : 1000	GE Healthcare; NA9340

2.2.6 Tissue culture techniques

2.2.6.1 Cell culture

Human embryonic kidney cell line HEK 293 (ATCC number CRL-1753) was maintained in Dulbecco's Modified Eagle's medium (DMEM + Gluta Max, Gibco) supplemented with 10% fetal bovine serum (Gibco) and 100 U/ml penicillin/streptomycin (Life Technologies). Cells were cultured in tissue culture dishes of 60 mm diameter. Cell passaging was carried out in sterile conditions using a hood (Microflow II Biological cabinet). Cell medium was removed and cells gently washed in 1 x PBS (PAA, UK) prior to adding 1.5 ml of 0.25% Trypsin (PAA, UK) in PBS. Cells were briefly incubated at 37°C in the incubator to promote the cells detaching. After around two to three minutes, 5 ml of fresh pre-warmed media was added to the cells and everything was transferred into 10 ml falcon tubes and briefly centrifuged. The supernatant was discarded without disturbing the cell pellet. Cells were re-suspended in fresh medium and plated out into fresh cell culture dishes. HEK 293 cells were cultured in a 37°C incubator with 5% CO_2 supply (Sanyo, MCO-18AIC, Japan).

2.2.6.2 Transfection and conditioned media

For transfection, cells at 70-80% confluency were used, usually in 30mm diameter culture dishes. 2.5 µg of the expression vector, 5 µl of Lipofectamine 2000 (Thermo Fisher) and 100 µl of Opti-MEM (Life Technologies) was incubated for 10 minutes at RT, before being added to the cell dish.

For the preparation of conditioned media, HEK293 cells were transfected with the chosen expression vectors and were incubated for three days in Opti-MEM (Life Technologies). Cells that had been separately cultured in serum-free conditions, were then treated with the conditioned media.

Cell extracts were prepared using TN-buffer (150 mM NaCl, 5 mM KCl, 0.5% NP-40 detergent, 10 mM Tris-HCl [pH 7.8]), supplemented with protease inhibitor cocktail (Roche) according to the manufacturer's protocol. For the analysis of phosphorylated proteins, 5 mM NaF and 1 mM Na_3VO_4 were added to inhibit dephosphorylation.

2.2.6.3 Immunoprecipitation

Immunoprecipitation (IP) was performed using Protein G Sepharose 4 Fast Flow (GE Healthcare). Cells were transfected with vectors separately and the cell populations then mixed on the following day. This was done to avoid non-specific intracellular binding of the secreted proteins analysed. Cell extracts of previously transfected cells with the appropriate expression vectors (each containing either a -myc, -HA or –FLAG tag) were prepared using TNEB-buffer (150 mM NaCl, 5 mM $MgCl_2$, 0.5% NP-40 detergent, 10 mM Tris-HCl [pH 7.8]), supplemented with protease inhibitor cocktail (Roche) (according to the manufacturer's protocol), 5 mM NaF and 1 mM Na_3VO_4. 25 µl of each sample was retained at this point for later western blot analysis. The remaining cell extracts were used for IP: 20 µl of TNEB cell extract was incubated with 20 µl of Protein G Sepharose beads and 2 µl of the chosen antibody for IP on a shaker, at 4°C over night. The next day, the IP samples were centrifuged at 13,000 rpm, the supernatant removed and beads washed in fresh TNEB buffer several times. IP samples were then prepared for direct use in SDS-PAGE gel analysis, along side the earlier retained control samples.

CHAPTER 3: ASPN INDUCES ECTOPIC EYES IN *XENOPUS LAEVIS* EMBRYOS

3.1 Introduction

Vision is certainly one of our most treasured senses and loss of sight is associated with a greatly reduced quality of life (Langelaan et al., 2007). So it is of no great surprise that the areas of eye induction, and growing eyes *in vitro*, are of great interest to the science and clinical community. So far, there have been great advances with growing differentiated eye and retinal tissues artificially (Eiraku et al., 2011, Gonzalez-Cordero et al., 2013, Nakano et al., 2012), but science is not yet capable of producing a full size human eye, ready for implantation into a human patient.

The "eye-maker" gene *Pax6* has long been known to be by it self sufficient to induce eye development. When the *Pax6* homolog *eyeless* was identified in *Drosophila* (Quiring et al., 1994), it was shown that the ectopic expression of *ey* in the imaginal discs of the fly resulted in the formation of ectopic eyes on wings, legs and antennae (Halder et al., 1995a, Nornes et al., 1998). Soon after, other groups showed that *Pax6* homologs from ribbonworm, squid and ascidians are also capable of inducing ectopic eye structures in *Drosophila* (Glardon et al., 1997, Kozmik et al., 2003, Loosli et al., 1996, Suga et al., 2010, Tomarev et al., 1997). This proved that a highly conserved group of genes must regulate eye development across both vertebrates and invertebrates. The tightly regulated signalling network, which regulates eye development in the fly, has since also been identified in vertebrates, where they are referred to as the 'eye field transcription factors' (as introduced in detail in chapter 1.4).

The frog model *Xenopus laevis* played a crucial role in this discovery. In response to overexpression of various EFTFs, frog can also display ectopic eye tissue (Zuber et al., 2003). Various overexpression studies, dating back to the 1990s, have been carried out in *Xenopus*, which show that several EFTFs are by themselves sufficient to expand endogenous eyes or induce ectopic eye tissue (Andreazzoli et al., 1999, Bernier et al., 2000, Chow et al., 1999b, Mathers et al., 1997a, Zuber et al., 1999). In more recent years, signals other than the EFTFs have been identified, which can also induce these ectopic-eye phenotypes in *Xenopus*, such as Frizzled 3 (fz3) (Rasmussen et al., 2001), purine signalling molecule E-NTPDase2 (Masse et al., 2007) and changes in transmembrane voltage (achieved through EXP1 and GlyR

overexpression) (Pai et al., 2012). These signals may be involved in the upstream regulation of the EFTFs during early development.

The family of SLRPs have become a popular research topic, as they have been shown to be involved in a range of important biological events such as development, growth and cancer. While they were once thought so be solely involved in collagen fibril organisation (Iozzo, 1999, Kalamajski and Oldberg, 2009, Keene et al., 2000, Kresse et al., 1997, Neame et al., 2000, Reinboth et al., 2006), the SLRP family of proteins are now well known for their ability to modulate many important intracellular signalling pathways. Most members of the SLRP family are expressed during the development of neural tissue (Le Goff and Bishop, 2007, Ohta et al., 2006). During embryogenesis, SLRPs have been shown to play important roles in a number of developmental processes such as germ layer specification, patterning and morphogenesis (Dellett et al., 2012, Moreno et al., 2005b, Morris et al., 2007, Ohta et al., 2004, Zagris et al., 2011). The overall expression patterns of SLRP family members throughout early development, and aforementioned findings from recent studies, certainly suggests a possible role in neural development and maintenance. SLRP family members are highly expressed in ocular tissues and mutations in many SLRPs are associated with eye malformations (Dellett et al., 2012, Lin et al., 2010, Majava et al., 2007, Singla et al., 2011, Wang et al., 2011).

Asporin (ASPN) is a relatively novel member of the SLRP family. To reiterate - ASPN was first identified in mice (Henry et al., 2001, Lorenzo et al., 2001), where it is mainly expressed in cartilage and bone during development. Like many other SLRPs, ASPN binds collagen and inhibits TGF-β signalling (Ikegawa, 2008, Kou et al., 2010, Nakajima et al., 2007) and it was found to play an important role in cartilage homeostasis (Torres et al., 2007). An aspartic acid repeat polymorphism in the N-terminal of the ASPN protein has been linked to osteoarthritis in humans (Kizawa et al., 2005). More recently, ASPN has become the focus of cancer research, where - depending on the type of cancer – it can have both a pro-invasive or tumour supressing effect (Maris et al., 2015, Satoyoshi et al., 2015). ASPN's role in early embryogenesis is however, still elusive.

During a functional screening of SLRP family members in *Xenopus laevis*, overexpression of ASPN mRNA caused a striking eye phenotype in the injected embryos. The embryos had developed enlarged and often ectopic eye-like tissue. ASPN's ability to induce this ectopic eye phenotype, prompted me to analyse the function of ASPN in more detail. The findings from the overexpression experiments, suggested that ASPN might play a crucial role in eye induction and possibly neural induction – at least in frog.

As a starting point for this project, it was important to get a better understanding of the *Xenopus* ASPN protein (e.g. sequence, special features and structure), as well as homologies and variations compared to other species, including humans. For this I carried out an *in silico* analysis, using the BLAST and Clustal Omega tools, to analyse and compare sequences.

Secondly, ASPN expression levels throughout early development, as well as expression patterns, needed to be investigated. Knowing where and when ASPN is expressed during *Xenopus* embryo development might already indicate potential roles and mechanisms through which ASPN elicits its actions. I initially used semi-quantitative PCR and qRT-PCR to determine ASPN expression levels at different developmental stages, as well as in different parts of the embryo. To visualise the patterns of expression throughout embryogenesis, I designed whole-mount *in situ* hybridisation ribo-probes for ASPN.

After establishing that ASPN is indeed expressed in the eye field region and at the time of eye induction, confidence grew, that this is indeed a novel and important factor for eye development. I proceeded to perform a detailed analysis of the eye phenotype observed upon ASPN overexpression, which will be covered in the second part of this chapter. Morphology of the expanded and ectopic eye-like tissue was examined, as well as antibody staining for eye specific cell types. Also, an attempt was made to visualise potential optic nerves extending from these ectopic eyes towards the brain. Finally, the effect of ASPN overexpression on several eye-field transcription factors and other developmental markers was investigated.

3.2 Structure and expression of *Xenopus* Asporin

3.2.1 *In silico* analysis

During a systematic investigation of SLRP family functions, we found that one of the clones demonstrated a strong ectopic eye formation activity upon forced expression and I became interested in its detailed molecular function. This clone encoded a polypeptide sequence similar to human ASPN belonging to the class 1 SLRP and drawing a phylogenic tree using the Clustal Omega phylogeny tool (http://www.ebi.ac.uk/Tools/msa/clustalo/) showed that this clone contained a *Xenopus* orthologue of the *ASPN* gene (Lorenzo et al., 2001, Henry et al., 2001) (Figure 3.1).

In detail, using protein BLAST (http://blast.ncbi.nlm.nih.gov) and based on the human amino acid sequences of various SLRP members, *Xenopus laevis* homologous genes were previously identified. IMAGE (Integrated Molecular Analysis of Genomes and their Expression; http://www.imageconsortium.org) clones were purchased from Source Biosciences and the produced clone mRNA injected into *Xenopus* embryos. The IMAGE clone #6931202 encoding *Xenopus* Asporin (NCBI Gene ID 495030) showed a strong eye inducing activity. In this study a very similar gene, which we termed *asporin-b,* was identified (registered in GenBank accession no.: LC056842). Its existence is probably due to the aforementioned allotetraploidy of *Xenopus laevis*. However, in this study 'Asporin' will refer to *asporin-a,* unless otherwise stated.

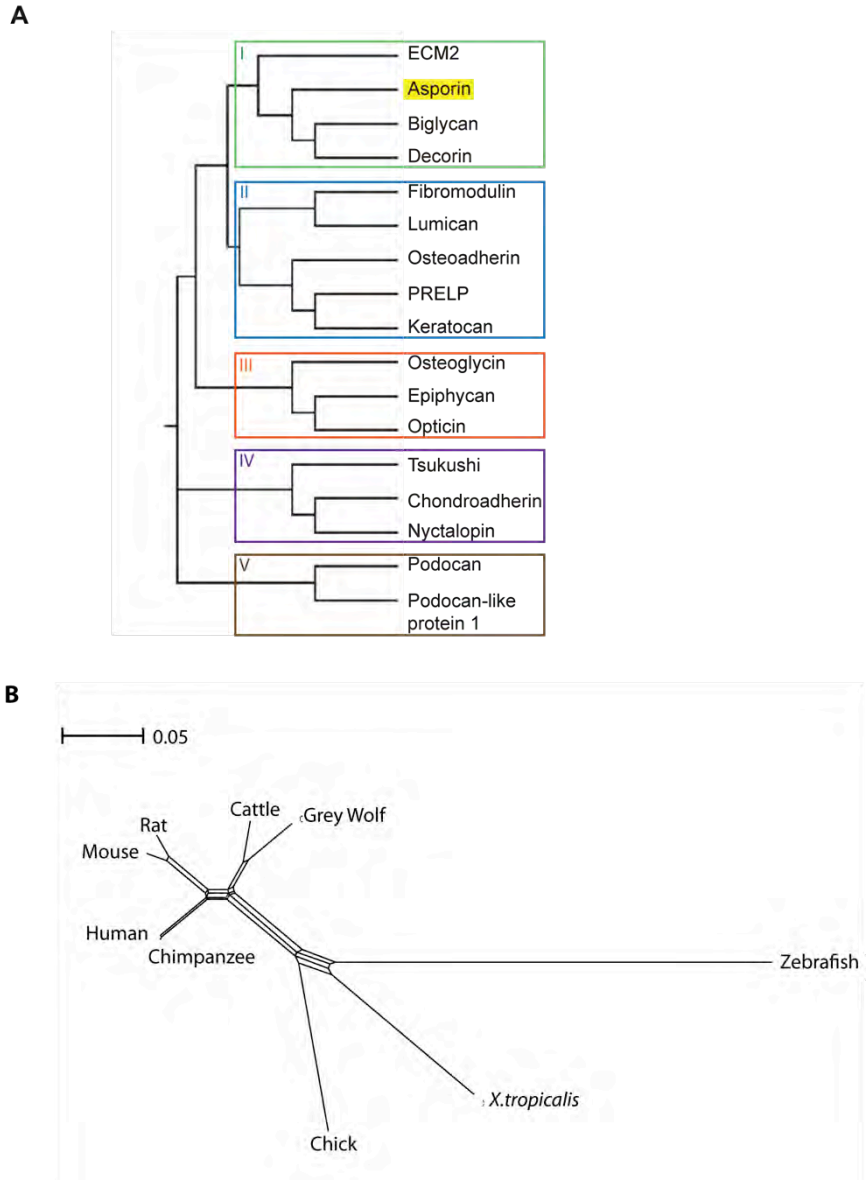


Figure 3.1: Phylogenic tree of SLRP family members and evolutionary relationship of ASPN orthologs in different vertebrate species. (A) The phylogenic tree shows the SLRP family members, which are divided into classes I-V. Asporin belongs to canonical class I, alongside Decorin and Biglycan (adapted from Dellet et al. 2012). (B) An evolutionary tree of ASPN orthologs from different vertebrate species, was created using the Clustal W2 Phylogeny tool (http://www.ebi.ac.uk/Tools/phylogeny/clustalw2_phylogeny/). The branch lengths are proportional to the amount of inferred evolutionary change. The length of the branch represents the amount of genetic change (line segment = genetic change of 0.05) and is represented in nucleotide substitutions per site (i.e. number of substitutions divided by the length of the sequence).

Sequence alignment using the Clustal Omega tool (http://www.ebi.ac.uk/Tools/msa/clustalo/) of ASPN in different species further revealed that *Xenopus* ASPN has some typically conserved characteristics. *Xenopus* ASPN has a signal peptide and a 13 amino acid stretch comprising aspartic acid and asparagine (Figure 3.2, B, red) in its amino-terminal region, which is how ASPN was named (Henry et al., 2001, Lorenzo et al., 2001). This stretch is followed by a cysteine cluster with the C-X_3-C-X-C-X_6-C pattern, which is conserved among the class I SLRPs ASPN, Biglycan and Decorin, where the second cysteine is replaced by arginine in *Xenopus* (Dellett et al., 2012). The characteristic stretch of eight leucine-rich repeats was also found to be conserved among vertebrate species (Figure 3.2, blue). mRNA similar to ASPN has been found in many vertebrate species such as zebrafish, which also contains stretches of aspartate residues. In mouse and fish poly-aspartate stretches are interrupted by other amino acids, but the domain is still conserved across species. This suggests that the aspartates are important to the function of ASPN (Lorenzo et al., 2001).

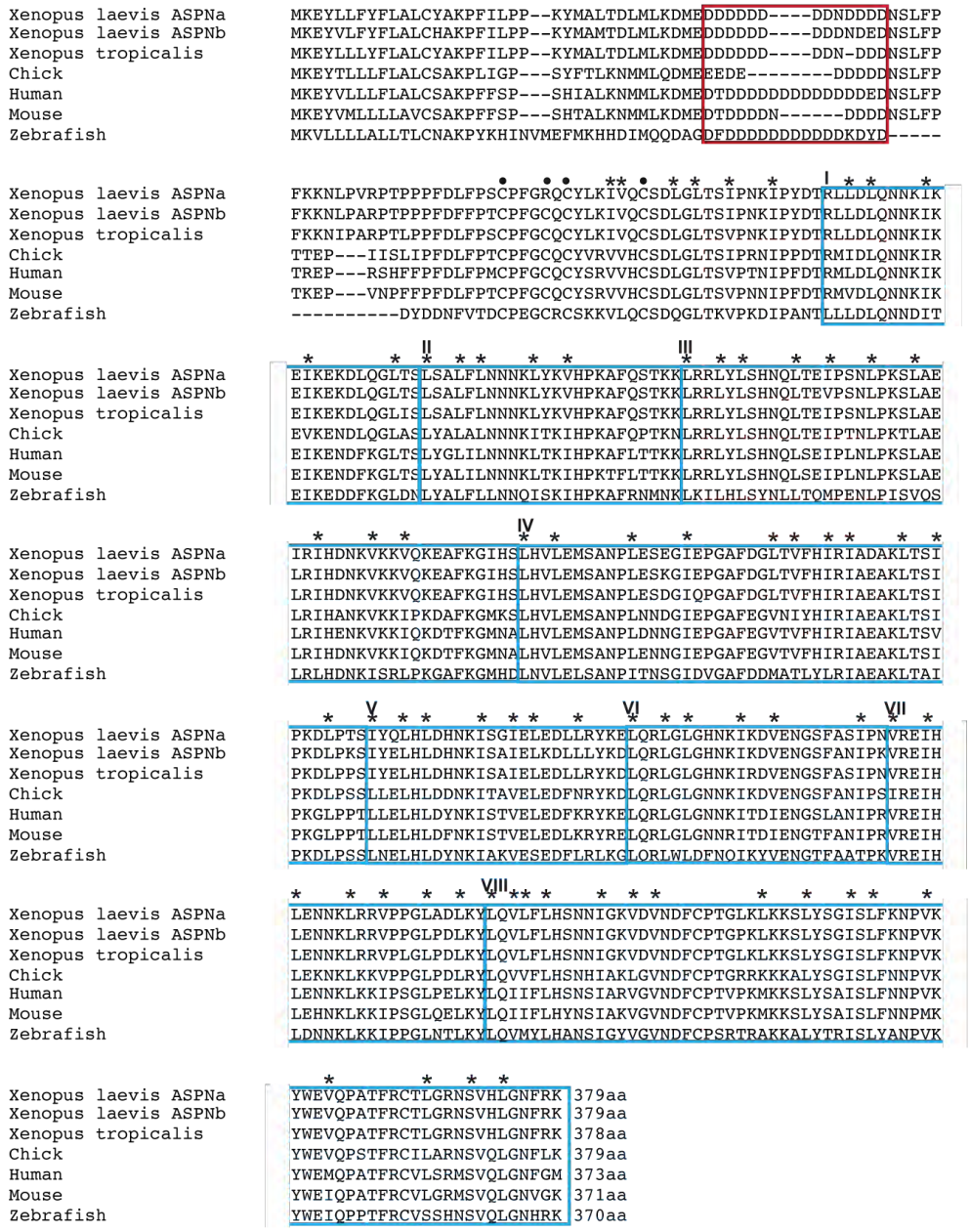


Figure 3.2: Alignment and comparison of ASPN amino-acid sequences in different species. The leucine-rich repeat domains (blue rectangles) and aspartic-acid rich repeat domain (red rectangle) seems to be conserved across vertebrate species, as evidenced in this sequence alignment of ASPN protein sequences of frog, chick, mouse, fish and human. Asterisks: isoleucine, leucine and valine, Black disc: cysteine (in the cysteine rich domain), I-VIII: leucine rich repeat, as predicted by a database search via LRR finder (http://www.lrrfinder.com).

3.2.2 Expression levels of ASPN during early development in *Xenopus*

To investigate the expression profile of ASPN during *Xenopus* laevis embryogenesis, I initially performed semi-quantitative PCR on whole embryos at different developmental stages (Figure 3.3, A). ASPN expression was already apparent in the unfertilized egg, but expression levels gradually increased during embryogenesis with higher levels being detected around stage 10, 13, 17 and then again at stage 28 (Figure 3.3, A).

In order to supplement this initial semi-quantitative PCR data, the expression of ASPN in different areas of the embryos was investigated. For this, explants of microinjected animal caps, dorsal marginal zone (DMZ) and ventral marginal zone (VMZ) were prepared and assayed for ASPN expression by means of qRT-PCR (Figure 3.3, B). To mimic the forebrain, embryos were injected with Chordin (Chd) mRNA (Sasai et al., 1995) into the animal blastomeres and animal cap explants cut (Figure 3.3, B (iii)). In addition, Chd and Wnt8 mRNA was co-injected and animal cap explants cut, which were to mimic more posterior neural domains (Figure 3.3, B (iv)) (Takai et al., 2010). Both Chd and Chd + Wnt8 injected animal caps showed enhanced ASPN expression compared to animal caps from un-injected control embryos (Figure 3.3, B (i)). ASPN expression in explants from DMZ (Figure 3.3, B (v)) and VMZ (Figure 3.3 (vi)), mimicking dorsal and ventral mesoderm respectively, showed low ASPN expression similar to that seen in the control animal cap. The DMZ and VMZ explants were cut at stage 10.5 and then cultured until stage 18 before they were assayed. The character of the explants was confirmed by long culture (see Figure 3.4) and Otx2 (Chow et al., 1999a) and Krox20 expression (Nieto et al., 1991) (Figure 3.3, C, D), where Otx2 expression was elevated in both IGF2 (Figure 3.3, C (ii)) and Chd (Figure 3.3, C (iii)) injected animal cap, as well as in the DMZ (Figure 3.3, C (v)). Krox20 was up regulated in animal caps injected with Chd and Wnt8 (Figure 3.3, D (iv)), including some expression in DMZ tissue (Figure 3.3, D (v)).

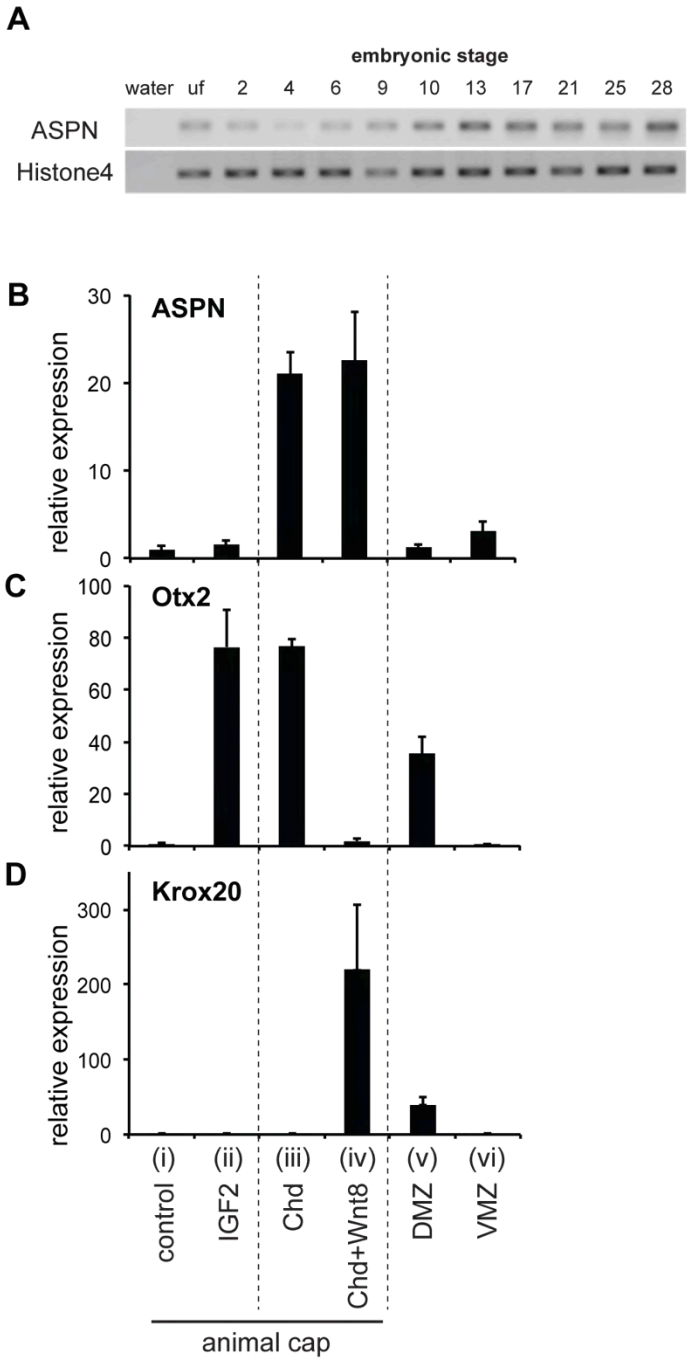


Figure 3.3: Expression of ASPN and other related genes in various explants. (A) Semi-quantitative RT-PCR of ASPN and Histone4 expression levels. Whole embryos were analysed by RT-PCR at various developmental stages. Low levels of ASPN expression can be detected already in the unfertilised egg. A marked increase in ASPN levels seems to occur at around stage 10, which continues through to later developmental stages. Expression levels of ASPN (B), Otx2 (C) and Krox20 (D) in various types of explants are shown, as assayed by qRT-PCR. Animal caps (control (i) or injected with mRNAs of 500 pg IGF2 (ii), 500 pg Chd (iii) or 500pg Chd + 100 pg Wnt8 (iv)) and dorsal marginal zone (DMZ; (v)) and ventral marginal zone (VMZ; (vi)) were prepared at stages 9 (animal caps) and 10.5 (DMZ, VMZ) and assayed at stage 18.

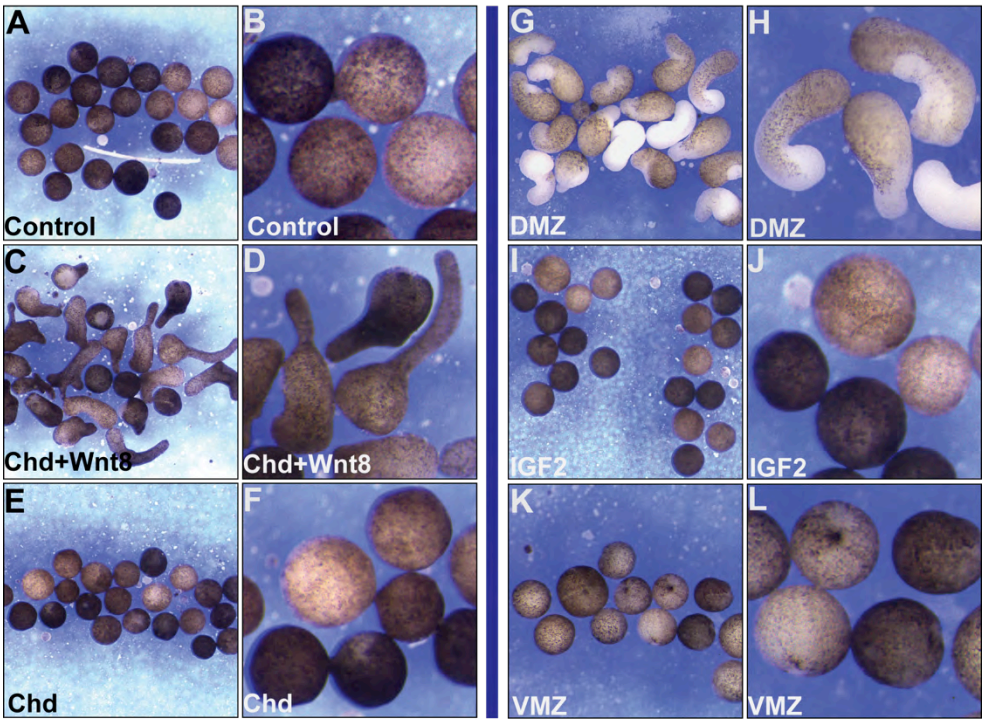


Figure 3.4: Cultured explants of injected animal caps, dorsal marginal zone (DMZ) and ventral marginal zone (VMZ). (A, B) Un-injected control animal cap explants formed the expected round ball shape of epidermal tissue, while Chordin (Chd) and Wnt8 injected animal caps (C, D) elongated in keeping with posterior neural domains. Chd injected (E, F) and IGF2 injected (I, J) animal explants show a spherical shape mimicking forebrain regions. DMZ (G, H) and VMZ (K, L) explants mimic dorsal and ventral mesoderm respectively.

3.2.3 Expression pattern of ASPN during early development in *Xenopus*

After establishing, that ASPN is indeed expressed during early *Xenopus* development, with increased levels in the forebrain region of the embryo, in the next step I wanted to visualise the expression patterns of ASPN in more detail.

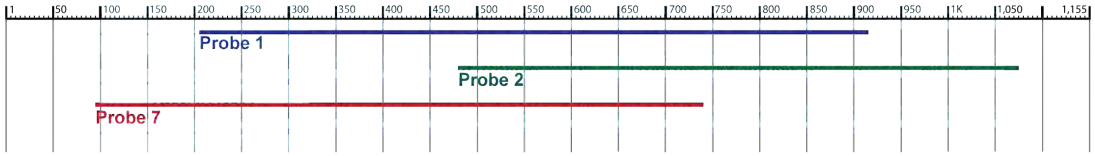
For this I designed *in situ* ribonucleotide probes, which I synthesised via the PCR method based on a technique published by David and Wedlich (2001). Primer pairs for *Xenopus* ASPN, which result in products larger than 600 base pairs (bp), were selected using Primer Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Care was taken to choose primer pairs that targeted different parts of the *ASPN* gene, to increase the chances for producing a working ribo probe. The targeted areas on the *ASPN* gene of the three chosen primer pairs 1, 2 and 7 are shown in Figure 3.5. The sequence for the T3 promoter was then added to the forward primers, and in the case

of reverse primer, the T7 promoter sequence was added (Figure 3.5). Using this approach the PCR product could be directly used for ribo probe synthesis, without the need to first subclone into a T3 and T7 promoter containing expression vector. As template for the PCR reaction, the ASPNa sequence was used. Lastly, an ASPN antisense probe was directly produced from the template ASPN plasmid (termed "ASPN 3") using the SP6 promoter.

A

	Forward Primer (5'->3')	Reverse Primer (5'->3')	Product Length
Asporin Probe 1	ACCTACGCCACCTCCATTTG	TAAATCTGCCAGCCCAGGTG	745
Asporin Probe 2	CGACGGTTGTACTTGTCCCA	TTGCTGGTTGAACCTCCCAG	631
Asporin Probe 7	ACATGGCACTGACTGACCTC	TTGTGGTCAAGGTGGAGCTG	679

B



C

	Forward Primer (5'->3')	Reverse Primer (5'->3')
Asporin Probe 1	AATTAACCCTCACTAAAGGGACCTACGCCACCTCCATTTG	TAATACGACTCACTATAGGGTAAATCTGCCAGCCCAGGTG
Asporin Probe 2	AATTAACCCTCACTAAAGGGCGACGGTTGTACTTGTCCCA	TAATACGACTCACTATAGGGTTGCTGGTTGAACCTCCCAG
Asporin Probe 7	AATTAACCCTCACTAAAGGGACATGGCACTGACTGACCTC	TAATACGACTCACTATAGGGTTGTGGTCAAGGTGGAGCTG

T3 Promoter primer sequence 5' AATTAACCCTCACTAAAGGG 3'
T7 Promoter primer sequence 5' TAATACGACTCACTATAGGG 3'

Figure 3.5: Method and design of whole-mount in situ probe for ASPN with PCR. (A) Primer pairs resulting in products larger than 600 bp were selected, using Primer Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Care was taken to choose primer pairs that targeted different parts of the ASPN gene, to increase the chances for producing a working riboprobe. The target areas on the ASPN gene of the three chosen primer pairs 1, 2 and 7 are shown in (B). To the forward primer the sequence for the T3 promoter was added and in the case of reverse primer, the T7 promoter sequence was added (C). As template for the PCR reaction, the ASPNa plasmid sequence was used, which is integrated in a pCS2+ vector. Lastly, a fourth ASPN probe was directly produced from the ASPN plasmid using SP6 promoter and polymerase.

Many different primer combinations and resulting probes were tested on the embryos, but only the three most promising shall be introduced in the following section. These three most efficient ribo probes will be referred to as 'ASPN 1', 'ASPN 2' (derived from primer pairs 1 and 2 respectively; Figure 3.5) and 'ASPN 3' (produced from ASPNa sequence containing plasmid).

The whole-mount *in situ* hybridization was carried out on embryos of stages between 13 and 42, to map the expression pattern throughout development. In the case of all three probes tested, embryos younger than stage 13 showed a uniform staining. It would be easy to assume that ASPN is ubiquitously expressed throughout the embryo at those very early developmental stages. But more likely when taking PCR results into account is that the *in situ* probes are not sensitive enough to detect ASPN expression at very low levels (Figure 3.6, A).

Overall probe ASPN 1 showed the best staining results (Figure 3.6, A-G), however only ubiquitous staining could be observed of embryos of stage 13 or younger (Figure 3.6, A). During neurula stages, *ASPN* was still rather ubiquitously expressed, however, there seems to be a little more abundance at the neural plate (Figure 3.6, B), including the presumptive eye field. This becomes more obvious when directly compared to the expression pattern of eye field transcription factor *Pax6*, as shown in Figure 3.6, C. The expression of *ASPN* is more evident around the presumptive eye field at the tailbud stage (Figure 3.6, D, E). At stage 35, strong expression can be observed in the whole head region (Fig. 3.6, F), while stage 42 sees expression only in selected head tissues (Figure 3.6, G). For the control sense probe no specific staining was found in embryos up to stage 28 (Figure 3.6, H). However, in older embryos such as shown in Figure 3.6, I at stage 42, some staining could be observed.

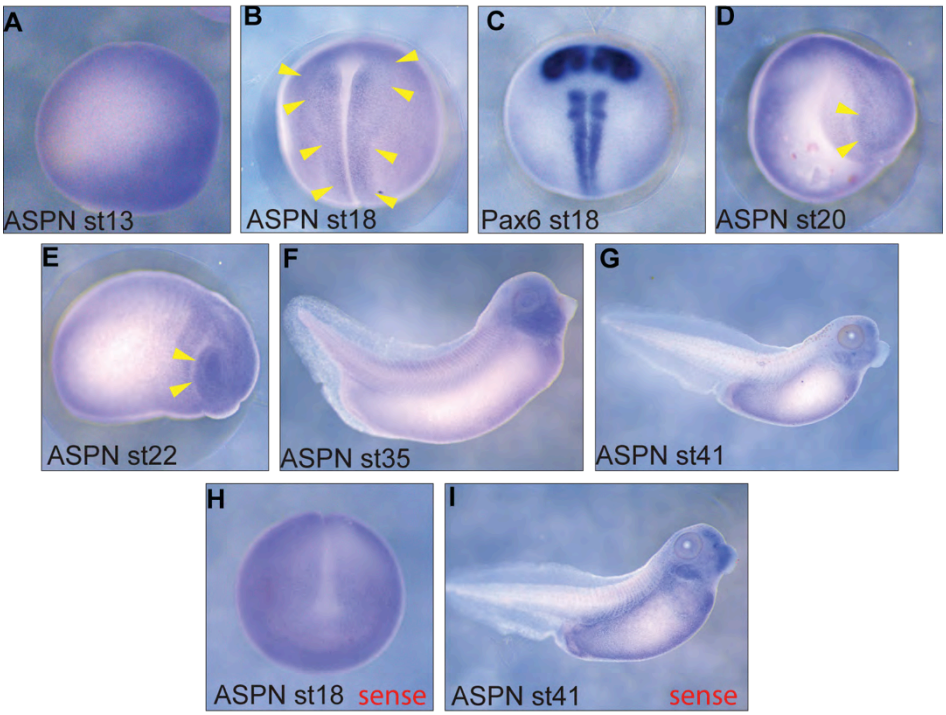


Figure 3.6: Whole-mount in situ hybridisation with probe ASPN 1. Antisense probe staining is shown in embryos from stages 13 – 41 (A, B, D-G) and sense probe control staining is shown (H, I). Specific staining can be observed from stage 18, where ASPN seems to be expressed around the neural plate and presumptive eye field (B). As a frame of reference, the Pax6 expression pattern at st18 is shown in (C) which shows some overlap with ASPN (B). From stage 22 (E) ASPN signal seems to be more condensed around presumptive eye field, while the whole head region is signal positive at higher stages (F, G).

Both probes ASPN 2 (Figure 3.7) and ASPN 3 (Figure 3.8) show very similar staining profiles compared to ASPN 1 probe (Figure 3.6). For both probes, again no staining patterns were observed in gastrula stage embryos. Late neurula stage embryos show a more focussed ASPN signal around neural plate and the presumptive eye field area (Figure 3.7, A, B; Fig. 3.8 A, B, C). At late tailbud stages, both ASPN 2 and ASPN 3 result in localised ASPN staining in the head region (Figure 3.7, C, D; Fig. 3.8, D, E). ASPN 2 sense probe gave some staining at late tailbud stages in the head region (Figure 3.7, F), which may just be a result on non-specific background staining.

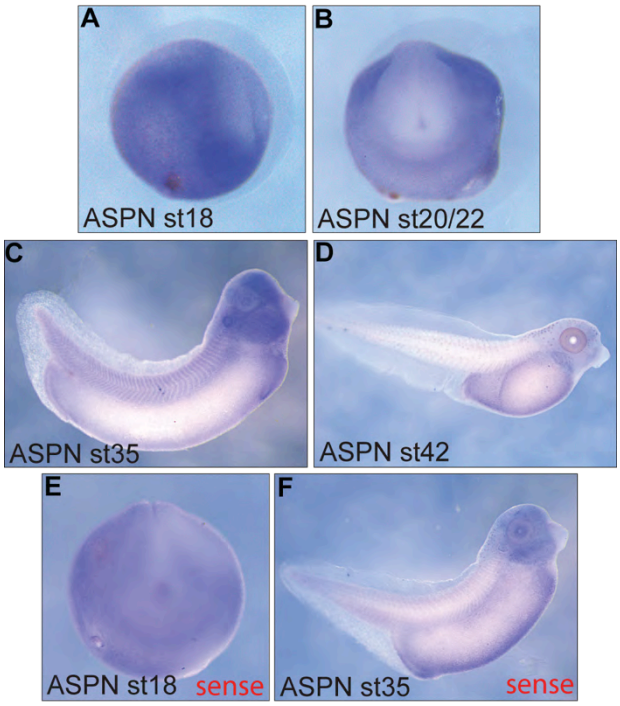


Figure 3.7: Whole-mount in situ hybridisation with probe ASPN 2. ASPN in situ staining is shown in embryos from stage 18-42. In embryos younger than stage 18, no specific areas of staining could be found. From stage 18 (A) to stage 20 (B), staining can be found in dorsal and anterior tissues. At later stages 35 (C) staining is mainly restricted to the head region and stage 42 (D) shows weak staining in certain head and proctodeum regions. Sense probe staining is shown in (E) and (F).

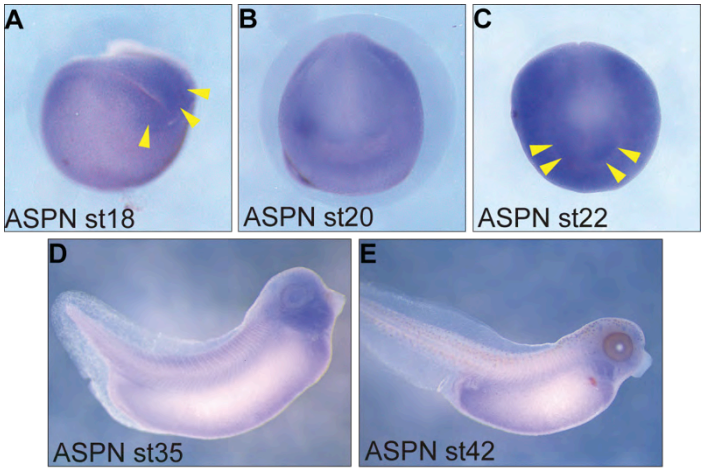


Figure 3.8: Whole-mount in situ hybridisation with probe ASPN 3. ASPN in situ hybridisation results are shown from stage 18-42 embryos. Again, embryos younger than stage 18 exhibited no specific areas of expression, but a more ubiquitous expression. Around stage 18 (A), 20 (B) and 22 (C) specific ASPN expression can be seen around the presumptive eye field and neural tube. At stage 35 (D), expression is found in the whole head region, while stage 42 exhibits staining in some head regions and the presumptive protodeum (E).

3.3 ASPN induces ectopic eyes in *Xenopus* embryos

3.3.1 ASPN overexpression phenotype

Gain-of-function experiments in *Xenopus* laevis embryos - achieved by injecting artificially high amounts of the protein's mRNA and subsequent phenotype analysis - can often give a good first indication of the protein's function during development. To find out more about ASPN's potential role in early frog development, varying concentrations of ASPN mRNA were injected into a dorsal or ventral animal blastomere at the four to eight cell stage. At the four to eight-cell stage, dorsal and ventral blastomeres in the animal pole of the embryo can be distinguished according to pigmentation (see Figure 3.9). Depending on where in the embryo ASPN mRNA was overexpressed i.e. in a dorsal or ventral, animal or vegetal blastomere, very different phenotypes could be observed. Dorsal animal injections of ASPN mRNA lead to striking eye phenotypes, while ventral animal overexpression resulted in extremely shortened and anteriorised embryos. These different ASPN induced phenotypes, shall be introduced and examined in the following section.

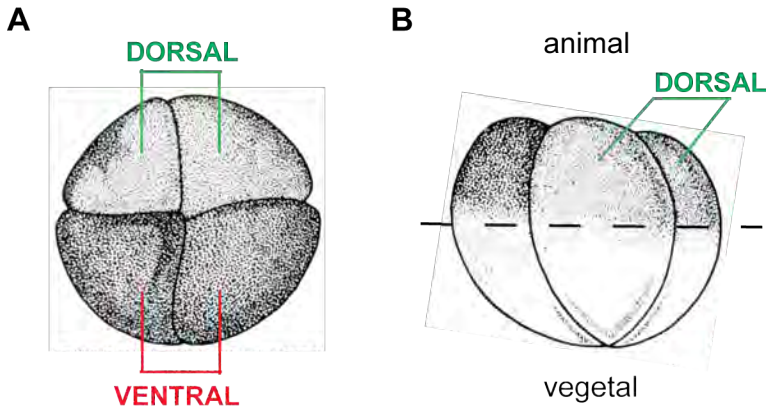


Figure 3.9: Schematic diagram of the four-cell stage Xenopus laevis embryo. (A) Shown is the animal pole of a four-cell frog embryo. The two dorsal blastomers show lighter pigmentation, while ventral blastomers are darker in colour. (B) The four-cell embryo is shown from a lateral view with the animal pole at the top and vegetal pole at the bottom. The lighter pigmented dorsal animal poles were the targets of ASPN mRNA injection for the purpose of ectopic eye induction. Pictures were adapted from Nieuwkoop and Faber (1994).

3.3.1.1 Dorsal Animal injections

For targeted ASPN overexpression in the dorsal animal blastomers of the embryo, increasing amounts of mRNA (0.5, 1 and 3ng) were microinjected at the four to eight cell stage of the embryo. The embryos were cultured until they reached developmental stage 42 and their phenotype then assessed. Control injections were carried out using β -gal mRNA. The resulting variety of mostly eye-related phenotypes, are represented in Figure 3.10.

There is a large variety in eye related phenotypes, following ASPN overexpression. The phenotypes can be broadly sub-divided into embryos displaying: a) ectopic eye-like tissue, which could include ectopic RPE, retina with lens; b) enlarged or expanded endogenous eyes and c) various defects of the endogenous eye, including missing eye structures, misshaped eyes, wrong eye location and very rarely ectopic heads.

The ectopic eye-like structures were of varying size from very small retina/RPE-like spots to ones of enormous size, which trumped the endogenous eye (Figure 3.10, C, C'). These ectopic structures were mostly observed in the midline of the head region, with some rarely occurring in posterior tail tissues (Figure 3.10, D, D') or the flank of the tadpole. Embryos displaying ectopic eye-like structures had an otherwise normal body, with no shortened or twisted body axes.

The enlarged eye phenotype was often uneven so that the concerned eye looked disproportionate and pulled out of shape (Figure 3.10, E, E'). In this category I count what others have referred to as proximal eye defects, i.e. a thin elongation of the retinal/RPE tissue towards the neural tube (Figure 3.10, F, F').

The most varied phenotype-subgroup, were the various eye defects that occurred upon forced overexpression of ASPN. In these embryos parts of the eye were missing (Figure 3.10, H, H'), thereby rendering the endogenous eye misshaped. As seen in Figure 10 (I, I') eyes also developed in the wrong place, such as more medially. The embryo in Figure 3.10, J, J' was a very rare find: it had an ectopic head structure including a pair of eyes and a cement gland, while the 'original' head

exhibited one large fused eye in the front of the head where the eye-field had failed to separate.

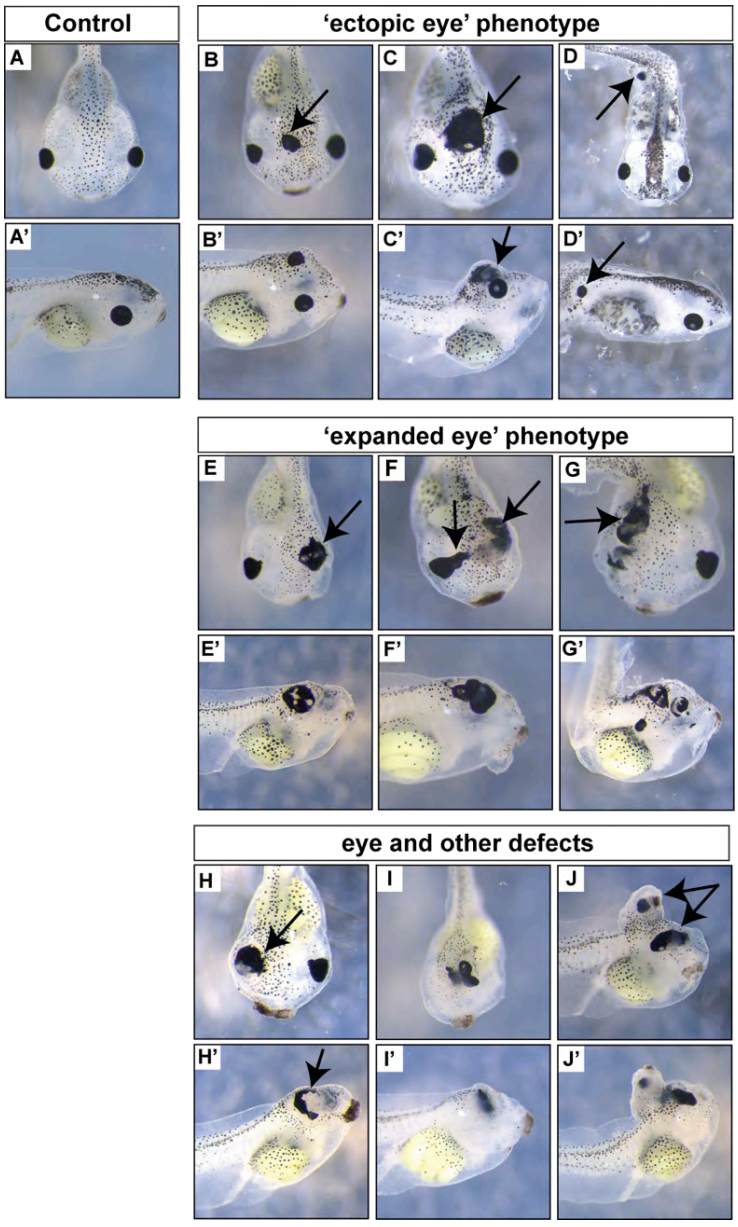


Figure 3.10: Phenotypes observed following ASPN mRNA injection into the developing X.laevis embryo. There is a great variety of different eye related phenotypes, which can be observed following an injection of 3ng of ASPN mRNA into one of the dorsal animal blastomeres at 4 or 8 cell stage, compared to control (A, A'). Ectopic eyes could be found in the head region (B, B', C, C') and sometimes also in tail and flank regions (D, D'). Expanded endogenous eye and ectopic retina was observed (E, E', F, F', G, G'). Additionally there were many eye related defects such as missing retina (H, H'), multiple eyes, which failed to divide (I, I') and very rarely ectopic heads with a set of eyes and cement gland (J, J').

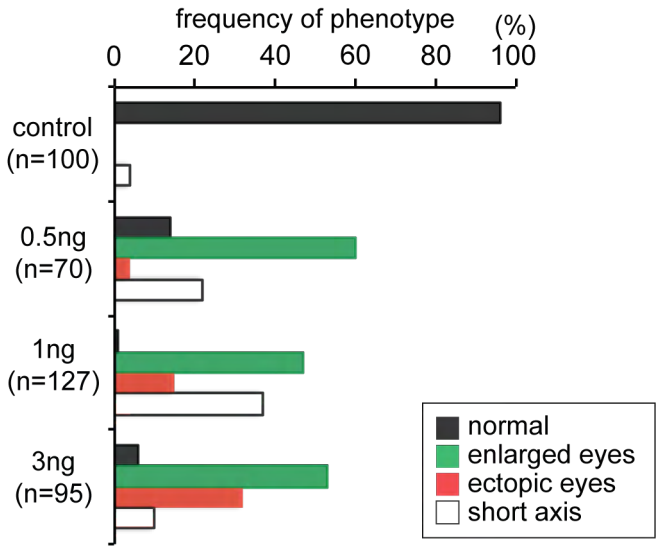


Figure 3.11: Quantification of the ASPN induced phenotypes. Following the injection of 0.5 ng, 1 ng or 3 ng of ASPN mRNA into a dorsal animal blastomere, phenotypes were quantified at developmental stage 42. The phenotypes were divided into four categories; embryos with normal eyes (black), with enlarged eyes (green), with ectopic eyes (red) and short axis (white). The number of ectopic eyes increased with increasing ASPN mRNA concentration injected.

Figure 3.11 shows the quantitative phenotype distribution following the ASPN mRNA injections with 0.5, 1 and 3ng. As expected, control injections with β-gal mRNA had no significant effect on *Xenopus* development (Figure 3.11). Incidences of tadpoles developing ectopic eye-like tissue increased with augmenting ASPN concentrations; from only 4% at 0.5ng ASPN to 15% at 1ng and finally 32% at the highest concentration tested of 3ng of ASPN mRNA. At the same time, the number of embryos with enlarged and expanded eyes was highest at the lowest ASPN concentration (60%), while slightly lower percentages of embryos were affected when 2 or 3ng mRNA was injected (47% and 53% respectively). The number of unaffected normal looking tadpoles decreased with increasing ASPN concentrations (14% for 0.5ng, 1% for 1ng and 6% for 3ng).

3.3.1.2 Ventral animal injections

Results of the ASPN overexpression experiment in dorsal animal blastomeres seem to suggest a role in eye development. The dorsal animal blastomeres have been shown to majorly contribute to eye and also lens tissue (Figure 1.5) and certain factors such as IGFs, purine-signalling molecule E-NTPDase2 and Frizzled3 have been shown to affect eye development when overexpressed in these tissues (Masse et al., 2007, Pera et al., 2001, Rasmussen et al., 2001, Richard-Parpaillon et al., 2002). IGFs also had a posteriorising effect on Xenopus embryos when injected in ventral animal blastomeres, which was determined to be due to the inhibition of Wnt signalling pathway. So in the next step, I wanted to explore if ASPN has a similar effect when overexpressed in the ventral blastomeres. In comparison to dorsal animal injection phenotype, when the ASPN mRNA was injected into one of the ventral animal blastomeres of the embryo, a very different phenotype could be observed. The embryos had an extremely shortened body (Figure 3.12, B, C, D) compared to the un-injected control (Figure 3.12, A). Posterior development seems to have been extremely inhibited, following ASPN injection, which might be due to Wnt-signal inhibition. Besides the dramatic shortening of the body, two-tails could often be observed, which might be a gastrulation defect due to general developmental toxicity caused by the high mRNA amount injected. Sometimes the tadpoles exhibited expanded eye-like tissue, reminiscent of the phenotype seen with dorsal animal injections (Figure 3.12, D).

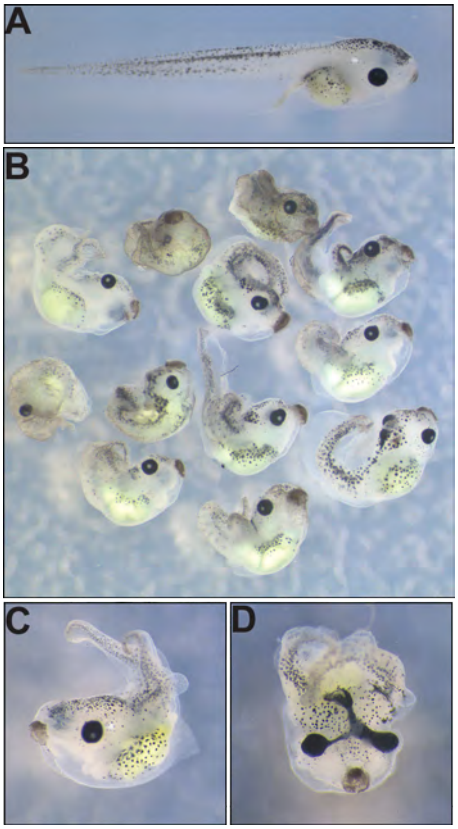


Figure 3.12: Phenotypes observed following ASPN overexpression in a dorsal vegetal blastomere. Compared to the un-injected control (A), embryos injected with ASPN mRNA in one of their dorsal vegetal blastomeres at the 4 or 8 cell stage exhibited a dramatically shortened body phenotype (B, C, D). This was sometimes accompanied by a double-tail and expanded eye-pigment (C, D).

3.3.2 Histological examination of ectopic tissue reveals eye-like structure

To further characterise the eye related phenotypes, tadpoles were embedded and sectioned in paraffin wax and then stained with Hematoxylin and Eosin (H & E staining) for histological analysis. Sectioned embryos displaying an expanded eye or ectopic eye phenotype can be seen in Figure 3.13, E and F, respectively. The extending endogenous retina and RPE seen in Figure 3.13, E, seems to merge with the neural tube, which itself is oddly shaped and shows signs of hyper-proliferation. It should be noted that no expansion of the cement gland was observed (Figure 3.13, A-C), which is the anterior-most structure in the embryo, suggesting that ASPN function is not entirely the same as IGF signalling (Pera et al., 2001), and thereby also differs from the effects of Cerberus (Bouwmeester et al., 1996).

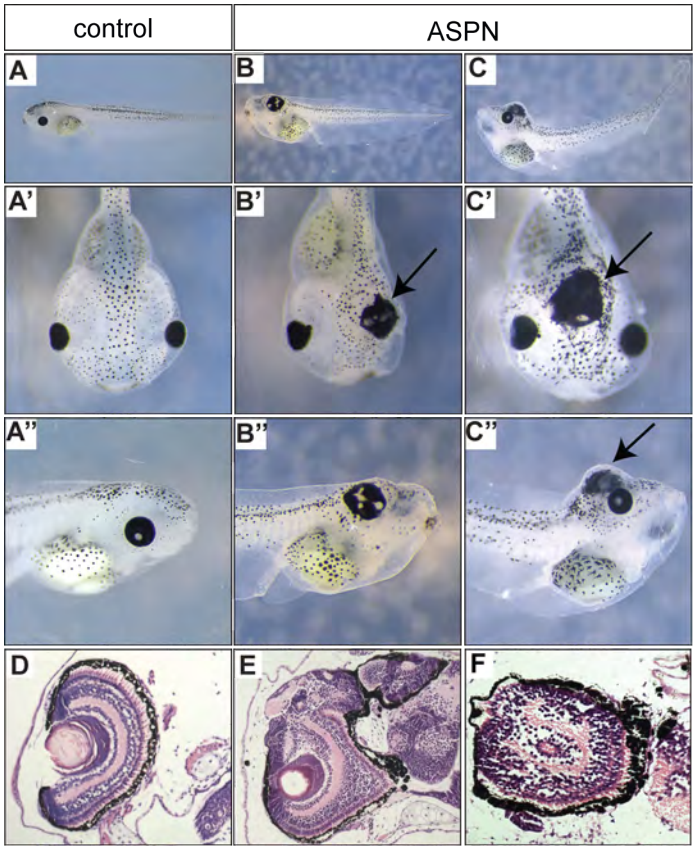


Figure 3.13: Paraffin sections of ASPN induced expanded eye and ectopic eye. Tadpoles with an enlarged endogenous eye (B, B', B'') and an ectopic eye (C, C', C'') were paraffin sectioned, followed by H&E staining (E, F). Control embryo and section are shown in A, A', A'' and D.

The presumed ectopic eye tissue shows a layered neural retina structure, with occasionally duplicated retinas and additional layering (Figure 3.14, C-H). The RPE found on the injected side (Figure 3.14, D, F, H) is thicker than on the un-injected side (Figure 3.14, B). Two cases of embryos with ectopic eye-like structures are shown in Figure 3.14, C, D and E, F. One of the ectopic structures is embedded in the neural tube (Figure 3.14, C, D), while the other induced ectopic tissue is adjacent, yet separate to, the neural tube (Figure 3.14, E, F). In both cases, the pigment can only be seen on the outside of the structure, which is presumably RPE, and the induced tissue has an overall epithelial character. Again, the presumed RPE is thicker than in the endogenous eye (Figure 3.14, B). Based on these observations from the histological data, I assumed that the condensed pigment structure surrounding the ectopic tissue is a pigmented epithelium of the retina (RPE), and the retinal structure was induced by the forced expression of ASPN. The enlarged endogenous eyes and

ectopically induced eyes show all the specific layers of the retina, which suggests the presence of all retinal cell types. The expansion and ectopic location of the induced eye tissue could be due to ASPN expanding the eye field at very early stages, through up-regulated EFTFs expression outside the normal eye field boundaries. The disorganisation of the ectopically induced and expanded retinas could be a result of the mRNA injection technique in *Xenopus* itself. Injected mRNA does not diffuse widely and evenly within the targeted embryo blastomere due to the inherent high lipid content of the embryonic tissue (lack of diffusion of injected material has been shown repeatedly with injection tracing agents such as β-gal). This could result in a mosaic cell population, where cells have different amounts of ASPN mRNA, which could then cause an uneven eye induction and explain faulty and disorganized layering and structure of the ectopic eye tissue.

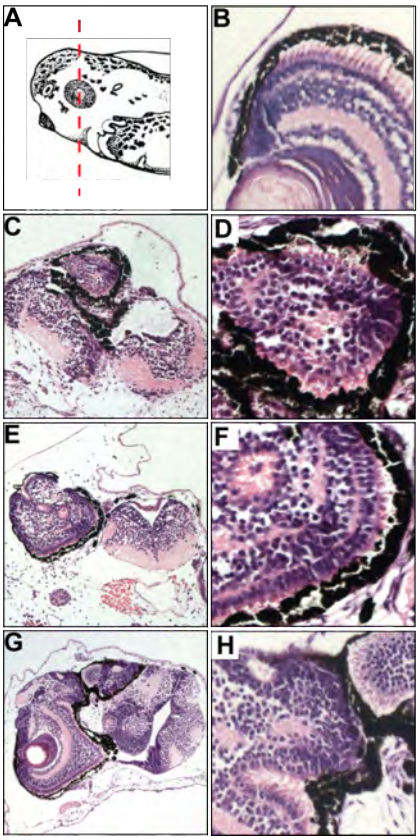


Figure 3.14: H & E stained paraffin sections of expanded and ectopic eye tissue. Sectioning plane is shown (A) and an un-injected control eye section (B). Also shown is an ectopic eye-like tissue, which developed within the neural tube (C, D) and thereby distorts the structure/appearance of the neural tube. Another ectopic eye is shown, which was found adjacent to the neural tube (E, F). Lastly, an expanded endogenous eye is shown in (G, H). The endogenous eye is fused with ectopic eye tissue and the neural tube.

3.3.3 Histological examination of brain reveals abnormal and enlarged CNS

Even without a fully developed ectopic eye structure being embedded or fused with the neural tube, ASPN injected embryos develop abnormal looking neural tubes, as seen in Figure 3.15, C, D. In these injected animals, the neural tube often shows signs of hyperplasia and is therefore often distorted with abnormal tissue layering and distribution. Often spots or circular inclusions of pigment could be found on top of the neural tube tissue and underlying the epidermis (Figure 3.15, C). This phenotype could be a result of ASPN's eye inducing activity and the expansion of eye field transcription factor expression domains, such as Rx1, which has previously been shown to modulate proliferation in the anterior neural plate (Andreazzoli et al., 1999, Zuber, 2010, Zuber et al., 2003). Also, overexpression of EFTFs Six3 and Six6 has been shown to affect the entire head region and leads to abnormal neural tissue structures. They also control eye precursor proliferation and are capable of converting midbrain cells to retinal progenitor cells (Bernier et al., 2000, Zuber, 2010).

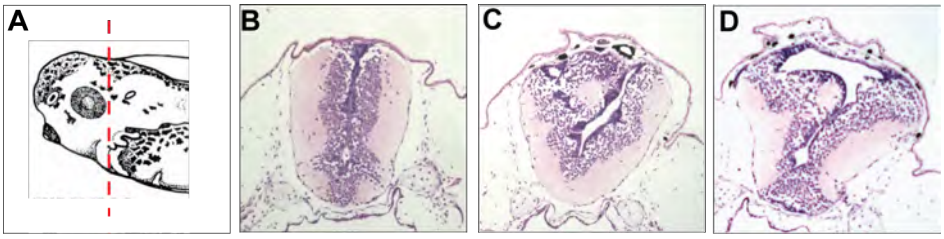


Figure 3.15: ASPN affects the development of the neural tube. Stage 42 tadpoles injected with ASPN (C, D) exhibit an abnormal neural tube structure compared to un-injected control embryo (B). Sectioning plane is shown in (A).

3.3.4 ASPN induced ectopic tissue is composed of eye specific cell types

The histological data suggested that the induced ectopic structures had an eye-like character. To further verify this hypothesis, I performed immunohistochemistry staining on cryo-sectioned ectopic and endogenous eyes (Figure 3.16). The antibodies used targeted eye specific cell types in *Xenopus*: β-crystallin for lens (Figure 3.16, B, C), glutamine synthetase for Müller glia (Figure 3.16, D, E), Hu-C/Hu-D for retinal ganglion and amacrine cells (Figure 3.16, F, G), and calbindin for cone photoreceptors (Figure 3.16, H, I). The ectopic structures stained positive for all

retinal specific antibodies tested. While β-crystallin positive lens was not always present, all ectopic structures examined tested positive for Müller glia, retinal ganglion and amacrine cells, as well as the cone photoreceptors (Figure 3.16, E, G, I). Where lens tissue was found, it was always correctly positioned on the epidermis facing side of the ectopic tissue (Figure 3.16, C). As previously mentioned, ectopic tissue often displayed layered, yet often unnaturally folded, retinal tissue. The Müller glia cells found in those induced structures still spanned the depths of the retina (Figure 3.16, E). However, due to the irregular overall layering, there is no parallel spatial pattern of Müller glia cells, as is the case in the endogenous eye (Figure 3.16, D). Layering can be observed for the population of Hu-C/Hu-D positive amacrine, the retinal ganglion cells, and also calbindin positive cone photoreceptors, but again due to folding of the ectopic tissue, the end location of the stained cells is not the same as in the endogenous eye (Figure 3.16, F-I).

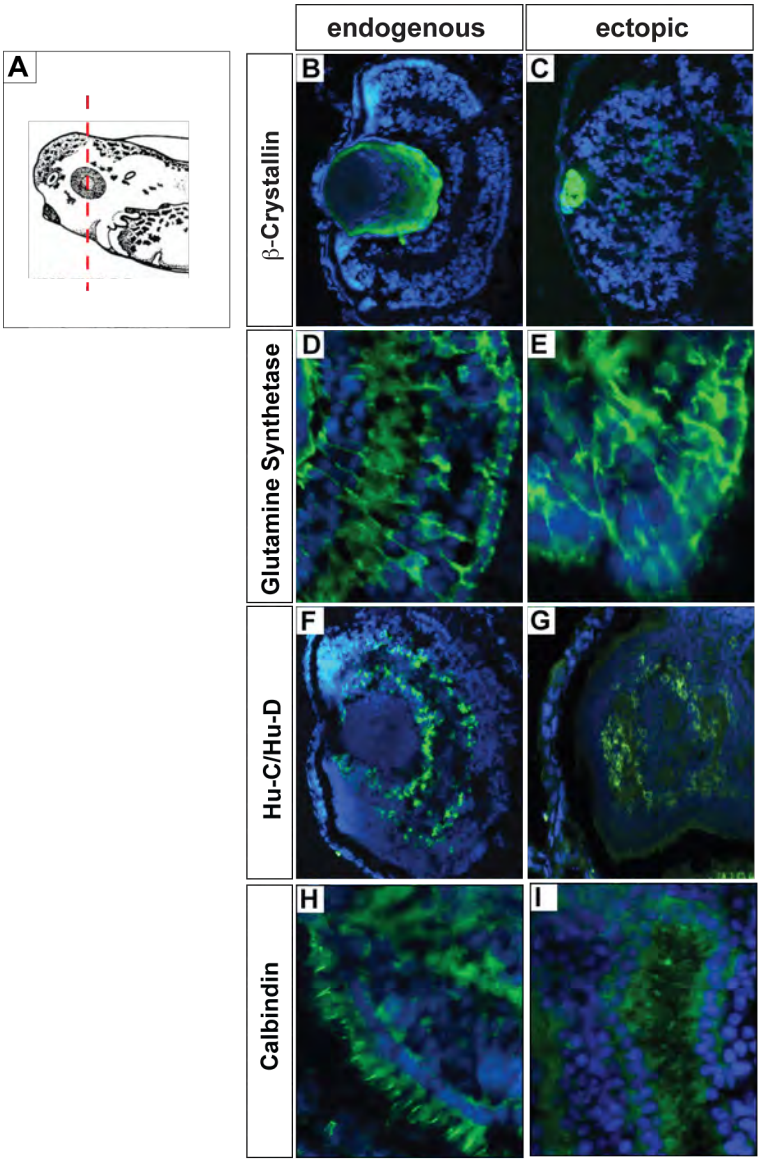


Figure 3.16: ASPN induced ectopic eye-like structures contain retina specific cell types. Tadpoles were injected with 3ng of ASPN mRNA and grown to stage 42. Ectopic eye-like structures (C, E, G, I) and control normal eyes (B, D, F, H) were cryo-sectioned and stained with β-crystallin antibody (lens; B, C), glutamine synthetase (Mueller glia; D, E), Hu-C/Hu-D (horizontal and amacrine cells; F, G) and calbindin (cone photoreceptors; H, I). Sectioning plane is shown in (A).

3.3.5 DiI staining cannot verify the existence of an optic nerve in ectopic eye structures

After histological and immunohistochemical data confirmed eye character for the ASPN induced structures, I wanted to find out if and how they might be innervated.

In vertebrates, axons often have to travel long distances to their final site of innervation. In the eye, the retinal ganglion axons from the retina normally grow along the well-defined optic tract, towards the tectum (Harris, 1986). In previous studies (Harris, 1986, Koo and Graziadei, 1995, Sedohara et al., 2003), it was shown that transplanted eyes in the cranial region, extend 'cellular bridges' and axons towards the optic tecta in the brain. These are maintained through metamorphosis and into adulthood. Also, transplanted eyes located in the area behind the head along the dorsal midline appear to extend axons, which enter the spinal cord (Giorgi and Van der Loos, 1978, Katz and Lasek, 1978).

To visualise the nerves, DiI (1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate) crystals where placed in the optic cup onto the retinas of 4 % PFA fixed embryos and then incubated in fixative for several days, before analysing. In Figure 3.17 (A-F) DiI staining of an endogenous eye of an un-injected embryo is shown. The retina stained strongly (Figure 3.17, B), as did the optic nerve leaving the retina (Figure 3.17, B, C, D). When dissected out of the tadpole and visualised by itself, the embryo's brain appeared to have also been stained (Figure 3.17, E). However, it was not possible for me to image the optic nerve extending and joining the tectum in the brain whilst keeping the embryo intact (Figure 3.16, F).

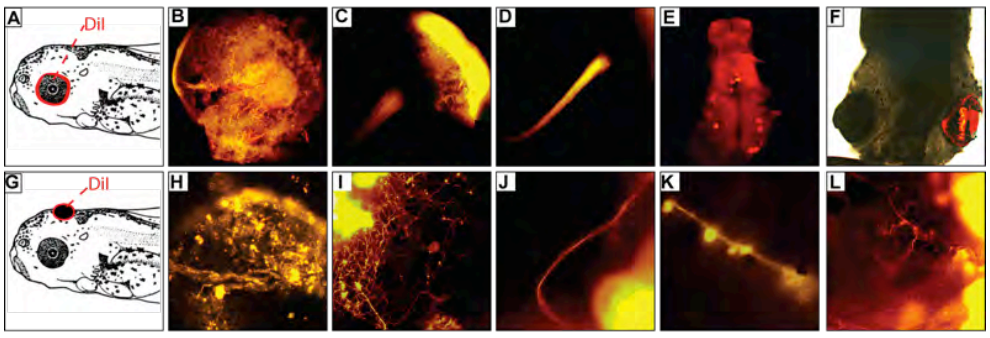


Figure 3.17: DiI staining of retina and optic nerve in control and ectopic eye tissue. (A-F) The lens of the endogenous eye was removed, and DiI crystals placed inside the optic cup of a 4% PFA fixed embryo at stage 42. The embryo was then incubated in fixative in the dark for 48 hours and the resulting staining analysed. (G-L) DiI crystals were also used to stain ectopic eye-like tissue. While no single optic nerve could be found, many smaller axons could be found, extending away from the ectopic eye tissue (I, J, K, L).

While the labelling of the endogenous eye and its optic nerve appeared to have worked, the labelling of ectopic structures turned out to be more difficult (Figure 3.17, G-L). Since the ectopic structures often did not display a cupped structure, placement and retaining of the DiI crystal for prolonged incubation and staining turned out to be tricky. Stained ectopic retina and RPE can be seen in Figure 3.17, H. While no single large optic nerve could be found leaving the ectopic structures, it does seem to be well innervated, with several axons extending away from it (Figure 3.17, I-L).

3.3.6 ASPN induces eye-field specific transcription factors (EFTFs) both *in vivo* and *in vitro*.

As previously described, the EFTFs play a crucial role in eye induction and their expression patterns have been well documented and characterized. The overexpression of several EFTFs causes the formation of ectopic eyes in *Xenopus*. It seemed therefore likely that overexpression of ASPN mRNA affects the expression of EFTFs. However, at what point in early development ASPN elicits its action and which EFTF members might be affected, remained to be seen.

In order to address the earlier effects of ASPN overexpression, I analysed gene expression patterns by whole-mount *in situ* hybridisation in embryos at stage 17 and 22. ASPN mRNA was injected into a single dorsal animal blastomere at the 4-cell stage and the embryos were cultured until the early tailbud stage 17. For EFTFs Rx1 and Pax6, embryos at stage 22 were also analysed.

At stage 17, the expression of Rx1 (Mathers et al., 1997b) (Figure 3.18, A, B; 90%, n=11) and Pax6 (Chow et al., 1999a) (Figure 3.18, E, F; 100%, n=11) was clearly expanded or appeared ectopically in the ASPN overexpressing side of the embryo, while the Otx2 expression pattern remained unchanged (Figure 3.18, I, J; 100%, n=11) (Blitz and Cho, 1995). In contrast, the telenchephalon marker XBF1 (FoxG1; telencephalon; (Bourguignon et al., 1998)) (Figure 3.18, K, L; 50%; n=20), En-2 (the midbrain-hindbrain junction; (Hemmati-Brivanlou et al., 1990)) (Figure 3.18, M, N; 81.5%, n=27) and Krox20 (hindbrain/rhombomere3 and 5; (Nieto et al., 1991)) (Figure 3.18, O, P; 100%, n=12) were down regulated. At stage 22, the ectopic and expanded expression of Rx1 (Figure 3.18, C, D; 93%, n=15) and Pax6 (Figure 3.18,

G, H; 78%, n=14) is still maintained. These observations suggest that ASPN specifically encourages retinal development *in vivo*.

Next, the function of ASPN *in vitro* was investigated. For this purpose, animal cap explants were prepared from embryos injected with the ASPN mRNA, and their gene expression analysed when the sibling whole embryos reached stage 22. ASPN increased the expression levels of Sox2 and NCAM (general neural), XAG1 (cement gland), XBF1 (telencephalon), Pax6 and Rx1 (forebrain and eye regions), Otx2 (forebrain and midbrain), while En-2 (midbrain and hindbrain junction), Krox-20 (hindbrain), Slug (neural crest) and cardiac Actin (mesoderm) were not affected by ASPN (Figure 3.18, Q). This suggests that ASPN can induce eye development on its own.

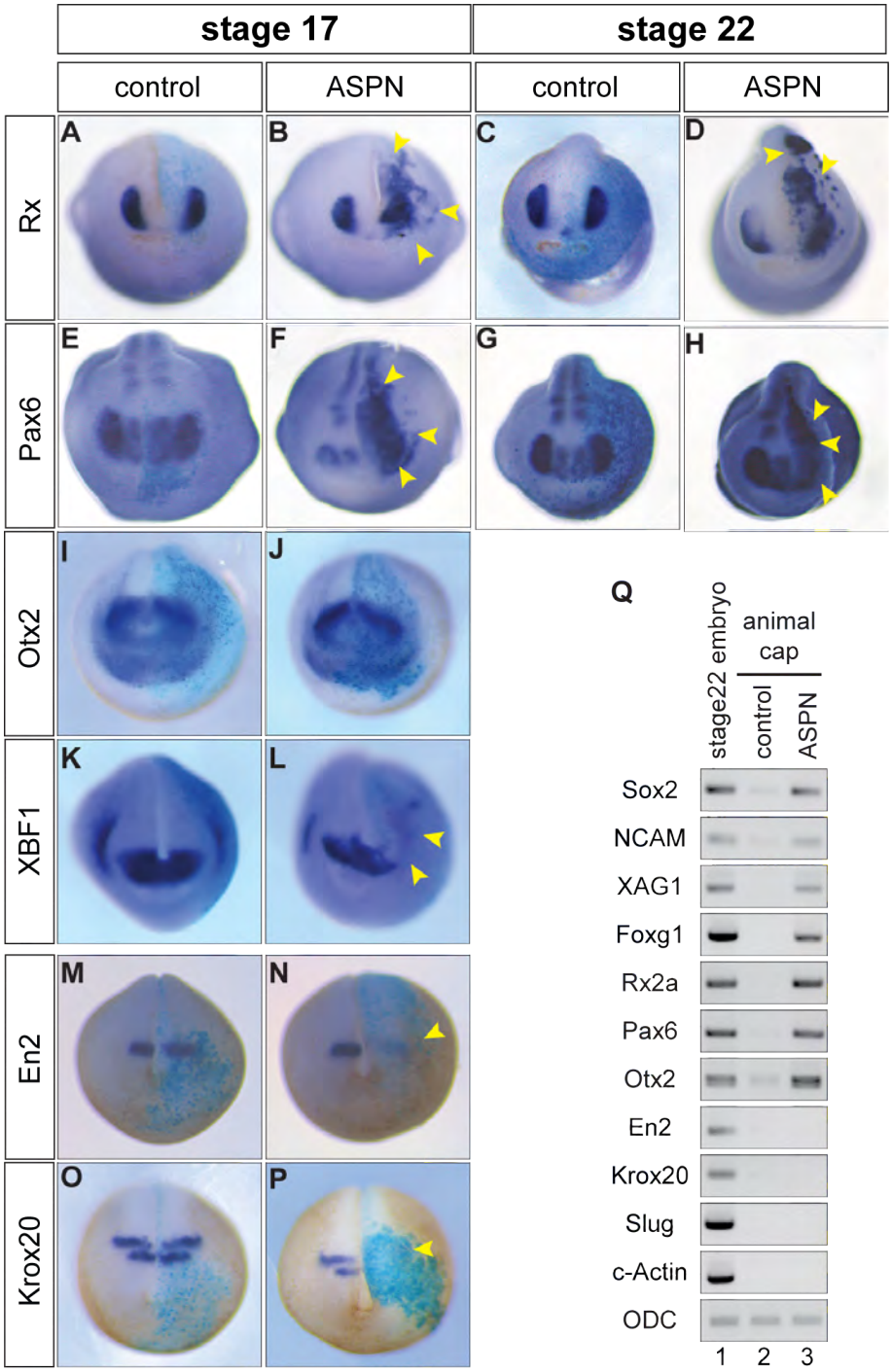


Figure 3.18: ASPN induces forebrain marker genes both in vivo and in vitro. (A-Q) Forebrain marker genes were increased at the expense of posterior markers in vivo, following unilateral ASPN mRNA injection. The tracer β-Galactosidase (light blue staining) was injected without (A, C, E, G, I, K, M, O) or with (B, D, F, H, J, L, N, P) ASPN mRNA (3ng). Embryos were analysed by in situ hybridisation with Rx (A-D), Pax6 (E-H), Otx2 (I, J), XBF1 (K, L), En2 (M, N) and Krox20 (O, P) probes at stages 17 and 22 (for Rx, Pax6). Affected areas are indicated with yellow arrowheads. (Q) Control (lane 2) or ASPN-injected (lane 3) animal cap explants were analysed by semi-quantitative RT-PCR. Whole embryos (lane 1) were used as positive control for the PCR.

3.4 Discussion

3.4.1 A *Xenopus* ortholog of ASPN plays an important role in eye development

The eye-inducing polypeptide of interest in this study, turned out to be a *Xenopus laevis* ortholog of ASPN. Multiple sequence alignment of ASPN in different vertebrate species, further revealed that it possesses many conserved features, such as the aspartic acid rich repeat and the number of leucine-rich-repeat domains (Figure 3.2). This suggests that these protein-features are important for its function. It is interesting that no ASPN ortholog could be found in *Drosophila* – its expression might be restricted to vertebrate species.

Until now, ASPN has been mostly associated with bone related disease, such as osteoarthritis, cartilage homeostasis and more recently with cancer (Kizawa et al., 2005, Satoyoshi et al., 2015, Torres et al., 2007, Maris et al., 2015). It is now recognized that SLRPs are not merely involved in the upkeep of the extra-cellular matrix and offering of structural support. This class of proteoglycans has been found to be involved in crucial cell signalling events, which direct developmental events such as migration, growth and development.

Most members of the SLRP family are well known to inhibit TGF-β and BMP signalling by directly binding to their respective molecules and thereby inhibiting receptor binding and cell-signal pathway activation. To demonstrate this point: Decorin, a class I SLRP, is known to bind to EGFR, IGF1R, WISP-1, LRP-1, c-MET, TFG-b and BMP4. It can regulate the downstream signalling of their associated signalling pathways (Brandan et al., 2006, Dellett et al., 2012, Desnoyers et al., 2001, Iozzo, 1999, Kolb et al., 2001, Schaefer et al., 2007) and is thought to be essential for convergent extension movements (Zoeller et al., 2009). Fellow class I SLRP biglycan regulates dorsal-ventral axis formation and has the ability to induce secondary axes in frog embryos, by modulating chordin mediated BMP4 inhibition (Dellett et al., 2012, Moreno et al., 2005b). Tsukushi (TSK) binds to and regulates the associated signalling pathways of at least nodal/Vg1, BMP4/chordin, FGF8, Frizzled4 and delta (Morris et al., 2007, Ohta et al., 2011). In *Xenopus* TSK is involved in germ layer specification and patterning events (Dellett et al., 2012, Morris et al., 2007) and in chick it was found to act as a neural inducer and modulate

organizer formation and neural induction (Ohta et al., 2004). Xenopus TSK further controls ectodermal patterning and neural crest specification, by modulating BMP and Notch/Delta signals (Dellett et al., 2012, Kuriyama et al., 2006).

SLRPs defects have long been known to cause various eye diseases (Bech-Hansen et al., 2000, Bredrup et al., 2005, Lin et al., 2010, Majava et al., 2007, Pellegata et al., 2000). The discovery that SLRP family member ASPN may play a crucial role in eye development is therefore not too surprising. Yet, so far, there have been no reports on ASPN's role in a developmental biology context.

Despite its wide popularity as a model organism, the genome of *Xenopus laevis* is still poorly understood. Genetic studies in *X.laevis* are challenging, as it is allotetraploid (also referred to as pseudotetraploid) with a large genome size of 3.1 Gbp (Pollet and Mazabraud, 2006). The allotetraploidy is thought to be due to a whole-genome duplication event after interspecific hybridisation of diploid species, which took place between 30-55 million years ago (Pollet and Mazabraud, 2006, Uno et al., 2013). While some genes are present in the frog in a diploid state, other genes were conserved as duplicated 'allogenes'. These can show different degrees of divergence (usually lower than 10%) (Pollet and Mazabraud, 2006).

Each animal model offers particular technical advantages and limitations (Harland and Grainger, 2011). Based on these, the researcher has to decide which model is best suited to investigate the scientific problem in question. The frog *Xenopus laevis* offers many advantages compared to other amphibian models (e.g. inducible egg laying, large robust eggs, etc.) and definitely has the jump on many other vertebrate models, especially mammalian models. Since there is a high conservation of many important developmental and cellular processes, as well as a high degree of genomic synteny with mammals, insights gained from work in *Xenopus* can be used to better understand development and disease in humans. The syntenic regions between *Xenopus* (*tropicalis* and *laevis*) with human are often 100 genes or more (Harland and Grainger, 2011). Hopefully, the insights gained from investigating the role of *Xenopus* ASPN are therefore transferable to mammalian models.

3.4.2 ASPN is expressed in the presumptive eye field of frog embryos around the time of eye induction

Since this study is based on an eye phenotype that was discovered following forced overexpression, the initial question that poses itself is whether ASPN plays a direct role in early eye induction? If this was the case, ASPN expression should be present around the time of eye induction and in the right tissues - i.e. anterior neuroectoderm and more specifically the presumptive eye field.

In human and mouse at adult stages, ASPN was found to be expressed in a variety of tissues via Northern blot analysis, however in varying amounts: high expression levels were found in the liver, heart, aorta and uterus, while only low levels of ASPN were detected in lung, bone marrow, trachea and no apparent ASPN expression in the human CNS, spleen and thymus (Henry et al., 2001, Lorenzo et al., 2001). The highest concentrations of ASPN mRNA were found in articular cartilage, as well as in aorta and uterus. This suggests that ASPN expression takes place in smooth muscle cells. Intermediate levels of mRNA were also found in other tissues, containing smooth muscle cells (Lorenzo et al., 2001). In developing mouse embryos, ASPN expression analysed by whole-mount in situ hybridisation, showed high expression levels in the developing skeleton (particularly in perichondrium and periosteum of cartilage and bone), as well as specialised connective tissues such as tendon and sclera (Henry et al., 2001). However, Henry et al. (2001) did not find ASPN expression at early developmental stages in the presumptive eye field region.

Since the 1960s it has been known that the eye anlagen are already specified at the neural plate stage, as shown in the salamander experiments by Lopashov and Stroeva (1964). As mentioned in the general introduction, EFTFs such as ET, Rx1, Pax6, Six3, Six6 and Lhx2 are expressed in a specific timed manner and pattern to eventually lead to the development of the eye. In *Xenopus laevis*, Six3 is the only EFTF that can be detected at egg stage, although this early expression seems to be only transient and disappears again by stage 10.5 (Zuber et al., 2003). ET, Pax6 and Rx1 are firstly detectable around stages 10, 10.5 and 11 respectively. A strong expression of most EFTFs can be seen to start nearly simultaneously around stage 12 to 12.5. While Zuber et al. (2003) found variability between samples, the relative time frame remained the same. Otx2 is required for the establishment of the

presumptive forebrain and midbrain region. It is therefore expressed in the entire presumptive neuroectoderm during gastrula stages. At the beginning of neurulation, Otx2 expression is down regulated in the central part to form an expression gap. This gap is the location of the presumptive eye field and shortly after EFTFs start to be expressed in partly overlapping patterns within this Otx2 free area (Pannese et al., 1995, Zuber et al., 2003).

In my PCR analysis of ASPN expression levels throughout early development of *X. laevis*, ASPN was already detected in the egg with weak expression prior to stage 10, at which point a marked increase took place (Figure 3.3, A). As mentioned previously, this is also the time at which the first eye field transcription factors ET, Rx1 and Pax6 start to be expressed. Therefore, the timing of this increased ASPN expression coincides nicely with the beginning of eye induction.

Since ASPN seems to be expressed at the correct time for eye induction, the question remained; whether spatial expression patterns further confirmed a potential role for ASPN in eye development? The qRT-PCR results performed on animal caps mimicking forebrain (Chordin induced), and other slightly more posterior neural domains (Chordin and Wnt8 induced) (Figure 3.3, B (iii), (iv)), clearly indicate that ASPN is expressed in anterior neuroectoderm and either directly or indirectly induced by Chordin. Since no ASPN expression could be detected in VMZ and DMZ (representing ventral and dorsal mesodermal tissue, respectively) the expression of ASPN in the broad anterior neuroectoderm seems to be specific, i.e. ASPN is not simply ubiquitously expressed throughout the embryo at the same level (Figure 3.3, B (v), (vi)).

In the hope of elucidating the exact expression pattern of *ASPN* during early *X. laevis* development, I set out to design and produce whole-mount *in situ* hybridisation probes. While ASPN 3 was directly synthesised from the ASPNa sequence in a pCS2+ plasmid, ASPN 1 and ASPN 2 were produced via a PCR method. Unfortunately, none of the *in situ* probes showed specific staining of ASPN expression patterns around the beginning of eye induction (i.e. stages 10-12). The absence of a visible ASPN *in situ* signal at the time of eye induction, stands in contrast to the detected ASPN qPCR signal. Since the gain-of-function experiments

suggest an early role for ASPN in eye development, this discrepancy in detected ASPN could be due to technical limitations of WISH. It is possible, that ASPN expression levels are simply too low to produce a visible signal in the *in situ* experimental setting around the time of eye induction. It is only from around stage 18 that specific areas of ASPN expression become visible around the neural plate and anterior regions. Particularly when compared to the expression pattern of EFTF Pax6 (Figure 3.6, B, C), it becomes apparent that the ASPN expression pattern shares some overlapping expression areas with Pax6. At later stages, specific ASPN expression in the eye region becomes apparent (Figure 3.6, D, E).

Together these observations revealed that ASPN is expressed during early embryogenesis around the time of eye induction. The detected ASPN expression levels throughout early frog development and specific expression patterns in the anterior neuroectodem, make it highly likely that ASPN plays a role in neural and more specifically eye development.

3.4.3 ASPN induced ectopic structures have eye character

The histological and immunohistochemical analyses showed that the ASPN induced ectopic structures have indeed eye character. Injected ASPN mRNA can either expand the endogenous eye on the injected side of the embryo, or even induce the formation of ectopic eye structures - mostly in the head region and occasionally in posterior tissues such as the tail or along the flank of the embryo (Figure 3.10).

While these ASPN induced structures, are definitely of an eye nature, they are abnormal in many ways: The retinal pigment epithelium, which surrounds the induced tissue, is thicker than normal (Figure 3.14, B, D, F). While the neural retina has a layered appearance and contains eye specific cell types (such as Mueller glia, horizontal and amacrine cells, cone photoreceptors and sometimes also lens) the layering is in parts doubled and inverted (Figure 3.14, D). The overall size of induced ectopic eyes was impressive and often outsized the endogenous eye (Figure 3.10, C, C'). With over 30% of embryos injected with 3 ng of ASPN mRNA, exhibiting ectopic eyes, ASPN is a very potent eye inducer (Figure 3.11).

It is still unclear if ectopic sensory organs, such as eyes, can connect to the CNS and become functional. It is possible that due to CNS plasticity, the brain can adapt and ectopic eyes may be able to function through novel pathways (Blackiston and Levin, 2013). In previous experiments, it was shown that transplanted ectopic eyes in the head region form 'cellular bridges' to connect with the optic tecta (Koo and Graziadei, 1995), while ectopic eyes transplanted in posterior tissues along the dorsal midline, also extend axons that seem to enter the spinal cord (Katz and Lasek, 1978, Giorgi and Van der Loos, 1978, Sedohara et al., 2003). In the case of ASPN induced ectopic eyes, it is not possible to draw conclusion as to their functionality. With DiI staining it could only be ascertained that the induced eye-structures are innervated and extend axons into the surrounding tissue. It was technically not possible to establish, if and where these axons found their postsynaptic partners (Figure 3.16, H-L). But attractants may be expressed and the presumptive retinal cells (either endogenous or ectopically induced) may be guided by those attractants.

Beyond establishing whether the right nerve connections are present, assessing the functionality of ectopic eyes in *Xenopus laevis* is generally very challenging. Frogs are notoriously difficult to use in cognitive studies and learning assays. In previous aversive training studies, frogs would rather die than adapt to avoid repeated exposure to electric shocks (Blackiston and Levin, 2012). Recently Blackiston and Levin (2012) developed a protocol for visual conditioning, which they used to assess the functionality of transplanted ectopic eyes.

3.4.4 ASPN induces eye field transcription factors (EFTFs) both *in vivo* and *in vitro*

When ASPN was overexpressed in a dorsal animal blastomere a spectrum of eye phenotypes could be observed as a result, ranging from enlarged endogenous eyes, to ectopic eye-like structures and various eye defects (Figure 3.10). The expanded or ectopic eye-like tissues were often connected with the animal's neural tube, which itself often showed signs of hyperplasia (Figure 3.14, G, H; Figure 3.15 C, D).

The observed phenotypes are reminiscent of those found upon overexpression of various EFTFs: Rx1 overexpression, for example, expands endogenous eye tissue and induces ectopic retina and RPE, while also leading to the expansion and

duplication of the neural tube (Andreazzoli et al., 1999, Mathers et al., 1997a). Depending on the injection site, Pax6 overexpression lead to ectopic eyes, lenses and RPE, as well as eye defects proximal to the neural tube (Chow et al., 1999b). Overexpressing Six3 in frog also lead to expanded endogenous eye and head tissues, and ectopic eyes, which seems to originate from transformed midbrain tissue. At very high concentrations Six3 disrupted eye morphology and lead to CNS hyperplasia (Bernier et al., 2000). When closely related Six6 is overexpressed in *Xenopus*, a dramatic increase in eye size at the expense of midbrain could be observed, along with retinas that are fused with the forebrain (Bernier et al., 2000, Zuber et al., 1999).

Based on these earlier studies, the working hypothesis was that ASPN is likely to directly affect the eye field transcription factors and elicit its effect by inducing their expression. This theory could be confirmed by the whole mount *in situ* hybridisation experiments, which showed an expansion of the expression domains of EFTFs Rx1 and Pax6 (Figure 3.18, B, D, F, H), following ASPN overexpression. Posterior markers FoxG1, En2 and Krox20 in contrast were down regulated (Figure 3.18, L, N, P). PCR analyses in animal cap, further confirmed that ASPN seems to induce forebrain specific marker genes both *in vivo* and *in vitro* (Figure 3.18, Q). ASPN did not expand the *Otx2* expression domain (Figure 3.18, I, J), even though in animal cap explants increased levels could be detected (Figure 3.18, Q). Otx2 is thought to 'prime' the anterior part of the embryo for the subsequent expression of eye specific genes (Zuber et al., 2003). There is a possibility that ASPN may not induce Otx2 *in vivo*, or it may potentially increase the intensity of Otx2 expression, while not expanding the area in which it can be detected. More likely though is that stage 17 and stage 22 are too late to detect any possible ASPN-induced changes in Otx2 expression patterns. While Otx2 is initially broadly expressed from the very anterior presumptive cement gland to the region of the midbrain, already by stage 12/13 Otx2 can no longer be detected in the eye field. In fact, in order for the eye to develop, Otx2 needs to be supressed within the eye field so that EFTFs can be expressed (Pannese et al., 1995, Zuber, 2010).

3.4.5 The ASPN induced phenotype in *Xenopus*, closely resembles the IGF overexpression phenotype but is not identical

Overexpression studies dating back to the 1990s, showed that several EFTFs are by themselves sufficient to expand endogenous eye tissue or induce ectopic eyes. Pera et al (2001) and Richard-Parpaillon et al. (2002) showed that IGF signalling plays an important role in anterior head and also eye development. Overexpression of IGFs resulted in expanded expression domains for various EFTFs and lead to ectopic eye phenotypes. The ASPN induced phenotypes are very reminiscent of the IGF induced eye phenotypes described by Pera et al. (2001) and Richard-Parpaillon et al. (2002), which raised the possibility of ASPN – an extracellular protein – conveying at least some of its eye inducing effects through the IGF signalling pathway.

However, there are also some differences. Pera and colleagues (2001) observed a drastic expansion of cement gland tissue upon overexpression of IGF1 and IGF2 in the embryos, and also Richard-Parpaillon et al. (2002) found ectopic cement glands when IGF1 was overexpressed in ventral animal blastomeres. While there was some induction of cement gland marker XAG in injected animal cap transplants (Figure 3.18, Q), no expanded or even ectopic cement glands could be observed in the whole embryos upon forced overexpression of ASPN (Figure 3.13, A'', B'', C'').

Richard-Parpaillon and colleagues observed ectopic eye like structures and hyperplasia of the anterior part of the neural tube, when IGF1 mRNA was injected into a dorsal animal blastomere (4-cell stage). The same is found following ASPN overexpression. However, in their study, forced expression of IGF1 in dorsal animal blastomeres resulted in a high number of embryos with severely truncated body phenotypes; a consequence of inhibited convergent-extension movements. While truncated embryos were also observed following ASPN overexpression, this phenotype was mainly observed when injections occurred in the ventral animal blastomeres (Figure 3.12). Richard-Parpaillon et al. (2002) observed expanded and ectopic Otx2 expression in the IGF1 injected side of the embryos. As previously described, in the ASPN overexpressing embryos no such ectopic Otx2 expression could be found (Figure 3.18, K, L). However in animal cap explants, ASPN was capable of inducing Otx2 expression (Figure 3.18, S). The comparison of IGF and

ASPN induced phenotypes suggests, that ASPN may transduce some of its actions through the IGF signalling pathway.

3.5 Summary

In this chapter, I show that the SLRP family member ASPN plays a role in early *Xenopus* eye development. It is expressed at the time and place of eye induction and has the ability to up-regulate the expression of eye field transcription factors Rx1 and Pax6, both *in vivo* and *in vitro*, at the expense of posterior neural markers. Forced overexpression of ASPN leads to the induction of ectopic tissue, which could be shown to have eye character, including eye specific cell types. While functionality of these induced ectopic eyes could not be confirmed due to technical challenges, ASPN certainly seems to play an important role in eye development.

CHAPTER 4: ASPN IS ESSENTIAL FOR EYE DEVELOPMENT AND UNIQUE AMONGST SLRPs

4.1 Introduction

While gain-of-function experiments are easily carried out in *Xenopus laevis*, thanks to the very accessible, large and robust embryos, loss-of-function studies are trickier. Due to the allotetraploid genome of *X.laevis*, loss-of-function can only be achieved through manipulation of expression and transient loss-of-function, by using dominant-negative constructs, antisense RNA and morpholinos (antisense-oligonucleotides). To address *X.laevis's* allotetraploidy, two morpholinos can be co-injected, each targeting one of the duplicated genes (allogenes). These may have slightly different sequences, but have a conserved function. True loss-of-function could not be accomplished until the use of the diploid relative *Xenopus tropicalis* (Harland and Grainger, 2011, Henry et al., 2008).

Morpholinos are synthetic oligonucleotides, which are complementary to, and therefore target, specific sequences like the 5'UTR (5' untranslated region) to prevent the initiation of mRNA translation. Morpholino antisense oligos (Gene Tools, 2016) bind complementary RNA and, depending on where they are targeted to, can block gene expression, modify RNA splicing or inhibit miRNA (micro RNA) activity. Morpholinos (MO) are short chains of typically 25 subunits, which each comprise of a nucleic acid base (i.e. A, C, G or T), a morpholine ring in the backbone (which replaces the ribose/deoxyribose) and a non-ionic phosphorodiamidate intersubunit linkage. MOs do not degrade the RNAs but act via an RNAse H-independent steric blocking mechanism. Because of their high target specificity, MOs are supposedly free of any off-target expression modulation. To better understand ASPN's role in early embryogenesis and eye development, I performed loss-of-function analysis by using morpholino oligonucleotides for ASPN, which is presented in this chapter. For this purpose morpholinos were designed, which block the translation initiation by targeting the 5'UTR sequence through the first 25 bases of the coding sequence.

The family of SLRPs share a lot of functions such as the binding to collagen and members of the TGF-β and BMP family of proteins (Iozzo and Schaefer, 2015, Iozzo and Schaefer, 2010). It is thought that class I-III arose due to chromosomal duplication, which may account for functional redundancy. Probably the most

thoroughly researched SLRPs are ASPN's fellow Class I members - Decorin and Biglycan. A lot of research had been carried out to find out more about their functions in cancer, angiogenesis and inflammation (Hsieh et al., 2014, Jarvinen and Prince, 2015, Neill et al., 2012, Sofeu Feugaing et al., 2013). A question posed itself as to whether ASPN is unique amongst SLRPs in its ability to induce the striking ectopic eye formation, or if it is a shared characteristic amongst this proteoglycan family? To investigate this further, I injected *Xenopus* Decorin, Lumican, Epiphycan and Chondroadherin mRNA into the developing embryos and analysed the phenotypes. The results are presented in the second half of this chapter.

As previously mentioned, the frog *Xenopus* is a good model to investigate eye development due to both practical reasons and a vertebrate species wide conserved gene network, which governs eye development/induction. The hope is that results obtained in frog are mostly transferable to human. While testing the transferability hypothesis in a mammalian model was beyond the scope of this study, I did test the frog ASPN mRNA for eye inducing effects in Zebrafish embryos, which will be described at the end of this chapter.

4.2 ASPN loss-of-function analysis

Morpholino ASPN-MO1 was designed to target the translational inititation site of both *ASPNa* and *ASPNb* (Figure 4.1). To confirm the specificity of ASPN-MO1, another set of morpholinos termed ASPNa-MO2 and ASPNb-MO2 were designed to be co-injected to ensure the duplicated genes translation was thoroughly blocked (Figure 4.1).

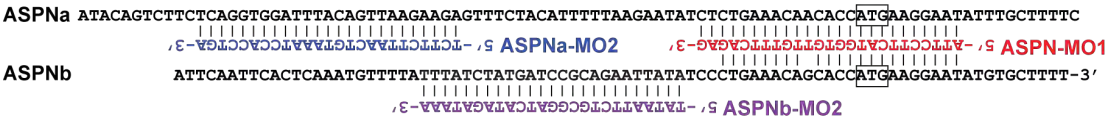


Figure 4.1: Design and target regions of morpholino oligonucleotides against ASPN. The nucleotide sequences (black characters) around the start codon (in rectangle) of Xenopus ASPN are shown. In addition to ASPNa, which this study is based on, we found another genome sequence (probably due to the pseudotetraploidity), and termed it ASPNb. The sequences and target regions for morpholinos ASPN-MO1 (red), ASPNa-MO2 (blue) and ASPNb-MO2 (purple) are also displayed.

4.2.1 ASPN Morpholino induced Phenotype

The ASPN morpholino (ASPN-MO1) was injected into one dorsal animal blastomere at 4-cell stage of the embryos, at concentrations of 10ng, 20ng and 40ng. The resulting phenotypes were analysed at developmental stage 42. As a control, I used a scramble morpholino, referred to as control-MO (sequence: CCTCTTACCTCAGTTACAATTTATA).

The injected tadpoles displayed a variety of eye defects on the injected side, ranging from partly missing eye structures and very reduced sized eyes, to a complete lack of eye development (Figure 4.2, C, E). While on the un-injected side, the endogenous eye developed normally. The control-MO injected embryos showed no phenotype (Figure 4.2, A). Paraffin sectioning and H & E staining of injected tadpoles further confirmed that ASPN-MO1 injection inhibited eye development compared to the control-MO (Figure 4.2, B, D, F). The majority of tadpoles injected with ASPN-MO1 that exhibited the small eye phenotype, had otherwise normal body length (Figure 4.2, H). The morpholino-induced small eyes exhibited – despite the overall size – normal retinal layering with RPE and lens. This suggests that ASPN may only be involved in the early stages of eye induction and may not play a role in later stages of retinal cell type specification.

The phenotype distribution at concentrations of 10ng, 20ng and 40ng is shown in Figure 4.3 (A, B, C). Inhibition of eye development increased, with increasing amounts of ASPN-MO1 injected. At a concentration of 10ng, 75% of injected tadpoles showed no phenotype, 19.4% of tadpoles displayed a slightly reduced eye

size and 5.6% had a very small or missing eye. At twice the injected ASPN-MO1 concentration (20ng, Figure 4.3, B) - only 1.8% of tadpoles remained completely unaffected, while the majority of injected tadpoles showed an extremely reduced eye size. At very high concentrations of 40 ng of ASPN-MO1, nearly all embryos showed severe eye phenotypes, with 89.4% of injected embryos showcasing the very small eye phenotype and 9% missing eyes (Figure 4.3, A-C). At 10 ng of ASPN-MO1 the overall embryo body length and shape was not affected, while 22% of embryos injected with 20 ng ASPN-MO1, and 79% of embryos injected with 40 ng of ASPN-MO1, had severely truncated body shape (Figure 4.4).

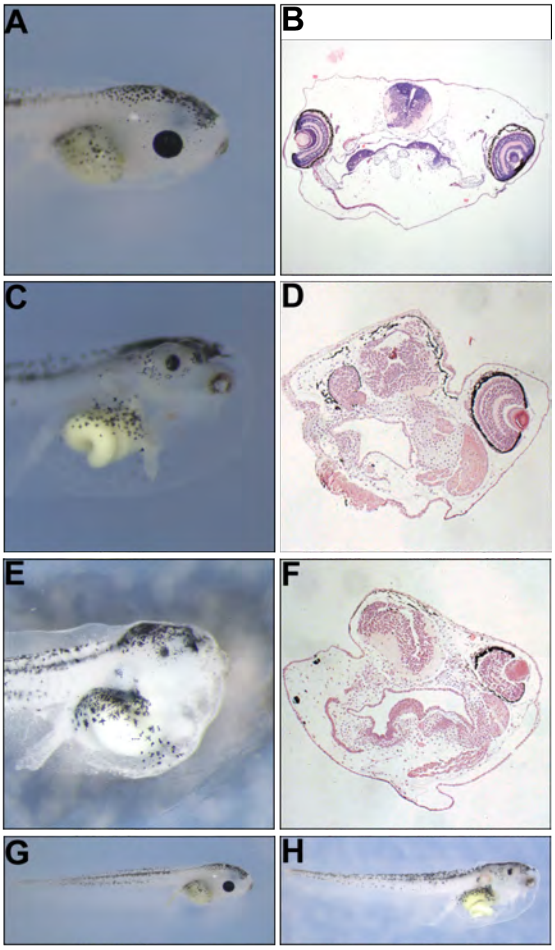


Figure 4.2: The ASPN-MO1 induced eye phenotype. (A) Shown are a control-MO injected tadpole with normal eyes, (C) a tadpole injected with 20ng of ASPN-MO1 with a reduced eye size on the injected side and (E) an embryo injected with 40ng of ASPN-MO1 with no eye on the injected side and severely truncated body. H & H stained paraffin sections of these embryos (B, D, F respectively) further confirmed the disrupted eye development. The majority of embryos displaying a 'small-eye' phenotype, showed normal body length (H) compared to control (G).

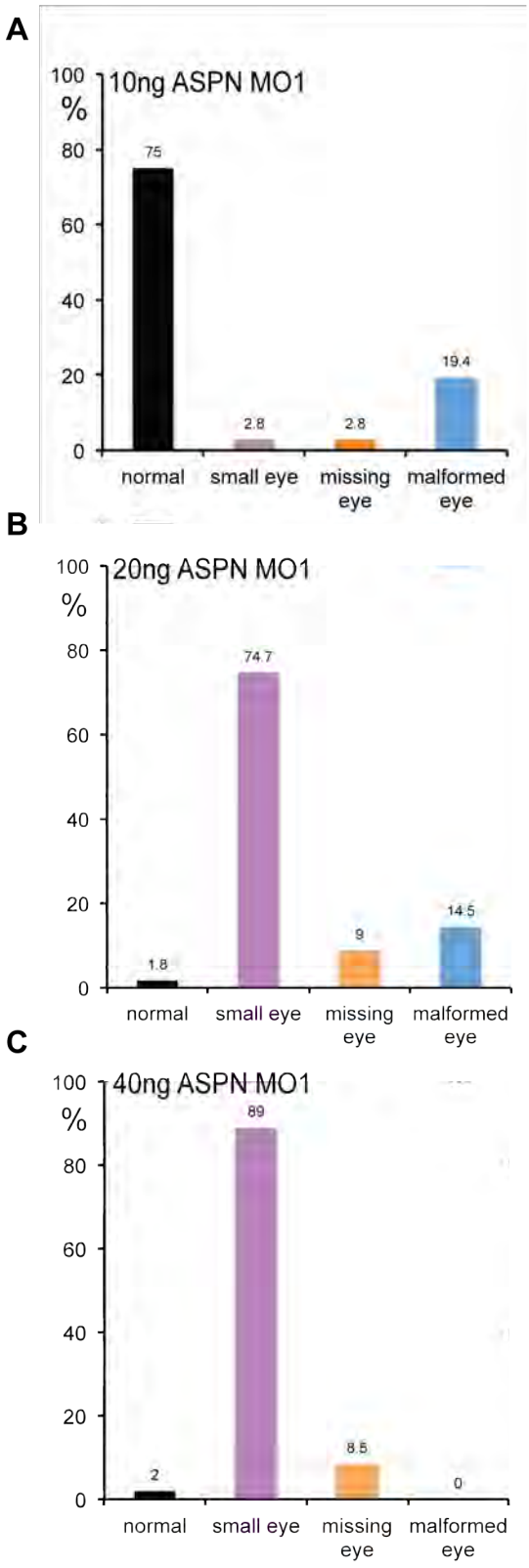


Figure 4.3: Eye phenotypes found, following ASPN-MO1 injections at different concentrations. Shown are the phenotype distributions amongst tadpoles injected with 10ng (A; n=36), 20ng (B; n=55) or 40ng (C; n=47) of ASPN-MO1 into one dorsal animal blastomere at the 4-cell stage. Embryos were analysed at stage 42.

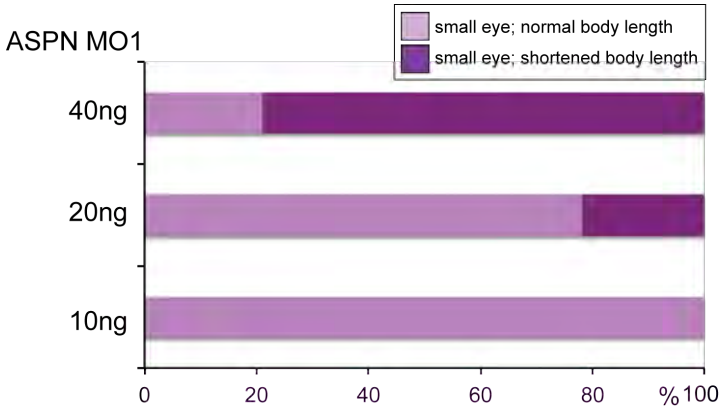


Figure 4.4: High doses of ASPN MO1 lead to embryos with shortened bodies. While embryos were unaffected at injections of 10 ng ASPN-MO1 (n=36), at higher concentrations of 20 ng (n=55) or 40 ng (n=47) of ASPN-MO1 increasing numbers of embryos with the 'small-eye' phenotype also displayed a severe truncation of their anterior-posterior body axis.

4.2.2 The ASPN-MO1 phenotype can be rescued

To confirm the specificity of ASPN-MO1 and show that the phenotype was not due to off-target effects, a rescue experiment was performed. For this experimental set-up mRNA, which is immune to the morpholino's effect, is used to 'rescue' the MO-induced phenotype. The eye defects induced by ASPN-MO1 could be rescued by co-injection of ASPN mRNA containing only the coding region ($ASPN_{CDR}$), further verifying the specificity of ASPN-MO1 (Figure 4.5). 20 ng of ASPN-MO1 was co-injected with either 1 ng or 3 ng of $ASPN_{CDR}$ mRNA and the resulting phenotypes analysed. With increasing $ASPN_{CDR}$ concentration, the frequency of ASPN-MO1 related phenotypes (small eye, missing eye) decreased, while ASPN overexpression related phenotypes (enlarged and ectopic eyes) increased (Figure 4.5, D).

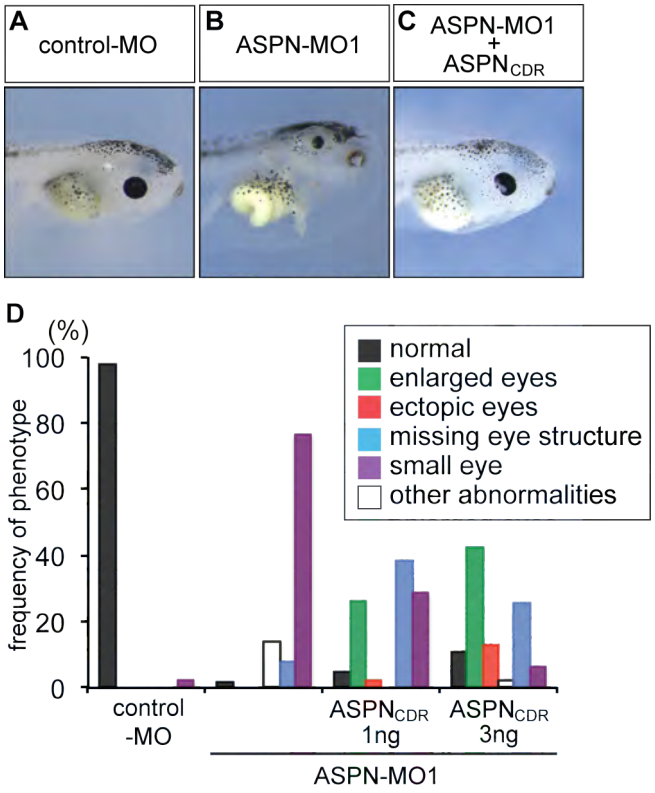


Figure 4.5: ASPN-MO1 effects can be rescued by addition of ASPN mRNA. (A-C) Representative images from the injection of control-MO (A; n=59), ASPN-MO1 (B, n=55) and ASPN-MO1 together with the coding region of ASPN (ASPN$_{CDR}$) mRNA (C). (D) Quantification of the phenotypes. For the rescue experiment, embryos were injected with either 20 ng ASPN-MO1 and 1 ng ASPN$_{CDR}$ (n=42), or 20 ng ASPN-MO1 and 3 ng ASPN$_{CDR}$ (n=47), and the phenotypes analysed at stage 41.

4.2.3 ASPN-MO2 confirms specificity

To further verify the specificity of ASPN-MO1, another set of ASPN morpholinos, termed ASPNa-MO2 and ASPNb-MO2 (Figure 4.1) was injected. To address the allotetrapleudity of *Xenopus laevis*, these two ASPN-MO2s were co-injected into the embryos, each targeting one of the duplicated genes (i.e. the allogenes). The ASPN-MO2s were further designed to target different areas of the mRNA compared to ASPN-MO1 (Figure 4.1).

ASPNa-MO2 and ASPNb-MO2 (referred to as ASPN-MO2) were co-injected into one animal blastomere of a 4-cell embryo at concentration of 10ng each, and the phenotype analysed at stage 42. ASPN-MO2 induced a very similar phenotype

compared to ASPN-MO1 injected embryos, such as inhibited eye development (Figure 4.6, B, C), but with overall less efficacy (Figure 4.6, D; Figure 4.3, B). Again, the phenotype induced by ASPN-MO2 could be rescued through co-injection of $ASPN_{CDR}$ as shown in Figure 4.7.

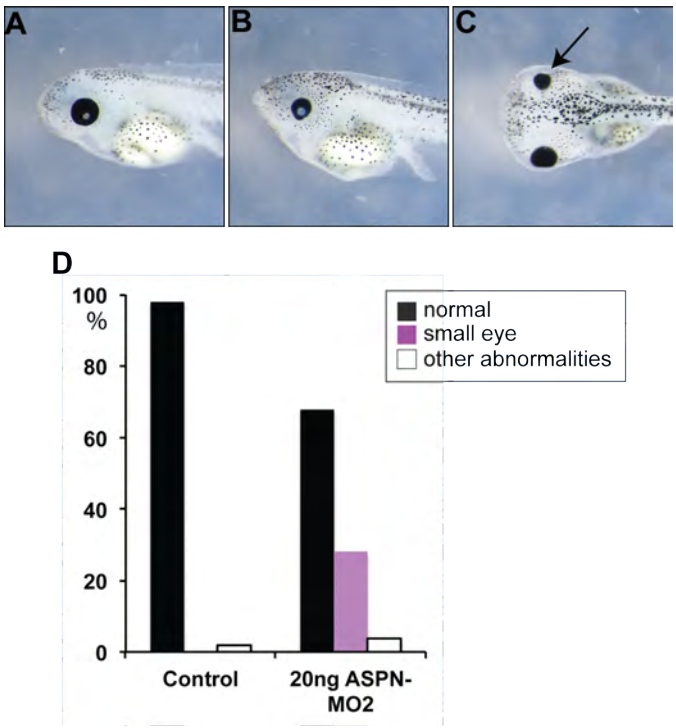


Figure 4.6: Eye phenotype observed following ASPN-MO2. (A-C) Representative images of an embryo injected with control-MO (A) or 20ng of ASPN-MO2 (10ng ASPNa-MO2 and 10ng ASPNb-MO2) (B, C). Following the injections of 20ng of ASPN-MO2, 28% of embryos developed a small eye on the injected side (D) (n=78).

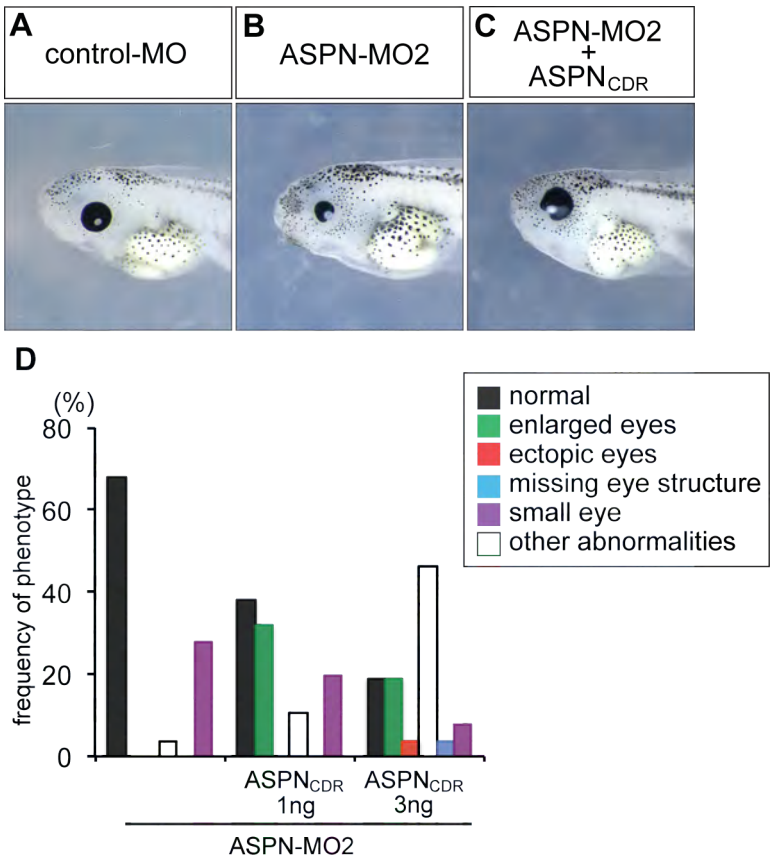


Figure 4.7: Effects of ASPN-MO2 can be rescued with co-injection of ASPN mRNA. (A-C) Representative images from the injection of 20ng control-MO (A, n=62), 20ng ASPN-MO2 (B, n=78) and 20ng ASPN-MO2 together with 1ng of the coding region of ASPN ($ASPN_{CDR}$) mRNA (C). (D) Quantification of the phenotypes. For the rescue experiment embryos were injected with either 20ng ASPN-MO2 and 1ng $ASPN_{CDR}$ (n=87) or 20ng ASPN-MO2 and 3ng $ASPN_{CDR}$ (n=47), and the phenotypes analysed at stage 42.

4.2.4 ASPN-MO1 changes expression of EFTFs

As I had determined that ASPN mRNA injection affects the expression of several eye field transcription factors (Chapter 3), I wanted to examine if the same holds true for ASPN-MO. So to describe the phenotype induced by ASPN-MO on a molecular level, I performed *in situ* hybridisation with probes for eye and other regional genes. For this either ASPN-MO1 or control-MO was injected (alongside β-gal tracing mRNA) into an animal blastomere at 4-cell stage and the expression patterns analysed at developmental stages 18 and 22.

ASPN-MO1 significantly affected the expression of the early eye-field transcription factors Rx (80%, n=10; Figure 4.8, B) and Pax6 (89%, n=11; Figure 4.8, F), while Otx2, En2 and Krox20 were unaffected at neurula stages (n=20 each; Figure 4.8, J, N, R). This tendency was maintained at early tailbud stages (stage 22) on the injected side of the embryos, as shown by the reduced expression of the second-stage eye-field transcription factors Six3 (67%, n=12; Figure 4.8, D) and Optx2/Six6 (56%, n=16; Figure 4.8, H) (Zuber et al., 2003), while other regional markers remained unaffected (n=20 each; Figure 4.8, L, P, T). In all cases, the control-MO injected embryos were completely unaffected and showed normal expression patterns (n=10 each; Figure 4.8, A, C, E, G, I, K, M, O, Q, S). These results suggest that ASPN is required for eye-field specification and eye development.

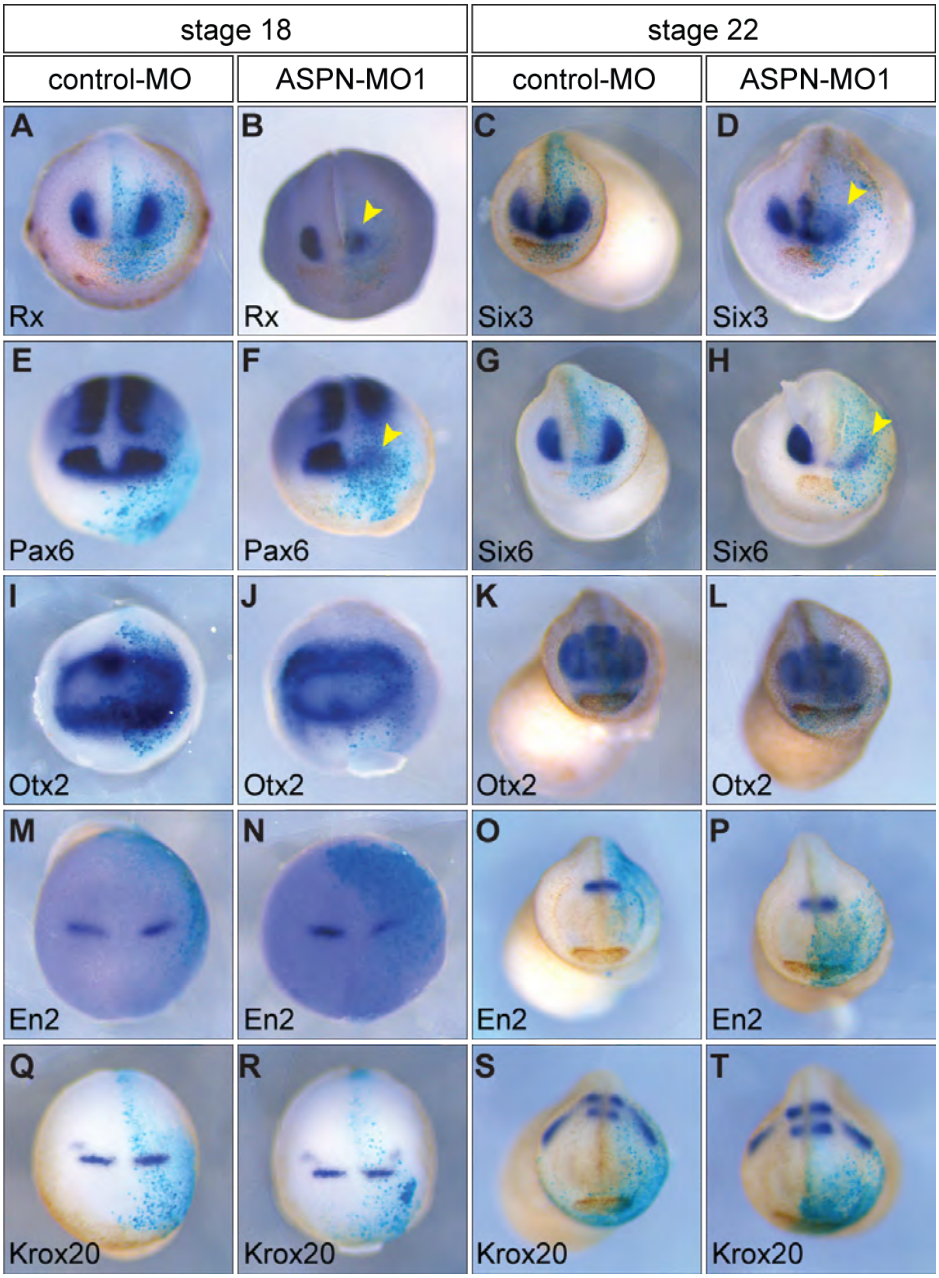


Figure 4.8: ASPN-MO1 affects the expression of EFTFs. Either control-MO (A, C, E, G, I, K, M, O, Q, S) or ASPN-MO (B, D, F, H, J, L, N, P, R, T) was injected together with β-Galactosidase mRNA as a tracer (light blue product) and embryos were analysed at stage 18 (A, B, E, F, I, J, M, N, Q, R) or stage 22 (C, D, G, H, K, L, O, P, S, T) by in situ hybridization with the probes of Rx (A, B), Six3 (C, D), Pax6 (E, F), Six6 (G, H), Otx2 (I-L), En2 (M-P) and Krox20 (Q-T). Arrowheads in B, F, D and H indicate affected areas.

4.2.5 ASPN and Chordin relationship

As shown earlier in Chapter 3 (Figure 3.3, B), animal caps injected with neural inducer Chordin showed elevated ASPN levels *in vitro*. As a further attempt to unveil the relationship between Chordin and ASPN, animal caps injected with Chordin (Sasai et al., 1994) were prepared, which showed the expected elevation in forebrain and eye gene *Rx2a* and *Pax6* expression (Figure 4.9 (ii)). In contrast, when ASPN-MO1 was co-injected with Chordin mRNA, the expression of these early eye marker genes was significantly down-regulated (Figure 4.9 (iii)), suggesting that ASPN acts downstream of the neural inducer Chordin. ASPN seems to be required for eye development, especially during the initial stages of the entire developmental process.

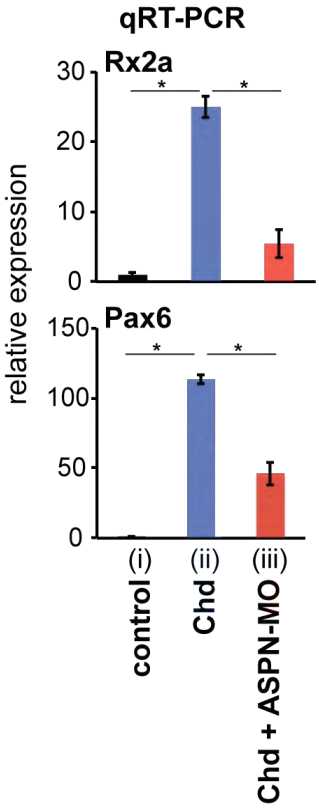


Figure 4.9: ASPN is essential for the induction of EFTFs by Chordin (Chd). Animal caps of control (i; black bars), Chd-injected (ii; blue bars) and Chd+ASPN-MO-injected (iii; red bars) embryos were prepared and the animal caps were analysed at stage 22 by qRT-PCR (*P<0.01; Student's t-test). Error bars represent s.e.m.

4.3 The ASPN induced eye phenotype is unique amongst SLRP members

To find out whether ASPN's ability to induce ectopic eyes is unique amongst the SLRP family of proteins, I overexpressed a selection of SLRPs and examined them with respect to eye forming activities. Representative proteoglycans from each of the first four SLRP classes were chosen: Decorin (class I), Lumican (class II), Epiphycan (class III) and Chondroadherin (class IV).

The mRNAs of these four SLRPs were then injected at a concentration of 3ng into a dorsal animal blastomere of a 4-cell embryo and the resulting phenotypes categorised at stage 42. In Figure 4.10 the phenotype distribution can be seen. We know that ASPN has the ability to induce ectopic eyes. This ability is not shared with the other tested SLRPs. Neither Decorin nor Chondroadherin overexpression induced an eye phenotype, but instead strongly truncated embryos. Lumican overexpression and Epiphycan overexpression lead to mild extension or enlargement of the endogenous eye (Figure 4.10).

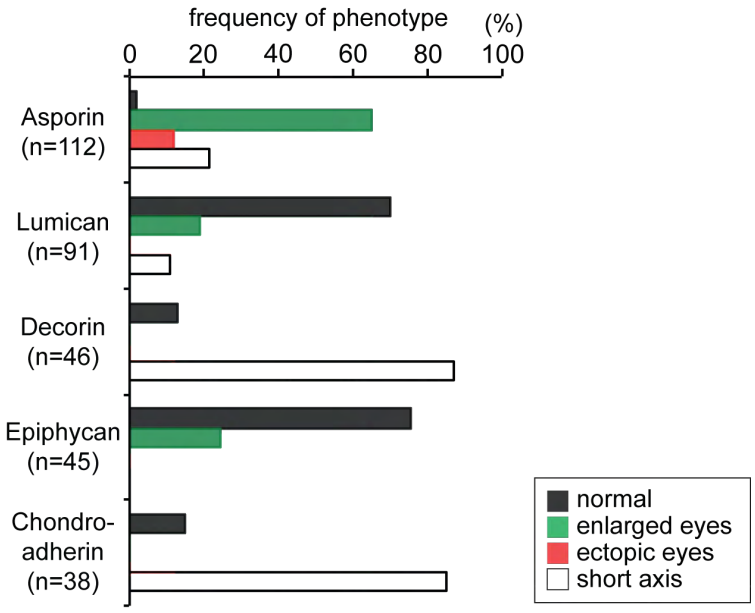


Figure 4.10: ASPN's ability to induce ectopic eyes is unique amongst SLRPs. The mRNA of SLRP family members ASPN, Lumican, Decorin, Epiphycan and Chondroadherin were injected at 3ng into a dorsal animal blastomere at the 4-cell stage and the phenotypes analysed at stage 42. Only ASPN overexpression resulted in embryos with ectopic eyes.

When 3ng of *X. laevis* Lumican was injected in a dorsal animal blastomere at 4-cell stage, some embryos developed a mild eye phenotype, which is shown in more detail in Figure 4.11. The endogenous eye on the injected side was enlarged, with either little wisps of retinal tissue extending away from the eye (Figure 4.11, A, C, D) or little spheres of retinal tissue developed adjacent to the eye (Figure 4.11, B). When the affected embryos were paraffin sectioned, the retina-like histology of the ectopic tissue confirmed eye character (Figure 4.11, E, H). Of course, to have certainty about what type of retinal cells are present in the Lumican-induced tissue, further immunohistochemical analysis would have to be carried out. The retina of the endogenous eye on the injected side of the embryo was sometimes found to show irregularities in layering, as seen in Figure 4.11, F. The neural tube section of a Lumican injected embryo, shown in Figure 4.11, G, also shows signs of hyperplasia and abnormal structure, which is reminiscent of the effect ASPN has on neural tube development.

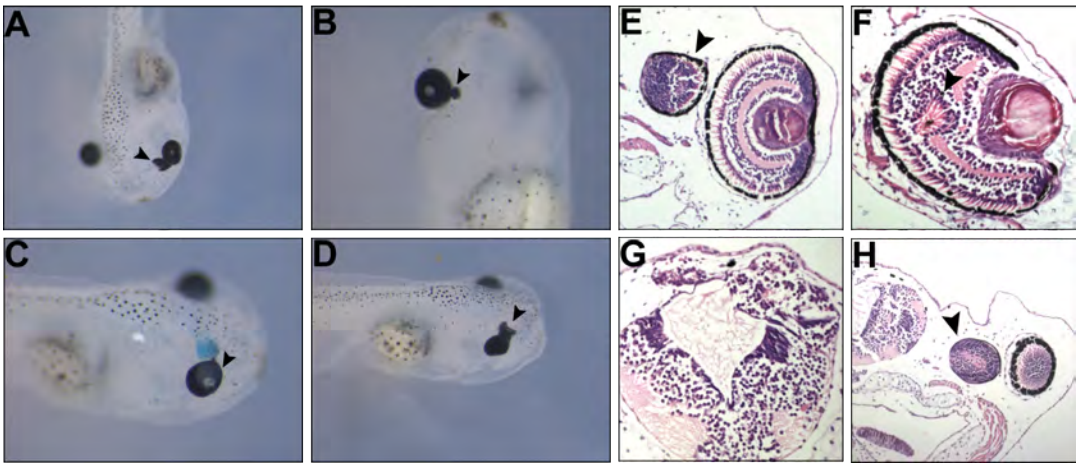


Figure 4.11: Lumican induces a mild eye phenotype. When 3ng of Lumican mRNA was injected into a dorsal animal blastomere at the 4-cell stage, some embryos exhibited an eye phenotype where retina was expanded or small spheres of ectopic retina developed adjacent to the endogenous eye (A-D). The retinal character of the extra tissue was confirmed in paraffin-sectioned samples (E, H). Endogenous retina on the injected side also appeared irregular (F). In some embryos, the neural tube had an abnormal structure (G).

In Figure 4.12 the overexpression phenotypes, following the injection of 3ng Epiphycan, 3ng Decorin and 3ng Chondroadherin, are characterised. Epiphycan

induces a very subtle enlargement of the endogenous eye on the injected side (Figure 4.12, B, C) in about a quarter of all injected embryos (Figure 4.10). No ectopic eye tissue was found in the Epiphycan injected embryos. The overall body length and shape was also not affected. No abnormalities were observed in the layering and morphology of both the embryo's neural tube or retina (Figure 4.12, D). When 3ng of Decorin was overexpressed in the animal blastomere of the frog embryos - 86% of embryos showed a shortened body, and were overall smaller in size, than un-injected controls (Figure 4.10; Figure 4.12, E). No eye related phenotype could be observed. Furthermore, the structure of the neural tube also seemed unaffected by Decorin overexpression (Figure 4.12, F). The overexpression of 3ng Chondroadherin resulted in 82% of embryos having severely truncated and twisted bodies (Figure 4.10; Figure 4.12, G, H). No ectopic eye tissue or eye enlargement could be observed in these embryos.

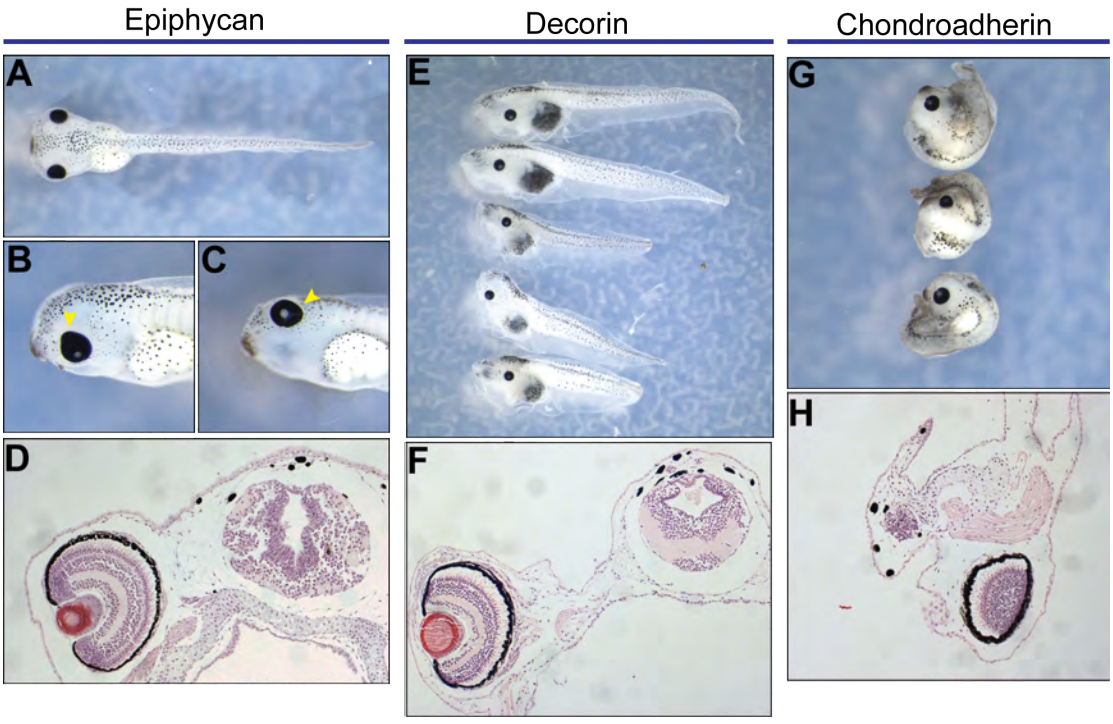


Figure 4.12: Overexpression phenotype of Epiphycan, Decorin and Chondroadherin. 3 ng of Epiphycan (A-D), Decorin (E, F) and Chondroadherin (G, H) was injected into a dorsal animal blastomere of the 4-cell embryo, and the phenotype categorised at stage 42. Epiphycan overexpression sometimes induced a very subtle enlargement of the endogenous eye on the injected side (B, C). Decorin overexpression did not result in eye phenotypes, but in overall shortened embryo bodies (E, F). Overexpression of Chondroadherin led to severely truncated and malformed embryos (G, H).

ASPN's striking eye phenotype is mediated through the IGF signalling pathway, as will be shown in Chapter 5. I was interested to see whether fellow class I SLRP Decorin shares this ability to activate the IGF downstream signalling. Moreover, the question posed itself whether class II SLRP Lumican induces its mild eye phenotype – like ASPN – through the IGF signalling pathway. For this, HEK 293 cells were transfected with ASPN-myc, Lumican-myc or Decorin-myc constructs, and the conditioned expression media applied to another set of HEK 293 cells for 20 minutes. Western blotting analysis was then performed with antibodies for phosphorylated ERK (p-ERK), ERK, phosphorylated AKT (p-AKT) and AKT (Figure 4.13, D). Based on these results, it seems that Decorin does not activate ERK or AKT phosphorylation. Lumican seems to induce AKT phosphorylation and, potentially to a much lesser extent, ERK phosphorylation. Since ASPN seems to activate ERK more strongly than Lumican, it could be assumed that p-ERK plays a more important role for ASPN's eye inducing effects, while p-AKT may play a more minor role. AKT and ERK signalling have both been previously shown to be important for eye development (Bugner et al., 2011, Li et al., 2013, Wu et al., 2006).

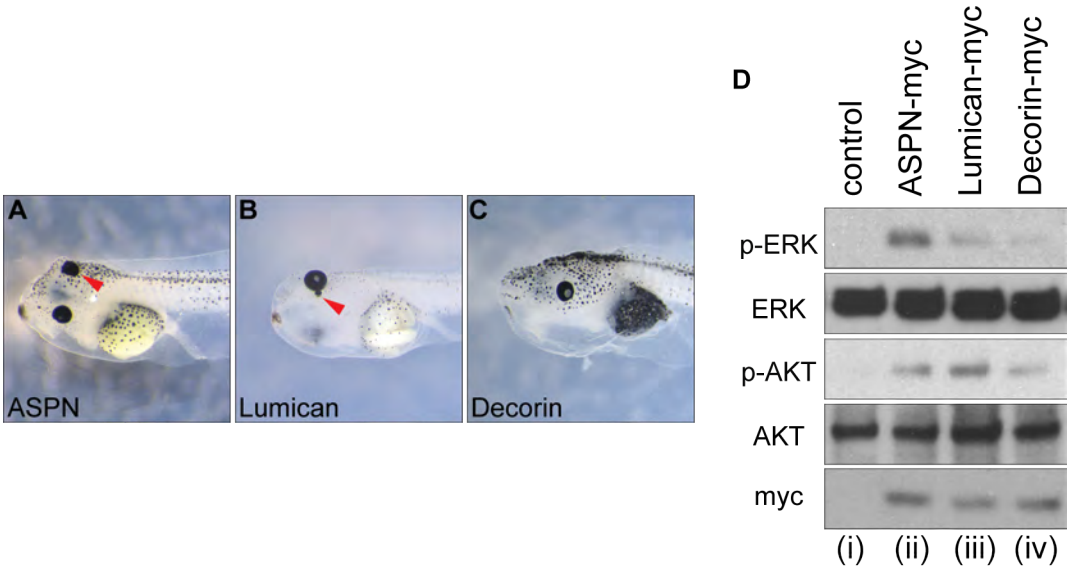


Figure 4.13: Characterisation of Lumican and Decorin. (A-C) Representative images of the embryos injected with 3ng ASPN (A), 3ng Lumican (B) and 3ng Decorin (C) mRNAs. (D) Differential activation of ERK and AKT by SLRP proteins. Control (i), ASPN-myc (ii), Lumican-myc (iii) or Decorin-myc (iv) expression media were prepared and applied onto HEK293 cells.

4.4 ASPN overexpression in Zebrafish

ASPN is an important factor in early eye development and it seems to be unique amongst SLRPs in its ability to induce large ectopic eyes in *Xenopus laevis*. But the question remains, whether ASPN fulfils the same role in other vertebrate species. Amphibians, such as the African clawed frog *Xenopus*, have some unique features that are not shared by other vertebrate classes, such as the ability to regenerate various body parts, including the eye, following injury (Vergara and Del Rio-Tsonis, 2009, Lee et al., 2013). So the ability to grow extra eyes may be a *Xenopus* specific trait. In fact, ectopic eyes and ectopic lenses in Zebrafish embryos have very rarely been reported (Cavodeassi et al., 2005, Kondoh et al., 2000), compared to those in *Xenopus* (Andreazzoli et al., 1999, Bernier et al., 2000, Chow et al., 1999b, Masse et al., 2007, Mathers et al., 1997a, Pai et al., 2012, Rasmussen et al., 2001, Zuber et al., 1999). To illustrate: while shown to play an important role in Zebrafish head and eye development, IGF overexpression did not result in an ectopic eye phenotype in the fish embryos (as found in frog). However, the gene network, which regulates early eye induction is known to be highly conserved amongst vertebrates. Unfortunately, testing ASPN on a mammalian model was beyond the scope of this current study. So in a first attempt to investigate the role of ASPN in another class or vertebrates, I analysed the effect of *X. laevis* mRNA on Zebrafish embryo development.

100pg, 200pg or 300pg of *X. laevis* ASPN mRNA was injected into Zebrafish embryos at 1-cell stage and the phenotypes then analysed 72 hours post fertilisation (hpf). At 100pg, ASPN injected fish embryos appeared largely unaffected, with around 94% of embryos showing no phenotype and 4.6% of embryos displayed an overall shortened body (Figure 4.14, C, D, E). Extraordinarily, one embryo was found to have developed, what appears to be a third eye in the middle of its head (Figure 4.14, A, B). Unfortunately, due to time constraints, it was not possible to investigate the potential eye character any further.

At 200pg of ASPN mRNA, half of the injected Zebrafish embryos showed various developmental defects, such as an overall shortened body and several eye malformations (Figure 4.15, D). Nearly a third of injected embryos displayed cyclopia with a single eye in the middle of their head (Figure 4.15, A, A', D).

Smaller proportions of embryos showed diminished eye size (Figure 4.15, B, B', D) or fused retinas (Figure 4.15, C, C', D). At the highest concentration tested (300pg ASPN mRNA), embryos increasingly showed a shortened body phenotype, with either cyclopia, small eyes or no eyes (Figure 4.16, A-E).

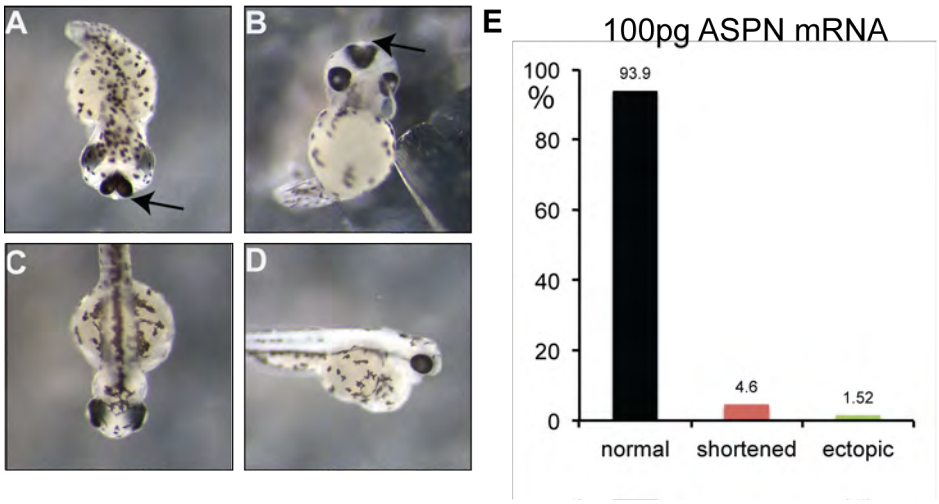


Figure 4.14: Phenotype in Zebrafish after injection of 100pg ASPN mRNA. Zebrafish embryos were injected with 100pg of Xenopus ASPN mRNA at the 1-cell stage and the phenotype analysed 72 hours post fertilisation. While the majority of embryos seemed unaffected by the injection (E), around 5 % had a truncated body often with reduced head size (C, D). Very rarely an embryo developed with what looked like a third eye located in the centre of the forehead (A, B, E) (n=66).

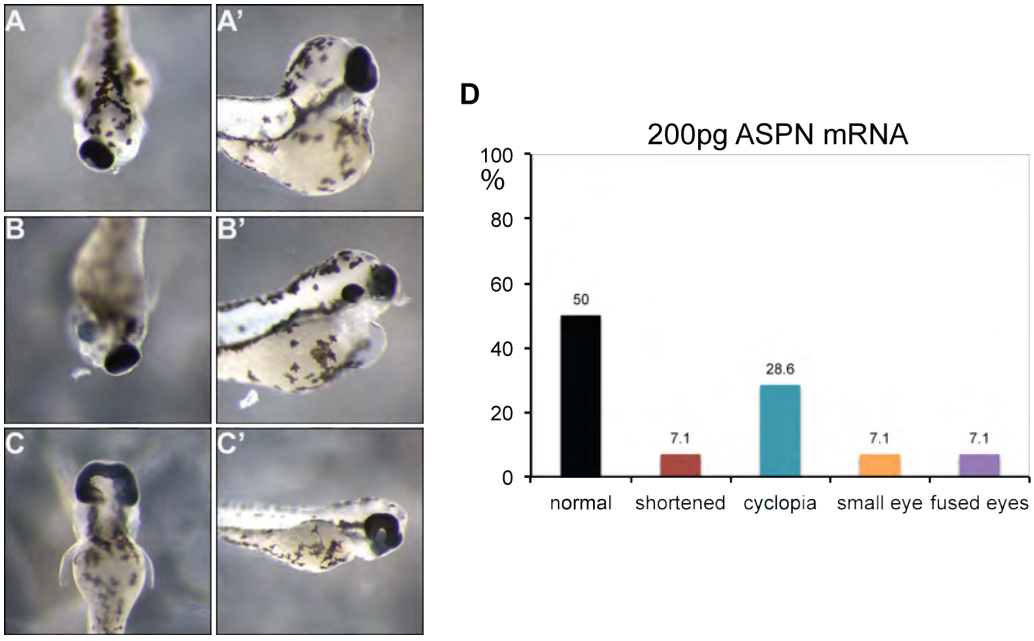


Figure 4.15: Phenotype in Zebrafish after injection of 200pg ASPN mRNA. Zebrafish embryos were injected with 200pg of Xenopus ASPN mRNA at the 1-cell stage and the phenotype analysed 72 hours post fertilisation. Half of the injected embryos exhibited a phenotype following injection (D). These ranged from cyclopia (A, A'), to decreased eye size (B, B') and fused retinas (C, C') (n=28).

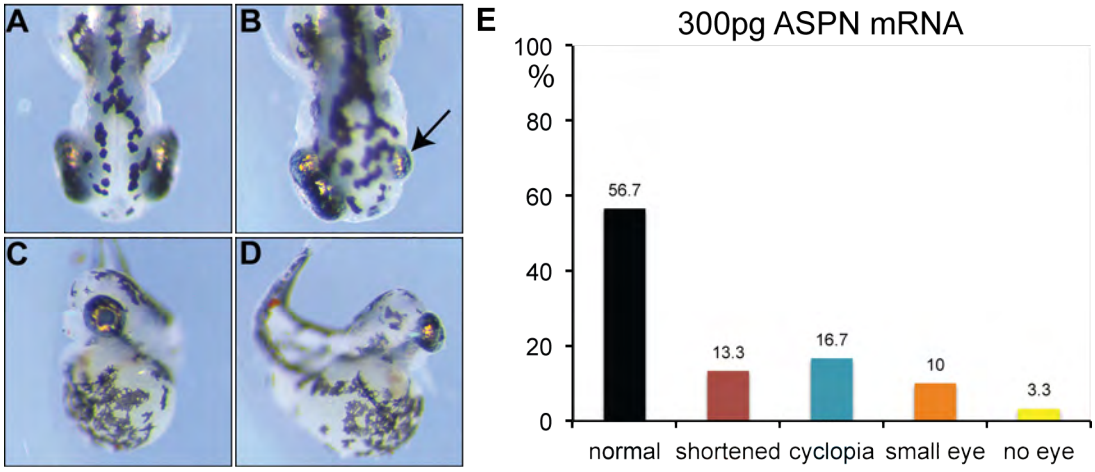


Figure 4.16: Phenotype in Zebrafish after injection of 300pg ASPN mRNA. Zebrafish embryos were injected with 300pg of Xenopus ASPN mRNA at the 1-cell stage and the phenotype analysed 72 hours post fertilisation. Nearly half of the embryos injected exhibited a phenotype (E). A high proportion showed cyclopia (C, D), with other embryos showing reduced eye size (B) compared to control (A) (n=30).

4.5 Discussion

4.5.1 ASPN is an important factor in *Xenopus* eye development

Following the gain-of-function study (Chapter 3), which indicated a role for ASPN in early eye development, I now wanted to test this hypothesis from a different angle. Through a transient loss-of-function, by use of specific morpholinos, I wanted to analyse what happens in eye development when ASPN is not present.

In keeping with the earlier overexpression experiment, the transient loss of ASPN activity through morpholinos ASPN-MO1 and ASPN-MO2 induced disrupted eye development in the injected embryos. The affected eyes were strongly diminished in size or even completely absent, as can be seen in the paraffin section where no eye rudiments are present (Figure 4.2, E, F; Figure 4.6). As expected from the aforementioned phenotype, ASPN-MO1 injections resulted in a down regulation of the eye specific genes Rx1 and Pax6 at stage 18. Second stage EFTFs - Six3 and Six6 - can also be seen to be down regulated on the morpholino injected side of the embryos at stage 22 (Figure 4.8).

While widely used in frog and Zebrafish, the reliability of morpholino-induced phenotypes has recently come under scrutiny (Eisen and Smith, 2008, Kok et al., 2015, Stainier et al., 2015) and many researchers have started to question the reliability of morpholino oligos as a loss-of-function system. Antisense strategies normally lead to some off-target effects. These can make it very difficult to assess whether the resulting phenotype is due to gene knockdown or these off-target effects. It is therefore extremely important to use proper controls to try and prove the specificity of the morpholino (Eisen and Smith, 2008). Morpholino oligos are still widely used in the *Xenopus* research community as a reasonable gene knockdown (gene function interfering) system, where genetic methods (such as gene knockout) have not yet been established as a common method. The recently developed CRISPR/Cas9 system may provide a powerful tool for achieving loss-of-function in the future (Ma et al., 2014, Wang et al., 2015). But as long as materials are overexpressed in the embryos, off-target problems cannot be completely resolved, whichever other methods we may use.

Nevertheless, there is an obvious need to ensure the specificity of the morpholinos used in this study. Therefore, as well as ASPN-MO1, an additional set of morpholino oligos was designed, which target independent areas of the ASPN mRNA (Figure 4.1), and it was confirmed they provided essentially the same phenotypes (i.e. the disruption of frog eye formation). Designing the second morpholino oligos necessitated us to isolate ASPN-b, the allogene of ASPN-a. The cDNA sequence of ASPN-b has been registered in GenBank (LC056842).

The two different ASPN morphlinos (ASPN-MO1 and ASPN-MO2) were shown to induce the same type of eye phenotype, albeit with different efficacies. To further validate their specificity, rescue experiments were carried out. These experiments are generally accepted as one of the means to show morpholino specificity (Eisen and Smith, 2008). For this experimental set up the morpholino phenotype is "rescued" by adding back mRNA, which is immune to the morpholino. This immunity was achieved by using ASPN mRNA, which only contained the coding region (and not the translational start site). For both ASPN-MO1 and ASPN-MO2 the rescue experiments showed that the induced phenotype can be rescued by co-injection of the ASPN mRNA (Figure 4.5; Figure 4.7).
To further verify morpholino activity, the reduced levels of the protein of interest should also be shown via Western Blotting. Unfortunately we could not find any commercially available antibodies, which gave a signal for *Xenopus* ASPN. While not ideal, by using two different morpholinos whose very similar phenotypes can be rescued by adding ASPN mRNA, I have hopefully convincingly proven the specificity of the morpholinos' actions.

Another interesting aspect is ASPN's relationship with the neural inducer Chordin. As shown in Chapter 3, Chordin injected in animal cap leads to increased levels of ASPN. When ASPN-MO1 was co-injected with Chordin, the expression levels of EFTFs Rx1 and Pax6 were down regulated compared to the strong induction observed upon Chordin injection (Figure 4.9). This might suggest that ASPN acts downstream of Chordin, but upstream of the EFTFs. When ASPN is transiently inhibited by ASPN-MO1 in the developing embryo, the EFTFs (at least Rx1 and Pax6) are not induced, which leads to disturbed eye development. In summary,

ASPN is required for frog eye formation, especially in the early stages of eye development.

4.5.2 ASPN's ability to induce ectopic eyes is unique amongst other SLRP family members

Class I ASPN, Decorin and Biglycan share similarities in terms of structure and amino acid sequences (Chapter 3, Figure 3.1). Whilst they also share certain properties that are mediated via their core proteins, such as the ability to bind to collagen, TGF-β and BMP family members, their biochemical characteristics differ from each other. Different binding affinities and binding partners result in a functional diversity (Kou et al., 2010). ASPN can bind type 1 collagen and competes with Decorin (but not Biglycan) for the collagen-binding site (Kalamajski et al., 2009). Furthermore, ASPN has been shown to bind directly to TGF-β and BMP-2 and inhibits them from binding to their respective receptors (Yamada et al., 2007, Nakajima et al., 2007, Tomoeda et al., 2008). Decorin interacts with the TGFβ1 and EGF receptors and either enhances or diminishes their signal intensities (Iozzo and Schaefer, 2010). Likewise, Biglycan binds to BMP4 and regulates early embryogenesis or osteoblast differentiation (Moreno et al., 2005a, Chen et al., 2004). This diversity is also reflected in the embryonic activities of each protein. The ASPN-induced strong eye phenotype could not be found when injecting other class I SLRPs in *Xenopus* embryos (Figure 4.10) (Kalamajski et al., 2009, Kizawa et al., 2005). SLRP members of other classes also did not elicit the ectopic eye phenotype when they were overexpressed – the exceptions being the class II SLRP Lumican and class III SLRP Epiphycan, which occasionally induced a subtle eye phenotype (Figure 4.11, Figure 4.12) (Kuriyama et al., 2006).

Due to the known importance of IGF signalling in the development of the anterior head and eye structures, as well as similarity in overexpression phenotype (Pera et al., 2001, Richard-Parpaillon et al., 2002), it was likely that ASPN elicits its effect through IGF signalling pathway (as indeed I will prove in Chapter 5). IGF is known to induce ERK and AKT phosphorylation *in vitro*. None of the SLRPs tested induced the ectopic eye phenotype observed upon ASPN overexpression. This is consistent with the fact that the levels of ERK and AKT activation by Lumican and Decorin are different (Figure 4.13, D). Therefore, each SLRP seems to have its own unique

functions and is not redundant with others. This demonstrates, that ASPN is unique amongst SLRPs in its role during frog eye development.

4.5.3 ASPN may play a role in Zebrafish eye development

The family of SLRPs seems to be conserved across vertebrate species, with ASPN orthologues identified in several species (Chapter 3, Figure 3.1, B). From an evolutionary point of view, the Zebrafish *Danio rerio* is the furthest relative to mammals. A close evolutionary ASPN orthologue to Zebrafish is the *Xenopus* version. The Zebrafish is a very popular model organism. Much like the frog, they are easy to keep and embryos are easily harvested and used for injections of mRNA or morpholinos (Chhetri et al., 2014, Glass and Dahm, 2004).

Since the techniques used in Zebrafish are very similar to those employed in *Xenopus* work, it seemed like a straightforward approach to test the effects of ASPN during early development in this vertebrate organism. For the injections, I used *Xenopus* ASPN mRNA, which may not be ideal. However, as previously mentioned, studies, which investigated the conserved function of Pax6 amongst vertebrate species initially used the Pax6 homologues of other species (e.g. ribbonworm and squid), and overexpressed them in Drosophila to assess their function (Glardon et al., 1997, Loosli et al., 1996, Tomarev et al., 1997). Also, the mRNA was injected into the fish embryos at the one cell stage, which results in a global up-regulated expression of ASPN, as opposed to the more targeted approach in frog.

At the lowest concentration tested of 100pg, one of the embryos displayed what looked like an ectopic third eye (Figure 4.14, A, B). In published literature ectopic eyes in Zebrafish embryos (Cavodeassi et al., 2005) or ectopic lenses (Kondoh et al., 2000) are rarely reported, compared to those in *Xenopus* (Andreazzoli et al., 1999, Bernier et al., 2000, Chow et al., 1999b, Masse et al., 2007, Mathers et al., 1997a, Pai et al., 2012, Rasmussen et al., 2001, Zuber et al., 1999). However, the Zebrafish embryo is very sensitive to injected material – a reason why it is increasingly used for toxicity testing (McCollum et al., 2011). Too much injected material can quickly lead to general toxicity in the embryos. This makes it often difficult to discern between specific phenotypes induced by the the material injected (e.g. mRNA or morpholino) and those induced by general toxicity (Rosen et al., 2009).

It is possible that ASPN plays a role in Zebrafish eye development. As will be shown in detail in Chapter 5, ASPN elicits its effect through the IGF signalling pathway. IGF signalling has also been shown to play an important role in Zebrafish development. A DN-IGF1R causes loss of head and eyes, as well as an absence of the notochord. Overexpressing IGF1 dorsalises the Zebrafish embryos with an expansion of the forebrain and a reduction of trunk and tail. In severe cases the embryos exhibited a complete lack of posterior and ventral tissues (Eivers et al., 2004). Regarding IGF overexpression, the Zebrafish phenotype is therefore very similar to that found in *Xenopus*, (Pera et al., 2001, Richard-Parpaillon et al., 2002).

Intended as a preliminary screen, I am aware that much more work needs to be carried out to say with certainty whether ASPN fulfils the same role in Zebrafish as it does in frog eye development. But it is a first step to showing a potentially conserved role for ASPN across other vertebrate species and is worth investigating further. To take this research forward, the endogenous Zebrafish ASPN should be used and overexpression analysis carried out on a larger number of embryos, with an optimisation of mRNA concentrations to exclude off-target effects. Again, the possible effects on eye field transcription factors in fish could be assessed using whole mount *in situ* hybridisation techniques. For loss-of-function studies, the CRISPR technique could possibly be employed, which has been shown to work well in Zebrafish (preferable to morpholino use, due to the range of off-target side effects that can occur).

4.6 Summary

Results presented in this chapter showed that ASPN is an essential factor in Xenopus eye development. The knockdown of ASPN by means of morpholino injections inhibited the expression of eye specific genes and resulted in a small eye or complete loss of eye development. Furthermore, ASPN appears to be unique amongst other SLRPs in its ability to induce the striking eye phenotype. Lastly, a first attempt was made to show a potentially conserved role of ASPN in eye development in Zebrafish *Danio rerio*.

CHAPTER 5 – ASPN ACTS THROUGH THE IGF PATHWAY AND INTERACTS WITH OTHER MAJOR SIGNALLING MOLECULES

5.1 Introduction

The studies by Pera et al. (2001) and Richard-Parpaillon et al. (2002) demonstrated the importance of IGF signalling in head and neural development. Their IGF gain-of-function experiments showed expanded and ectopic expression domains of several EFTFs such as Otx2, Pax6 and Six3, which suggests that IGF signalling modulates the expression of EFTFs and thereby contributes to eye induction. The ASPN induced ectopic eyes observed in this study were strongly reminiscent of the IGF induced phenotypes in *Xenopus* (Pera et al., 2001, Richard-Parpaillon et al., 2002). As previously discussed, while very similar, the IGF and ASPN induced phenotypes are not identical. For example IGF overexpression leads to an expansion of Otx2 expression domain in the early embryo, as well as an expansion of the cement gland – the embryo's most anterior structure. Neither of these features could be found upon ASPN overexpression (see Chapter 3). Nevertheless, similarities were great enough for me to hypothesise that at least some of ASPN's effects are mediated through the IGF signalling system. In addition, previous studies showed that fellow class I SLRP Decorin can bind to, and activate, the IGF1R (Schaefer et al., 2007, Schonherr et al., 2005). Decorin seems to regulate cell death and synthesis of matrix components, by acting as a signalling molecule for the IGF signalling system in epithelial and renal cells (Reed et al., 2005, Santra et al., 2002). Receptor tyrosine kinases, such as IGF1R and FGFR, play central roles in eye development. Signalling through the IGF1R receptor can lead to the activation of the two downstream signalling cascades Ras/MAPK and PI3/AKT, which have previously been shown to be important in correct eye formation (Bugner et al., 2011, La Torre et al., 2015, Wu et al., 2006).

With this in mind, I started to investigate how ASPN is associated with the IGF signalling pathway, and the results are presented in this chapter. In the first instance, I show that ASPN can induce IGF mediated AKT and ERK phosphorylation in an *in vitro* system. By means of co-immunoprecipitation, I also show that ASPN associates with the IGF1R and forms a complex. Furthermore, I attempted to elucidate the relationship of ASPN and IGF signals in the context of eye development. Finally, an attempt was made to explain the necessary spatial and temporal selectivity of ASPN/IGF signals, which must contribute to the correct placement of the eye.

Many other signalling pathways are known to play a role in early eye development. BMP and Wnt signals need to be inhibited in the anterior neural plate for the eye field to be able to develop. As part of their study, Richard-Parpaillon et al. (2002) found that IGF1 inhibits Wnt-signalling and affects Wnt target genes - *Siamois*, *Xnr-1*, *Wnt-8* and β-catenin. Naturally ASPN's relationship with other major signalling pathways, such as the Nodal, BMP and Wnt pathways, needed to be investigated. SLRPs, including ASPN, are known to interact with TGF- β family members and also BMP molecules. So in the second half of this chapter I will present my results regarding ASPN's relationship with molecules of the Wnt, BMP and Nodal signalling pathways. By analysing ASPN's effect on these signalling pathways on a molecular level - by means of reporter assay, qRT-PCR analysis, Western Blot and co-immunopreciptiation - I will present evidence that ASPN binds to and inhibits Wnt, BMP and Nodal signalling.

5.2 ASPN induces eye development via the IGF receptor mediated signalling pathway

As mentioned previously, due to the similarity in phenotypes induced by ASPN and IGF overexpression, as well as the prominent role of the IGF signalling pathway in eye development (Pera et al., 2001, Richard-Parpaillon et al., 2002), we hypothesised that ASPN may elicit its effects, at least in parts, through the IGF signalling pathway. To investigate this theory, I first wanted to find out whether ASPN can activate the same signalling pathway as IGF.

5.2.1 ASPN activates the IGF downstream signalling pathway

IGF has been shown to induce phosphorylation of ERK and AKT both in cultured cells and in animal cap explants (Wu et al., 2006, Rorick et al., 2007). Therefore, it was examined whether ASPN activates the same intracellular signalling molecules. This was done in an *in vitro* experimental setting using the cell line HEK 293 – a human embryonic kidney cell line. HEK 293 is widely used and popular as a transient expression system, due to its easily transfectable nature and effectiveness at producing the gene products of the artificially incorporated expression constructs (Lin et al., 2014, Thomas and Smart, 2005). Initially, to ensure that the transfection

technique was working, a YFP containing construct (pCS2+ YFP) was transfected into HEK 293 cells. The successfully transfected cells, which consequently started to fluoresce in green, were imaged and are shown in Figure 5.1. The transfection efficiency was determined to be around 70-80%.

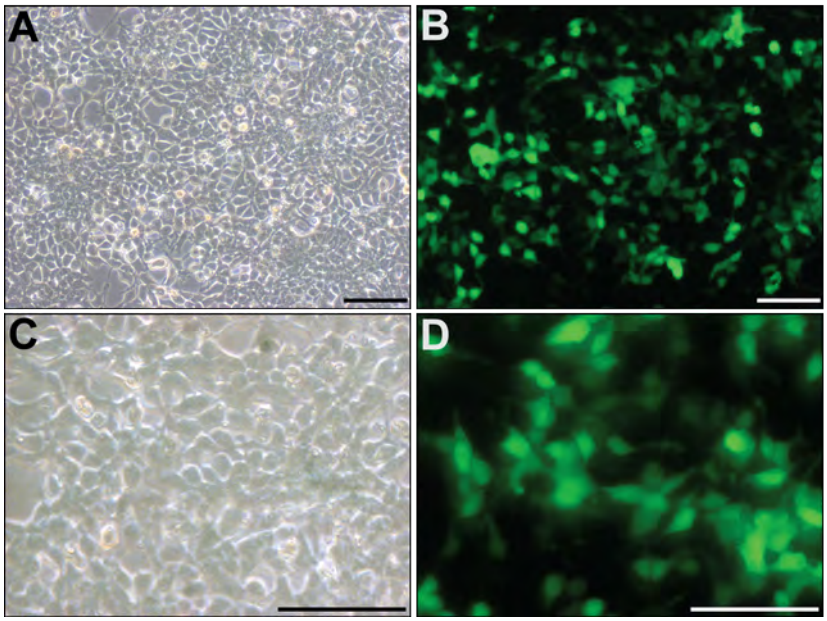


Figure 5.1: Transfection of HEK293 cells with pCS2+ YFP after 24hrs incubation. Bright field image of transfected HEK293 cells (A) and the successfully YFP transfected cells (B) under fluorescent light both at 10x magnification. (C) shows the same cell population in bright field and (D) under fluorescence at 20x magnification. Scale bars shown equal 100μm.

For the actual experiment, HEK 293 cells were separately transfected with ASPN and IGF2. Following three days of culturing in Opti-MEM medium the resulting conditioned media (which contained secreted ASPN and IGF2) was then applied onto another set of HEK293 cells. The cells treated with either ASPN or IGF2 activated the phosphorylation of AKT and ERK within 20 minutes of the treatment (Figure 5.2, lanes 2,3), suggesting that ASPN and IGF share the same downstream intracellular signalling pathways.

However, the phosphorylation of ERK and AKT was only submaximal, presumably because the conditioned medium did not contain maximum levels of the proteins. The normalised up-regulation of the intensities of the phosphorylated ERK and AKT

in the ASPN-treated cells was 1.85 times for pERK and 4.27 times for pAKT.

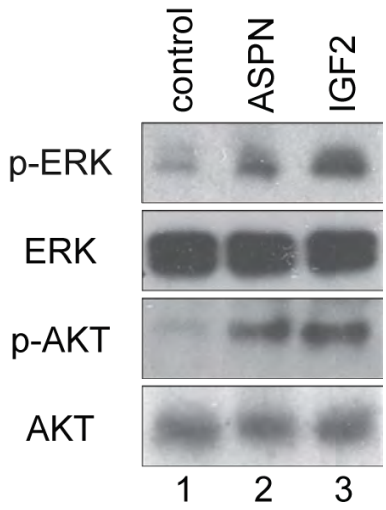


Figure 5.2: ASPN activates ERK and AKT. Conditioned media taken from control GFP (lane 1), ASPN (lane 2) or IGF2 (lane 3) expressing cells were applied to HEK293 cells for 20 minutes. Western blotting analysis was performed with antibodies for phosphorylated ERK (p-ERK), ERK, phosphorylated AKT (p-AKT) and AKT.

5.2.2 ASPN forms complex with IGF1R

The next question was - does ASPN form a complex with the IGF1 receptor? To address this an immunoprecipitation assay was performed. For this purpose, HEK 293 cells were co-transfected with plasmids encoding ASPN (which had a myc tag attached) and the IGF1-receptor (IGF1R), and cell lysates analysed 24 hours post-transfection. The co-immunoprecipitation analysis was carried out using the Protein G sepharose system with the IGF1R antibody (Cell Signaling Technology, #9750), and the complexes detected with the myc antibody (Cell Signaling Technology, #2276) following SDS-PAGE (Figure 5.3). The results seem to indicate that ASPN does indeed establish a complex with the IGF1R.

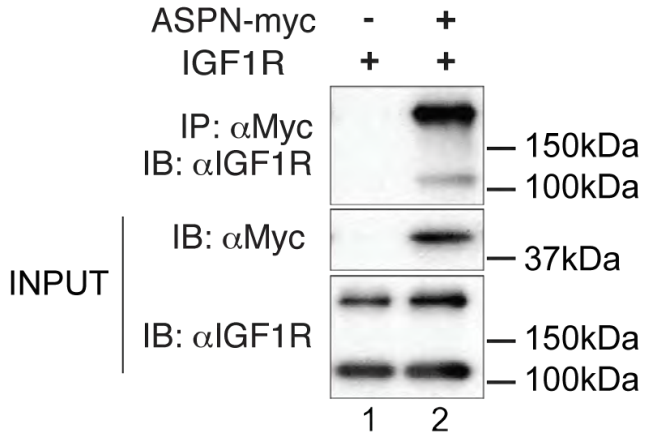


Figure 5.3: ASPN forms a complex with IGF1R. HEK293 cells were transfected with expression vectors carrying IGF1R (lanes 1,2) and ASPN (lane 2) and co-immunoprecipitation analysis was performed using the IGF1R antibody and detected with the myc-antibody. IB: immunoblotting. IP: immunoprecipitation.

5.2.3 ASPN signals through IGF1R and both ASPN and IGF are required for signal transduction

After showing that ASPN forms a complex with the IGF1R, the question remained whether ASPN actually activates and transduces its signal via the IGF1 receptor. To examine this, a dominant-negative version of the IGF1 receptor (dnIGF1R) (Pera et al., 2001) was injected, together with ASPN mRNA, into a dorsal blastomere of the 4-cell embryo and the eye phenotype observed at tadpole stage (Figure 5.4, A, for control, n=20). Upon the injection of ASPN mRNA only the typical ectopic formation could be observed (Figure 5.4, B, 12%, n=112). In contrast, the combined injection of ASPN and dnIGFR mRNAs significantly decreased the size of the eyes on the injected side of the embryo (Figure 5.4, C, 22.6%, n=62).

To further elucidate the relationship between ASPN and IGF, we conversely perturbed the function of ASPN with ASPN-MO1. As reported previously, IGF2 injection caused enlarged eyes (Figure 5.4, D, 90%, n=22) (Pera et al., 2001). However, when ASPN-MO1 was co-injected alongside IGF2 mRNA, the expected eye enlargement was blocked (Figure 5.4, E, 91%, n=23). These results suggest that eye development in *Xenopus* embryos requires both ASPN and IGF signals. IGF2 was used here rather than IGF1 following communication with Dr. Kuroda (co-

author of the Pera et al. (2001) paper), who suggested that IGF2 had the strongest effect out of IGF1 - 3.

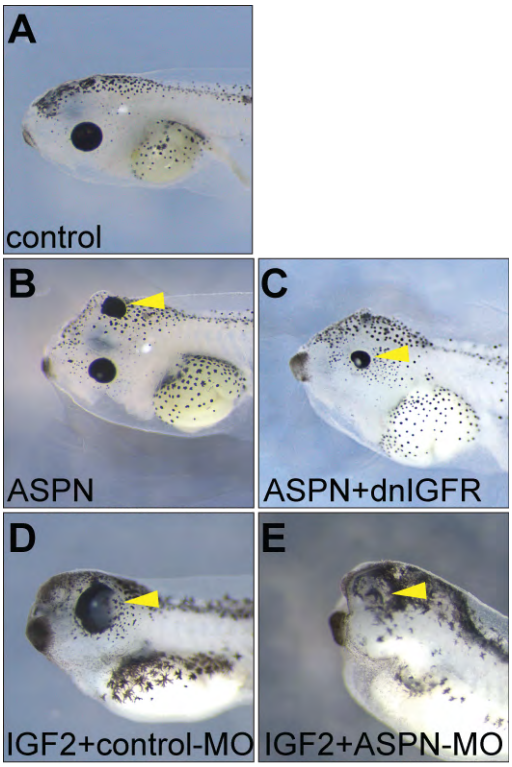


Figure 5.4: Embryonic eye formation requires both ASPN and IGF signals. Embryos were injected with 3 ng β-Galactosidase mRNA (control: A), 1 ng ASPN mRNA (B), 1 ng ASPN + 3 ng dnIGF1R mRNAs (C), 1 ng IGF-2 mRNA + 20 ng control-MO (D) or 1 ng IGF-2 mRNA + 10 ng ASPN-MO1 (E) into the dorsal animal blastomere at the 4-cell stage, and phenotypes were evaluated at stage 42. Affected areas are indicated with yellow arrowheads.

To further confirm the necessity of both ASPN and IGF activity in eye development, on a molecular level, an animal cap assay was performed. IGFs and IGF1R are widely expressed in the early *Xenopus* embryo and were therefore assumed to be present in the ectodermal tissue of the excised animal caps. ASPN and dnIGFR mRNAs, or IGF2 mRNA and ASPN-MO1, were co-injected and the expression of EFTFs *Pax6* and *Rx2a* assayed through quantitative RT-PCR. Un-injected control animal caps showed no induction of either Pax6 or Rx2a expression (Figure 5.5A(i), B(iv)). As expected, ASPN and IGF2 mRNA injected separately were both capable of up-regulating Pax6 and Rx2a expression in the animal caps (Figure 5.5A(ii),

B(v)). The expression of both EFTF genes was however down-regulated when ASPN and IGF2 were co-injected with the inhibiting constructs dnIGF1R and ASPN-MO1, respectively (Figure 5.5A(iii), B(vi)). These results again underpin the concept that ASPN and IGF are both required for the early steps of eye development. Together, these data demonstrate that ASPN induces eye development by regulating the IGF signalling pathway through an association with the IGF1-receptor.

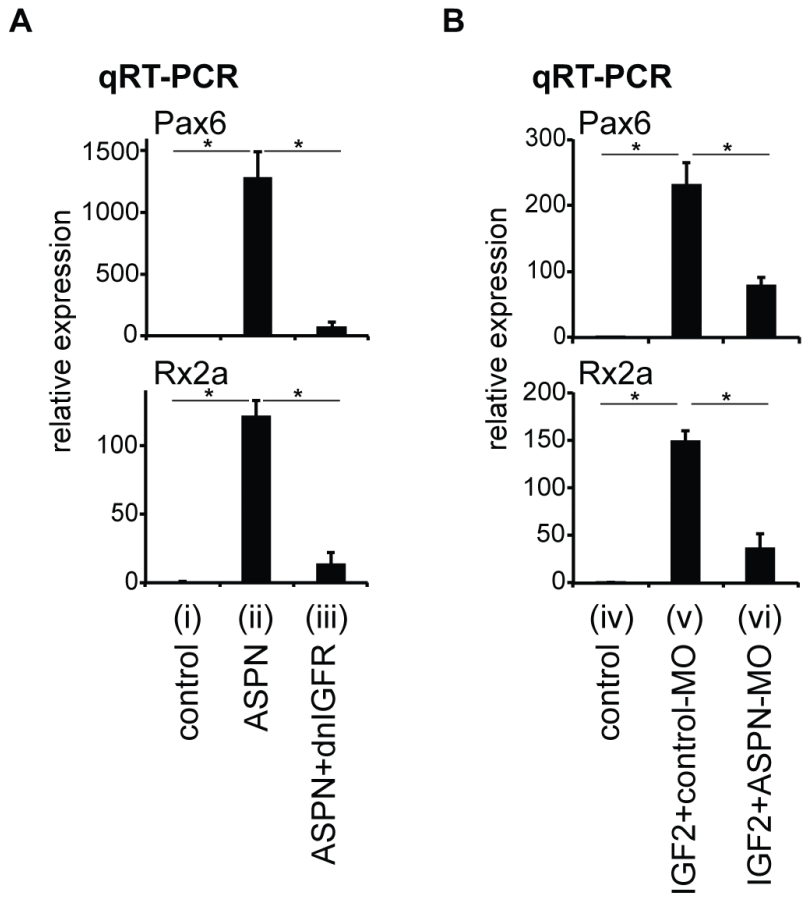


Figure 5.5: ASPN and IGF signals are both required for the early steps of eye development. (A, B) Embryos were injected with 3 ng β-Galactosidase mRNA (control (i), (iv)), 1 ng ASPN mRNA (ii), 1 ng ASPN + 3 ng dnIGF1R mRNAs (iii), 1 ng IGF-2 mRNA + 20 ng control-MO (iv) or 1 ng IGF-2 mRNA + 10 ng ASPN-MO1 (vi) into the dorsal animal blastomere at the 4-cell stage. Animal caps were prepared and analysed at stage 22 for Pax6 and Rx2a expression with qRT-PCR (*P<0.01, Student's t-test). Error bars represent s.e.m.

Bugner and colleagues (Bugner et al., 2011) recently showed that IGF1R substrate IRS-1 plays an important role in *Xenopus* eye development. They showed that IRS-1

is specifically expressed in the anterior neural plate including the eye field, around the time of eye induction. In a recent publication by Gao et al. (2014), IGF1R was shown to be able to transcriptionally up-regulate IRS-2 levels. Some functional redundancy has been reported for IRS-1 and IRS-2 (Bugner et al., 2011), which made me curious whether ASPN-activated IGF1R can up-regulate IRS-1 transcription in the animal cap explant. In a preliminary attempt to determine if the ASPN/IGF1R signal is transduced via IRS-1; I compared IRS-1 levels in un-injected and ASPN injected animal caps by means of semi-quantitative RT-PCR. The results were unfortunately inconclusive, as some ASPN injected animal cap samples showed an increase and others no elevation of IRS-1 levels (Figure 5.6).

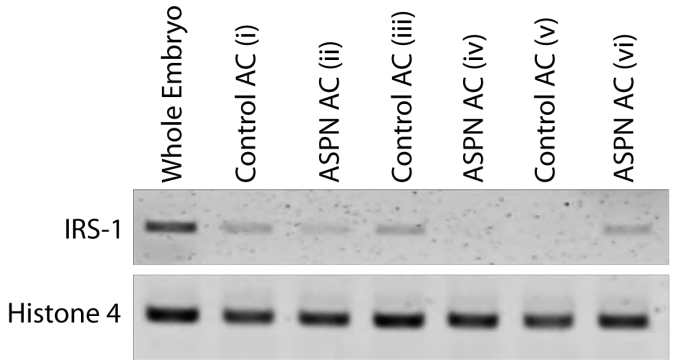


Figure 5.6: Attempt to elucidate ASPN's downstream effects on IRS-1 expression levels. Embryos were either un-injected (i), (iii), (v) or injected with 3ng of ASPN mRNA (ii), (iv), (vi) and animal cap explants prepared and analysed with semi-quantitative RT-PCR for expression levels of IRS-1.

5.3 ASPN interacts with and antagonises Nodal, BMP and Wnt molecules

There is a range of signalling molecules that are involved in the early development of the vertebrate eye (Ikeda et al., 2005). It has been shown that SLRP family members can interact with, and inhibit, the function of a number of signalling compounds in a context-dependent manner (Dellett et al., 2012). With that in mind, I investigated ASPN's ability to affect the important Nodal/Activin-, BMP- and Wnt–signalling pathways.

5.3.1 ASPN inhibits Nodal, BMP and Wnt signalling in luciferase assay

In the first instance, it was important to find out how, and if, ASPN influences these other signalling pathways. For this purpose, reporter constructs of either the Activin-Response Element (ARE; for Nodal/Activin), BMP-Response Element (BRE; for BMP signals) or TOPFLASH (for Wnt) were injected together with mRNAs of Activin (for ARE), BMP4 (for BRE) or Wnt8 (for TOPFLASH) into the embryos. This acted as a positive control and confirmed the reporter activities were elevated at early gastrula stage. When ASPN mRNA was co-injected with either of these signalling molecule mRNAs their reporter activity was significantly reduced (Figure 5.7). This suggests an inhibitory effect of ASPN on Nodal/Activin-, BMP- and Wnt–signalling pathways.

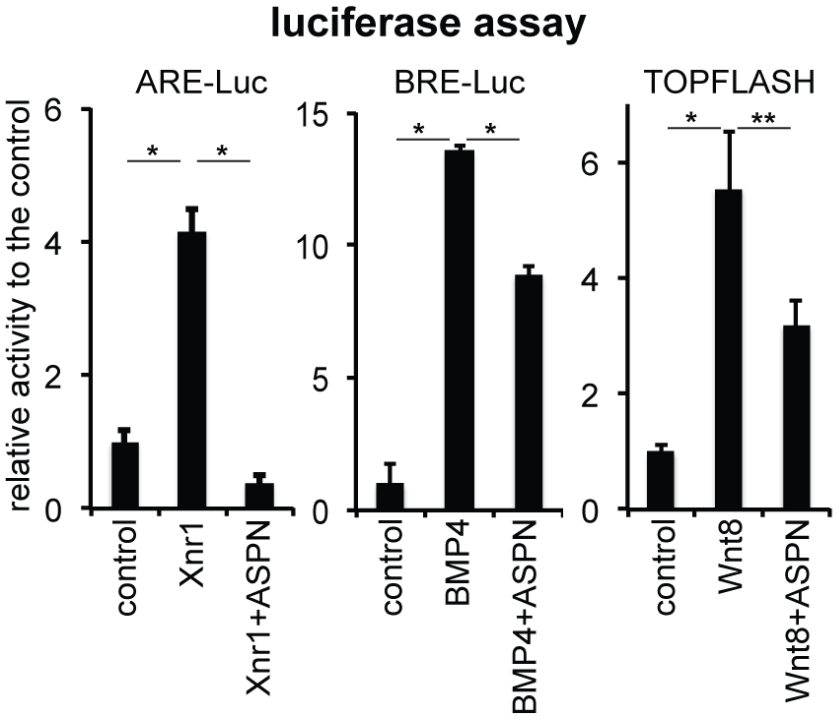


Figure 5.7: ASPN blocks endogenous Activin, BMP and Wnt signals, as examined by luciferase assays. ARE-luc, BRE-luc or TOPFLASH reporter constructs were injected with 1 ng β-Galactosidase mRNA (control), 100 pg Xnr1 mRNA (for ARE), 100 pg BMP4 mRNA (for BRE), 100 pg Wnt8 mRNA (for TOPFLASH), 100 pg Xnr1 + 1 ng ASPN mRNAs (for ARE), 100 pg BMP4 + 1 ng ASPN mRNAs (for BRE) or 100 pg Wnt8 + 1 ng ASPN mRNAs (for TOPFLASH) and were assayed at stage 12.

In order to further confirm that ASPN indeed has the ability to inhibit these signals, expression analyses were performed using either whole embryos or animal cap extracts. The animal caps were injected with ASPN mRNA and then treated with Nodal for 2 hours. The expression of the Nodal target gene Mix.2 (which is up-regulated following treatment with Nodal) was analysed with qRT-PCR. The expression level of Mix.2 was found to be down regulated in the ASPN injected animal caps, compared to control explants (Figure 5.8).

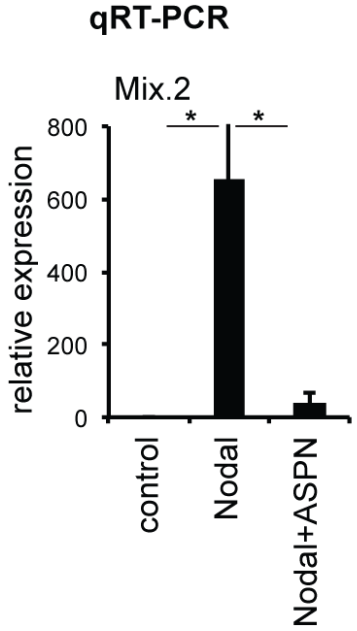


Figure 5.8: ASPN down regulates the Nodal target gene expression Mix.2. Un-injected or ASPN mRNA injected animal caps were treated with human recombinant Nodal for 2 hours at a concentration of 10 ng/ ml and then the relative expression of Nodal target gene Mix.2 determined via qRT-PCR (*P<0.01, Student's t-test). Error bars represent s.e.m.

Next, ASPN's inhibitory effect on the BMP signal was investigated more closely. Either Chordin, which is a well-known BMP inhibitor (Sasai et al., 1995), or ASPN mRNA was injected into embryos and the expression of general neural markers analysed at early neurula stage. Sox2 and NCAM were expressed at stage 14 in both chordin and ASPN injected animal cap explants. This is consistent with the idea that ASPN inhibits the BMP signalling pathway and thereby promotes a neural fate (Figure 5.9).

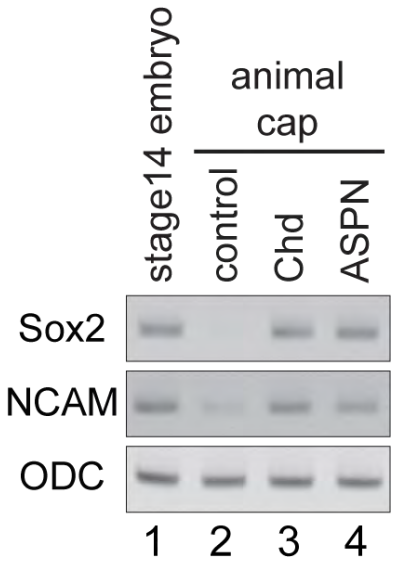


Figure 5.9: ASPN induces general neural markers Sox2 and NCAM. When known BMP-inhibitor Chordin is injected into Xenopus embryos general neural markers NCAM and Sox2 are found to be up-regulated at neural stage (lane 3), compared to un-injected control animal cap (lane 2). When ASPN mRNA was injected Sox2 and NCAM were also found to be up-regulated (lane 4), which further suggests that ASPN may act as a BMP inhibitor.

ASPN's relationship with the Wnt signalling pathway was also investigated further. Wnt8 mRNA was injected either on its own or together with ASPN mRNA and the animal cap explant then analysed for the expression of Xnr3, which is one of the target genes of the Wnt signalling pathway (Yang-Snyder et al., 1996). As expected, the expression of Xnr3 was induced when Wnt8 was injected. However, when ASPN was co-injected, Xnr3 levels were found to be significantly reduced (Figure 5.10).

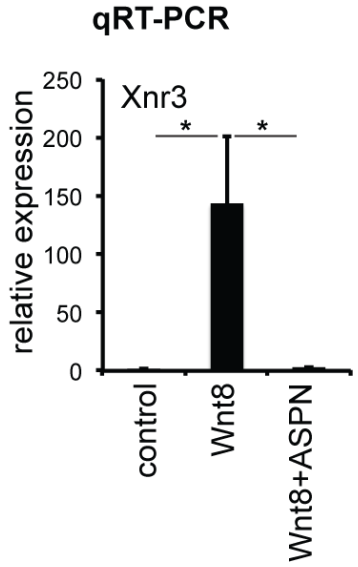


Figure 5.10: ASPN inhibits Wnt signalling pathway target Xnr3. Animal caps injected with Wnt8 mRNA were analysed at stage 10.5 and showed an up-regulation in Wnt pathway target gene Xnr3. When ASPN mRNA was co-injected Xnr3 induction in the explants was found to be inhibited (*P<0.01, Student's t-test). Error bars represent s.e.m.

5.3.2 ASPN binds to Xnr-1, BMP4 and Wnt8

The previous results revealed that ASPN acts as a multiple inhibitor for Nodal, BMP and Wnt signals. As ASPN is a secreted factor, I hypothesised that ASPN forms complexes with the other signalling molecules. In an attempt to find out how ASPN exerts its inhibitory effect on the aforementioned signalling molecules binding assays were performed.

To perform this, tagged versions of expression constructs encoding Xnr-1, BMP4 or Wnt8 were transfected into HEK 293 culture cells together with a tagged version of ASPN, and a co-immunoprecipitation analysis was performed. The results show that ASPN does indeed form complexes with all three tested molecules BMP4 (Figure 5.11, A), Xnr-1 (Figure 5.11, B) and Wnt8 (Figure 5.11, C), suggesting that ASPN interacts with these molecules in the extracellular space and thereby impedes their activities.

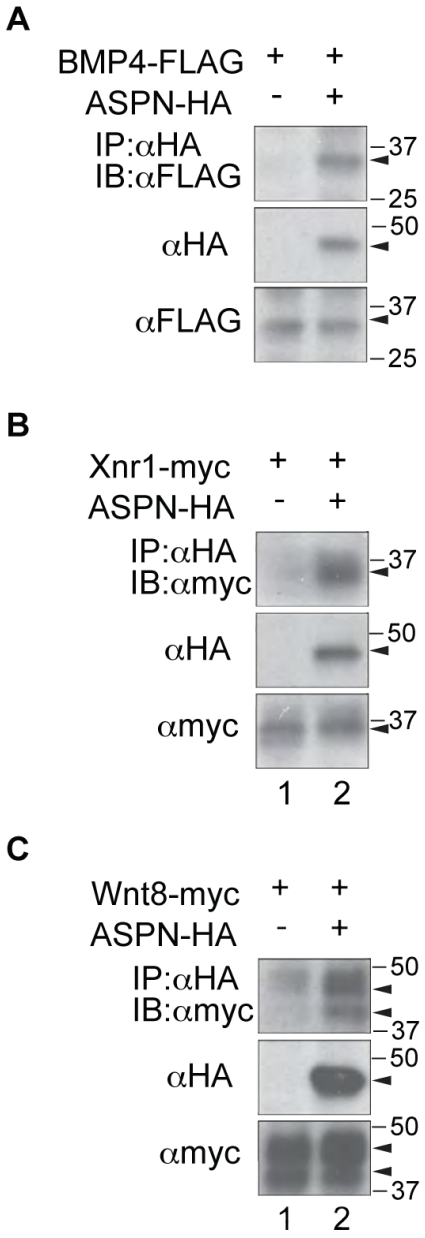


Figure 5.11: ASPN forms complexes with BMP4, Xnr1 and Wnt8 proteins. In order to avoid artificial interactions within the same cells, each expression construct was separately transfected into HEK 293 cells. Cells were then combined on the following day as indicated: (A) BMP4-FLAG and ASPN-HA; (B) Xnr1-myc and ASPN-HA; (C) Wnt8-myc and ASPN-HA. The cell lysates were collected after two additional days of culturing and immunoprecipitation (IP) was performed with the flag (A) or myc (B,C) antibodies.

5.4 Discussion

5.4.1 ASPN acts via the IGF signalling pathway

The work presented in this chapter revolved around the relationship of ASPN with some of the major signalling pathways, thought to be important in early eye development. Due to similarities in phenotypes, and known importance of IGF signals for anterior neural development, there was a big focus on investigating ASPN's potential association with the IGF pathway.

Firstly, I showed that ASPN can activate the phosphorylation of ERK and AKT *in vitro*, which are known to be downstream targets of the IGF signalling pathway (Figure 5.2). Following IGF treatment, cells have been shown to develop strongly elevated levels of AKT phosphorylation (Romanelli et al., 2007, Ye et al., 2010). The IGF-PI3-AKT pathway is known to be important for neural proliferation and survival (Johnson-Farley et al., 2007), as well as other crucial cell activities such as motility, metabolism and differentiation (Bugner et al., 2011). More recently, its importance for eye field specification has been unveiled (La Torre et al., 2015). IGF signalling through the Ras-Raf-MAPK-ERK is well studied and thought to be key for IGF mediated cell proliferation. In neural cells this pathway is believed to contribute mainly to cell maturation and survival processes (D'Ercole et al., 1996, Ye et al., 2010).

I have also shown, that ASPN forms a complex with the IGF1R *in vitro*, as seen in Figure 5.3. While the results show that ASPN co-immunoprecipitates with the IGF1R, it cannot be excluded that ASPN actually binds to IGF instead. Bound ASPN might in turn promote the binding of IGF to the receptor. My colleague Dr. Sasai has shown in a separate experiment that ASPN also binds to IGF2 (Appendix Figure 7.2). However, we did not find a significant difference of IGF2 binding to its receptor in the absence and presence of ASPN. This needs to be analysed in a more quantitative way to be able to draw definite conclusions, which is beyond the scope of this project. However, all the co-immunoprecipitation experiments were performed with overexpressed constructs and therefore the environment was stoichiometorically in favour of the expressed constructs and not the endogenous proteins. It is therefore unlikely that the binding and precipitation of the two proteins

involved endogenous proteins. Also, the co-immunoprecipitation assays in this study were performed in HEK 293 cells (i.e. non-physiological conditions), which might be perceived as sub optimal and not as relevant as *in vivo* data would be. While experiments in physiological conditions would have been ideal, there were unfortunately no antibodies commercially available that gave significant signals in either Western blotting or immunohistochemistry.

ASPN seems to mediate its effect on eye development via the IGF1R, as seen when ASPN function was inhibited by the co-injection of the dominant-negative form of the IGF1 receptor, both in the long cultures and the animal cap assay. Without a functioning form of the IGF1R, the ability of ASPN to induce ectopic eyes in the embryos was drastically reduced in whole embryos (Figure 5.4, B, C). In the animal cap injected ASPN mRNA in combination with dn-IGF1R lead to only a minimal induction of EFTFs Pax6 and Rx2a (Figure 5.5, A).

Results indicate that ASPN and IGF are both required for eye development. When IGF2 was co-injected with ASPN-MO1, the expected IGF2 mediated expansion of the embryos' eyes was diminished or eye development even inhibited (Figure 5.4, D, E). Also, IGF2 mediated induction of EFTFs Pax6 and Rx2a in the animal cap was inhibited when ASPN-MO1 was co-injected (Figure 5.5, B). This suggests that both ASPN and IGF are necessary and important in the early stages of eye development in *Xenopus laevis*.

Lastly, I wanted to make an attempt in finding out how ASPN and IGF transduce their specific eye development actions, in the right temporal and spatial fashion. IGF and ASPN are both ubiquitously expressed in anterior regions at the time of eye induction in *Xenopus laevis*. However, they seem to fulfil crucial roles in specific tissue, and perform these roles in a very regulated timely fashion, during early eye development. The question poses itself - how this specific ASPN and IGF signalling is achieved at the right place and time, especially when both ligands and IGF1R are present in a large area at that crucial developmental stage?

Adaptor proteins such as Kermit2 or IRS1 could play a role in coordinating specific spatial/temporal signalling patterns of ASPN and IGF (Bugner et al., 2011, Wu et al.,

2006). IGF1R's substrate IRS-1 in particular was shown to play an important role in *Xenopus* eye development, via activation of PI3/AKT signalling pathway. Bugner and colleagues showed a specific expression pattern for IRS-1, which was limited to the anterior neural plate and the eye field, around the time of eye induction. Loss-of-function of IRS-1 through the use of a morpholino, resulted in the downregulation of EFTF expression domains and overall small eyes. It is imaginable that such a specifically expressed substrate of the IGF1R receptor ensures that the ASPN/IGF signal contributes to eye development, in the right tissues, at the correct developmental stage. To make a first attempt at evaluating this, I analysed un-injected and ASPN injected animal caps for possible elevations in IRS-1 levels. Unfortunately my preliminary experiment proved inconclusive (Figure 5.6).

In summary, ASPN seems to induce eye development by regulating the IGF signalling pathway through a direct or indirect interaction with the IGF1R. Both ASPN and IGF are required for the induced eye development.

5.4.2 ASPN antagonises Nodal, BMP and Wnt proteins

The results also show that ASPN interacts with other major signalling molecules such as Nodal, BMP and Wnt proteins. In the luciferase assay ASPN inhibited the Nodal, BMP and Wnt signalling pathways (Figure 5.7). After investigating each of these three pathways more closely, it turned out that ASPN down-regulates both the nodal target gene Mix.2 (Figure 5.8) and Wnt target gene Xnr3 (Figure 5.10) in animal cap explants. Furthermore, it could be shown indirectly that ASPN inhibits BMP signalling. Much like well-known BMP-inhibitor Chordin, ASPN induced general neural markers Sox2 and NCAM and therefore promotes neural fate (Figure 5.8).

Consistent with the notion that ASPN inhibits Wnt and BMP signalling, my colleague Dr. Kurosawa found that ASPN mRNA injections at the equator region resulted in a reduction of Xbra (Xenopus orthologue of Brachyury) expression (Appendix Figure 7.3) at gastrula stage, suggesting that mesoderm determination was severely disrupted by ASPN. The shortened body axis phenotype, which was exhibited at the tadpole stage upon ventral animal ASPN injection (Figure 3.12),

could be caused by disrupted Xbra expression. Xbra is a crucial factor for the promotion of convergent extension in the developing embryo (Kwan, 2003).

ASPN also forms a complex with Xnr-1, BMP4 and Wnt8 in co-immunoprecipitation assay (Figure 5.11, A-C). To exclude the notion that ASPN acts as a general ligand-binding proteoglycan, Dr. Sasai analysed the potential binding of ASPN to some major receptor proteins, which are shown in supplementary Figure 7.2 (Appendix). He found that ASPN did not bind to the Activin receptor, BMP receptor or Frizzled receptor. While these results obviously do not exclude the possibility that ASPN may bind to other receptor proteins, it does suggest that ASPN binds to proteins in a selective manner.

Together these data suggest that ASPN interacts with major signalling molecules that antagonise the eye formation in the extracellular space and blocks those activities.

5.5 Summary

Results indicate that ASPN interacts with many other regulatory molecules including BMP, Wnt and Nodal in addition to IGF and IGF1R, but has no (or very little) affinity to bind other receptor proteins, such as Activin receptor, BMP receptor and Frizzled receptor (supplementary Figure 7.2). It suggests that ASPN acts as a multiple inhibitor for Nodal, BMP and Wnt proteins. ASPN therefore interacts with important signalling molecules, which antagonise eye development and inhibits their function. This means that ASPN is not just an extracellular matrix protein that randomly binds to proteins, but instead an active modulator for other signal molecules. Furthermore, ASPN can apparently regulate bound proteins in different ways. BMP, Nodal and Wnt are inhibitied by ASPN while in contrast the IGF signal is promoted. This finding may account for the phenotypic differences following overexpression of IGF and ASPN; ASPN exhibits a strong phenotype specifically in the eye, while IGF induces the whole head structure including cement grand.

CHAPTER 6 - GENERAL DISCUSSION

6.1 Summary of findings in this study

When screening SLRP family members for their effects on early development, the Xenopus ortholog of ASPN induced a striking eye phenotype in the developing tadpoles. I decided to analyse this phenotype more closely and to try and determine ASPN's potential role in eye development, as well as its underlying molecular mechanisms. The aim of this study was to investigate the ASPN induced eye phenotype more closely and elucidate the molecular mechanism underlying ASPN's action.

In Chapter 3, I introduced ASPN as a novel factor involved in *Xenopus* eye development. ASPN is expressed in the area of the presumptive eye field around the time of eye induction, although detailed expression patterns around stage 11/12 could unfortunately not be visualised. I characterised the induced phenotype more closely and could show evidence that the induced structures indeed have eye character. The question of whether these ectopic eyes possess any functionality cannot be answered with certainty at this point. Furthermore, I showed that ASPN induces eye field transcriptions factors both *in vivo* and *in vitro*. Overall, the ASPN induced phenotype has many similarities to the IGF overexpression phenotype, described in detail by Pera et al. (2001) and Richard-Parpaillon et al. (2002), while not being identical.

Results presented in Chapter 4 showed that ASPN is indeed essential for frog eye development. Experiments using ASPN morpholino oligos resulted in reduced or complete inhibition of eye formation. The ASPN morpholino injected embryos showed reduced levels of EFTFs on the injected sides. I investigated if ASPN's effects are unique amongst SLRPs and showed that the ability to induce ectopic eye development is not shared with other SLRP family members. Fellow class I SLRP Decorin and class IV Chondroadherin had no effect on the tadpoles' eye development, while Lumican and Epiphycan induced mild eye phenotypes.

Molecular mechanisms were investigated in Chapter 5. I could show that ASPN activates the IGF signalling pathway and can bind to the IGF1R. Additionally, ASPN antagonises nodal, BMP and Wnt protein signalling.

6.2 The proposed model of ASPN's actions during eye development

Based on the data collected in this study, I propose the following mode of action for ASPN in the context of *Xenopus* eye development (as illustrated in Figure 6.1): At a specific time and in specific tissue, around the time of eye induction, ASPN (possibly induced by Chordin) activates the IGF signalling pathway. To enable this ASPN forms a complex with the IGF1R, either directly or possibly while bound to IGF. The ASPN/IGF1R signal then induces EFTFs, such as Rx1 and Pax6, which leads to eye field specification. At the same time ASPN also inhibits Wnt, Nodal and BMP signalling in a temporal and spatial specific manner – presumably by binding to the signalling proteins and thereby inhibiting them from attaching to their respective receptors.

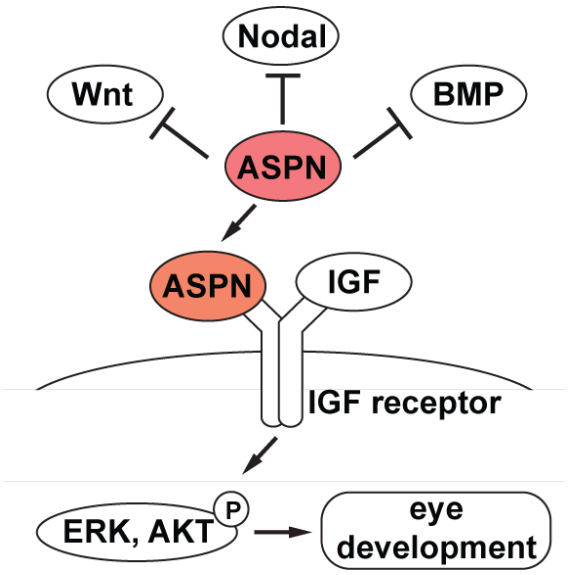


Figure 6.1: Proposed molecular mechanism of ASPN in eye development. ASPN binds to signalling molecules from the Wnt, Nodal and BMP families and thereby antagonises these signalling pathways. ASPN also binds to the IGF1R and activates downstream signalling, which leads to increased ERK and AKT phosphorylation. The ASPN/IGF1R signal leads to an up-regulation of EFTFs, such as Rx1 and Pax6, eventually leading to eyefield specification and eye formation.

The experiments presented here have demonstrated that ASPN interacts with IGF and IGF1R, activates the downstream signalling pathways (Figure 5.2; Figure 5.3) and that both IGF and ASPN are required for the activation of these pathways, which

leads to eye development (Figure 5.4; Figure 5.5; Appendix Figure 7.1). Since both IGF and ASPN need to be present, this might suggest that they bind to the IGF1R at the same time. Interestingly, neural inducer Chordin activated ASPN expression, while IGF did not (Figure 3.3 B (ii), (iii)). This suggests that two independent regulatory pathways (Chordin/ASPN and IGF) are involved in the induction of eye development.

The importance of IGF1 was further shown by Mellough et al. (2015) in hESC (human embryonic stem cells). The addition of exogenous IGF1 to the cultured cells induced the formation of three-dimensional eye-like structures and primitive lens and cornea. However, the inhibition of IGF1R reduced the formation of these ocular structures. They concluded that IGF signalling must be important for both the early stages of eye development, as well as later stage photoreceptor maturation processes.

More evidence that both an active IGF signal and inhibition of BMP, TGF-β and Wnt signals, are important for proper eye development, comes from recently published work: Zhou and colleagues (2015) showed that simultaneous inhibition of BMP, TGF-β and Wnt signalling is needed for eye development. Coco (Dand5), a Cerberus family member, is expressed in the developing and adult mouse retina. In human embryonic stem cells (hESC), Coco exposure was shown to induce differentiation into S-cone photoreceptors. Coco exhibited a synergistic, dose-dependent activity with IGF-1 in blocking BMP, TFG-β and Wnt signalling. IGF-1 greatly enhanced Coco's inhibition (Zhou et al., 2015).

The IGF1 receptor and IGFs are expressed widely during development and are involved in many cellular processes such as proliferation, maturation, survival and growth (O'Kusky and Ye, 2012). ASPN *in situ* data also showed ubiquitous expression around the time of eye induction. So how can be ensured that eye induction only takes place in specific tissues and the right time? Specification of the presumptive eye region may rely on spatial and temporal coordination of ASPN and IGF, i.e. when and where the two molecules' signals intersect. IGF-related proteins, such as IGFBPs, are known to modulate IGF signalling and may contribute to achieving the specification. Furthermore, IGF mediator proteins IRS-1 and Kermit2 (GIPC2) have been shown to play important roles in eye development (Bugner et al.,

2011, Wu et al., 2006). Both are intracellular proteins that interact with the IGF1R and transduce downstream signals, such as the PI3/AKT and MAPK/ERK pathways, and may selectively modulate the IGF1 receptors downstream response to ASPN/IGF binding. Detecting levels of IGF, ASPN, ERK/AKT activation and mediators IRS-1/Kermit2 *in vivo* will help to better understand how the distribution of these molecules relates to eye development.

6.3 Are these insights translatable to mammals and humans?

ASPN obviously plays an important role in the *Xenopus laevis* eye development. The question remains, whether the same holds true for other vertebrates, in particular mammals and humans?

I carried out some preliminary overexpression experiments in the Zebrafish *Danio rerio*. While not statistically significant, due to a low n-number, one embryo exhibited an ectopic eye-like structure (Figure 4.14). Evidence that IGF signalling also plays an important role in anterior development in fish comes from work carried out by (amongst others) Eivers et al. (2004): Injection of DN-IGF1R caused the loss of head and eyes, as well as an absence of the notochord. The overexpression of IGF1 dorsalised the embryos, resulting in an expansion of the forebrain and a reduction of trunk and tail. In severe cases the embryos exhibited a complete lack of posterior and ventral tissues. The Zebrafish phenotype is similar to that found in *Xenopus*. The question as usual is whether the same is true for mammals. Mouse mutants only exhibit an overall decreased size, which might suggest only a limited role for IGF in differentiation and cell fate regulation in the mouse (Eivers et al., 2004, Zuber et al., 2003). Another possibility is that there is a high degree of redundancy in the highly complex mammalian IGF signalling system. The phenotype variation could also be due to differences in experimental technique. The use of dominant negative receptors causes a more global and unspecific blocking of the signalling pathways (i.e. IGF1, IGF2, In, InR). For example mice with a deficiency in both IGF1R and InR show a more severe phenotype (Eivers et al., 2004).

All components of the IGF signalling system are widely expressed in human brain and cerebrospinal fluid (CSF) (Chesik et al., 2006, Mashayekhi et al., 2010, O'Kusky and Ye, 2012). However, there is still very little information available regarding the role of IGF signalling in human neural development. Individuals with mutations in both *igf1* or *igf1r* gene have been described, and the overall phenotype seems to be consistent with those observed in rodents: intrauterine growth retardation, microcephaly, severe deafness and mental retardation. While smaller brain size was observed, the overall brain architecture and myelination was found to be normal. Individuals with mutated *igf1* gene had severely increased IGF2 serum levels, which might indicate a level of redundancy (Ye et al., 2002a, Ye et al., 2002b). The findings of relatively normal cell differentiation and development, upon IGF1R knockout, stands in stark contrast to the results obtained in *Xenopus* in this study, as well as by Pera et al. (2001) and Richard-Parpaillon et al. (2002), and in Zebrafish (Eivers et al., 2004). It might be possible that the role of IGF signalling in anterior neural induction and development was retained in *Xenopus* and Zebrafish, while it has been lost in mice or masked through other functions. Since the mammalian IGF system is very complex, one can expect a high rate of functional redundancy to occur.

Very recently, work carried out on ASPN knockout mice has been published (Awata et al., 2015). The *ASPN* $^{-/-}$ mice were created using homologous recombination in embryonic stem cells. While the *ASPN* $^{-/-}$ mice phenotype is not described in detail, the animals were reported to be fertile and without any apparent developmental defects until 5 weeks after birth. Also, when ASPN was first characterised by Henry et al. (2001), they did not find ASPN expression in the eye of the developing mouse until 15.5 dpc (days post coitum). ASPN expression could then only be detected in the mouse embryos sclera (Henry et al., 2001). This might suggest, that ASPN does not play the same role in eye development in mouse.

On the other hand, much like the mammalian IGF system, SLRP signalling might just be more complex in mammals. Functional redundancy and compensating mechanisms have often been observed. Despite their distinct functions, some SLRPs are similar enough, to be able to rescue the effects of another SLRP, which had been knocked out. For example increased levels of Lumican were found in Fibromodulin

deficient tendon (Svensson et al., 1995) and Decorin and Biglycan can compensate for each other in a bone phenotype with a synergistic effect in double knockout (Ameye and Young, 2002). Mutations in several SLRPs and loss-of-function has been shown to affect the human eye. For example mutated forms of Nyctalopin cause congenital stationary night blindness (Bech-Hansen et al., 2000), abnormal forms of Keratocan cause a corneal disorder (Pellegata et al., 2000) and changes in Opticin, PRELP, Lumican and Fibromodulin lead to high myopia (Lin et al., 2010, Majava et al., 2007). This indicates, that SLRPs are involved in human eye development, which might mean that ASPN also plays a role.

6.4 Future directions

There are still many questions left to answer. ASPN clearly interacts specifically with many different ligands and receptors. It would be interesting to try and identify other possible binding partners and interactions, via high through put screening. On a molecular level, more detailed analyses will be required to better understand the association between ASPN, IGF, IGF1R and downstream signalling components such as phosphorylated ERK and AKT. This will further help to understand, how these different players work together to facilitate frog eye development.

To take this project one step further it would be important to characterise ASPN's potential role in eye development in a mammalian model. Prof. Makoto Asashima reported in 2003 (Sedohara et al., 2003) the generation of whole *Xenopus* eyes *in vitro* using tissue culture. These *in vitro* eyes exhibited layered retina, lens and, once implanted into host tadpoles, successfully innervated the host's tectum. More recently ground-breaking work was carried out by Prof. Yoshiki Sasai and his team, who showed that both human and murine ES cells are capable of generating self-organizing optic cups in three dimensional cultures (Eiraku et al., 2011, Nakano et al., 2012).

As previously mentioned, IGF signalling has been shown to drastically improve *in vitro* eye formation in human embryonic stem cells, as well as simultaneous inhibition of Wnt and TGF-β signalling (Mellough et al., 2015, Zhou et al., 2015). It

is imaginable that if ASPN acts in a similar fashion in mammalian cells it could prove a useful tool for *in vitro* eye induction. A range of 'eye-maker' proteins have been identified over the years. Being an extracellular protein, ASPN would have the advantage of easy application to treat cells to induce differentiation *in vitro*.

Another interesting aspect relates to how the forebrain and retinal lineages are separated from each other during development. The work presented here suggests that tissues/cells that are exposed to anti-Wnt, anti-Nodal and pro-RTK (IGF) signals, in addition to anti-BMP signals, tend to acquire a retinal cell fate (as facilitated by Asporin in frog). Apart from the role of ASPN in this process *per se*, this represents an important principle. Although ASPN may not be expressed in the early retinal area in the mouse or human, the same mechanism may still be conserved in those organisms, but facilitated through other factors. My colleague Dr. Noriaki Sasai is currently pursuing this possibility in his laboratory, by chemical manipulation of the Wnt and Nodal levels in chick explants. Future studies will be focused on isolating such gene(s) in other species and human.

Appendix

The data presented in this section were acquired by my colleagues Dr. Noriaki Sasai (Figure 7.1 and 7.2) and Dr. Maiko Kurosawa-Yoshida (Figure 7.3).

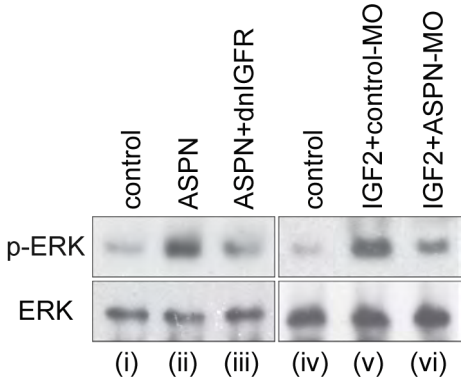


Figure 7.1: Both IGF and ASPN are required for the full activation of ERK. Animal cap explants were prepared from 3 ng control β-Galactosidase (i,ii,iv), 3 ng dnIGFR mRNA (iii), 20 ng control-MO (v) or 20 ng ASPN-MO (vi) injected embryos and were incubated with the conditioned media expressing control (i,iv), ASPN (ii,iii) or IGF2 (v,vi) for 20 minutes. The explants were analysed by western blotting using phospho-ERK or ERK antibodies.

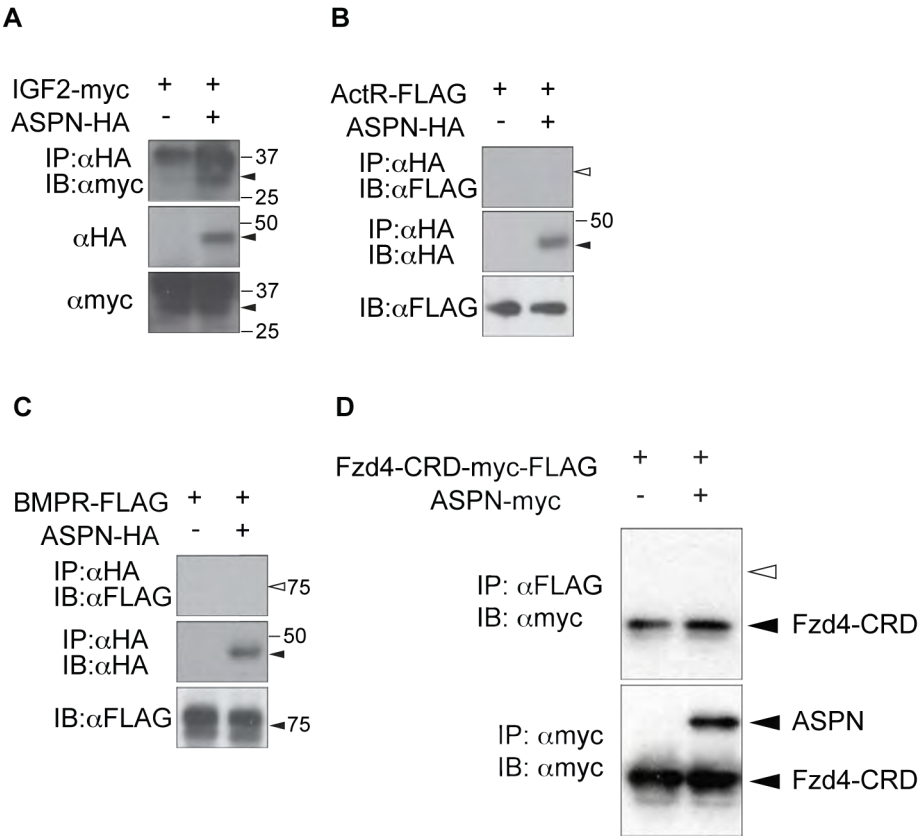


Figure 7.2: Interactions between ASPN and other molecules. The expression plasmids encoding ASPN-HA and IGF2-myc (A), Activin receptor (ActR)-FLAG (B), BMP receptor (BMPR)-FLAG (C) and Fzd4-CRD (the cysteine-rich domain in the extracellular part of Frz4)-myc-FLAG (D) were transfected into HEK293 cells. The cell extracts were analysed by co-immunoprecipitation assays.

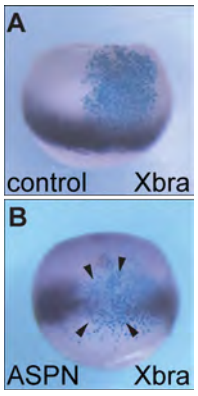


Figure 7.3: Xbra expression was inhibited by ASPN, as analysed by in situ hybridisation. The β-Galactosidase mRNA (light blue product) was injected without (A) or with (B) ASPN mRNAs into one blastomere at the equator region of 4-cell stage embryos and embryos were cultured until stage 10.5. Affected areas are indicated with arrowheads.

BIBLIOGRAPHY

ABERG, N. D., JOHANSSON, U. E., ABERG, M. A., HELLSTROM, N. A., LIND, J., BULL, C., ISGAARD, J., ANDERSON, M. F., OSCARSSON, J. & ERIKSSON, P. S. 2007. Peripheral infusion of insulin-like growth factor-I increases the number of newborn oligodendrocytes in the cerebral cortex of adult hypophysectomized rats. *Endocrinology,* 148**,** 3765-72.

ACAMPORA, D., BOYL, P. P., SIGNORE, M., MARTINEZ-BARBERA, J. P., ILENGO, C., PUELLES, E., ANNINO, A., REICHERT, H., CORTE, G. & SIMEONE, A. 2001. OTD/OTX2 functional equivalence depends on 5' and 3' UTR-mediated control of Otx2 mRNA for nucleo-cytoplasmic export and epiblast-restricted translation. *Development,* 128**,** 4801-13.

ALI, S. A., HOSAKA, Y. Z. & UEHARA, M. 2011. Expression of small leucine-rich proteoglycans in the developing retina and kainic acid-induced retinopathy in ICR mice. *J Vet Med Sci,* 73**,** 439-45.

ALTMANN, C. R., CHOW, R. L., LANG, R. A. & HEMMATI-BRIVANLOU, A. 1997. Lens induction by Pax-6 in Xenopus laevis. *Dev Biol,* 185**,** 119-23.

AMEYE, L. & YOUNG, M. F. 2002. Mice deficient in small leucine-rich proteoglycans: novel in vivo models for osteoporosis, osteoarthritis, Ehlers-Danlos syndrome, muscular dystrophy, and corneal diseases. *Glycobiology,* 12**,** 107R-16R.

ANDONIADOU, C. L. & MARTINEZ-BARBERA, J. P. 2013. Developmental mechanisms directing early anterior forebrain specification in vertebrates. *Cell Mol Life Sci,* 70**,** 3739-52.

ANDREAZZOLI, M., GESTRI, G., ANGELONI, D., MENNA, E. & BARSACCHI, G. 1999. Role of Xrx1 in Xenopus eye and anterior brain development. *Development,* 126**,** 2451-60.

ANDREAZZOLI, M., GESTRI, G., CREMISI, F., CASAROSA, S., DAWID, I. B. & BARSACCHI, G. 2003. Xrx1 controls proliferation and neurogenesis in Xenopus anterior neural plate. *Development,* 130**,** 5143-54.

ARSENIJEVIC, Y. & WEISS, S. 1998. Insulin-Like Growth Factor-I Is a Differentiation Factor for Postmitotic CNS Stem Cell-Derived Neuronal Precursors: Distinct Actions from Those of Brain-Derived Neurotrophic Factor. *The Journal of Neuroscience,* 18**,** 2118-2128.

ARSENIJEVIC, Y., WEISS, S., SCHNEIDER, B. & AEBISCHER, P. 2001. Insulin-Like Growth Factor-I Is Necessary for Neural Stem Cell Proliferation and Demonstrates Distinct Actions of Epidermal Growth Factor and Fibroblast Growth Factor-2. *The Journal of Neuroscience,* 21**,** 7194-7202.

AWATA, T., YAMADA, S., TSUSHIMA, K., SAKASHITA, H., YAMABA, S., KAJIKAWA, T., YAMASHITA, M., TAKEDACHI, M., YANAGITA, M., KITAMURA, M. & MURAKAMI, S. 2015. PLAP-1/Asporin Positively Regulates FGF-2 Activity. *J Dent Res,* 94**,** 1417-24.

AYASO, E., NOLAN, C. M. & BYRNES, L. 2002. Zebrafish insulin-like growth factor-I receptor: molecular cloning and developmental expression. *Mol Cell Endocrinol,* 191**,** 137-48.

AYER-LE LIEVRE, C., STAHLBLOM, P. A. & SARA, V. R. 1991. Expression of IGF-I and -II mRNA in the brain and craniofacial region of the rat fetus. *Development,* 111**,** 105-115.

BAE, S., REID, C. D. & KESSLER, D. S. 2011. Siamois and Twin are redundant and essential in formation of the Spemann organizer. *Dev Biol,* 352**,** 367-81.

BAKER, J., LIU, J. P., ROBERTSON, E. J. & EFSTRATIADIS, A. 1993. Role of insulin-like growth factors in embryonic and postnatal growth. *Cell,* 75**,** 73-82.

BALLARD, F. J., FRANCIS, G. L., ROSS, M., BAGLEY, C. J., MAY, B. & WALLACE, J. C. 1987. Natural and synthetic forms of insulin-like growth factor-1 (IGF-1) and the potent derivative, destripeptide IGF-1: biological activities and receptor binding. *149,* 2**,** 398-404.

BANDTLOW, C. E. & ZIMMERMANN, D. R. 2000. Proteoglycans in the developing brain: new conceptual insights for old proteins. *Physiol Rev,* 80**,** 1267-90.

BARON-VAN EVERCOOREN, A., OLICHON-BERTHE, C., KOWALSKI, A., VISCIANO, G. & VAN OBBERGHEN, E. 1991. Expression of IGF-I and insulin receptor genes in the rat central nervous system: a developmental, regional, and cellular analysis. *J Neurosci Res,* 28**,** 244-53.

BATEMAN, J. F., BOOT-HANDFORD, R. P. & LAMANDE, S. R. 2009. Genetic diseases of connective tissues: cellular and extracellular effects of ECM mutations. *Nat Rev Genet,* 10**,** 173-83.

BECH-HANSEN, N. T., NAYLOR, M. J., MAYBAUM, T. A., SPARKES, R. L., KOOP, B., BIRCH, D. G., BERGEN, A. A., PRINSEN, C. F., POLOMENO, R. C., GAL, A., DRACK, A. V., MUSARELLA, M. A., JACOBSON, S. G., YOUNG, R. S. & WELEBER, R. G. 2000. Mutations in NYX, encoding the leucine-rich proteoglycan nyctalopin, cause X-linked complete congenital stationary night blindness. *Nat Genet,* 26**,** 319-23.

BELFIORE, A., FRASCA, F., PANDINI, G., SCIACCA, L. & VIGNERI, R. 2009. Insulin receptor isoforms and insulin receptor/insulin-like growth factor receptor hybrids in physiology and disease. *Endocr Rev,* 30**,** 586-623.

BERNIER, G., PANITZ, F., ZHOU, X., HOLLEMANN, T., GRUSS, P. & PIELER, T. 2000. Expanded retina territory by midbrain transformation upon overexpression of Six6 (Optx2) in Xenopus embryos. *Mech Dev,* 93**,** 59-69.

BIJSTERBOSCH, J., KLOPPENBURG, M., REIJNIERSE, M., ROSENDAAL, F. R., HUIZINGA, T. W., SLAGBOOM, P. E. & MEULENBELT, I. 2013. Association study of candidate genes for the progression of hand osteoarthritis. *Osteoarthritis Cartilage,* 21**,** 565-9.

BILITOU, A. & OHNUMA, S. 2010. The role of cell cycle in retinal development: cyclin-dependent kinase inhibitors co-ordinate cell-cycle inhibition, cell-fate determination and differentiation in the developing retina. *Dev Dyn,* 239**,** 727-36.

BIRK, D. E., NURMINSKAYA, M. V. & ZYCBAND, E. I. 1995. Collagen fibrillogenesis in situ: fibril segments undergo post-depositional modifications resulting in linear and lateral growth during matrix development. *Dev Dyn,* 202**,** 229-43.

BLACKISTON, D. J. & LEVIN, M. 2012. Aversive training methods in Xenopus laevis: general principles. *Cold Spring Harb Protoc,* 2012.

BLACKISTON, D. J. & LEVIN, M. 2013. Ectopic eyes outside the head in Xenopus tadpoles provide sensory data for light-mediated learning. *J Exp Biol,* 216**,** 1031-40.

BLITZ, I. L. & CHO, K. W. 1995. Anterior neurectoderm is progressively induced during gastrulation: the role of the Xenopus homeobox gene orthodenticle. *Development,* 121**,** 993-1004.

BOIVIN, W. A., SHACKLEFORD, M., VANDEN HOEK, A., ZHAO, H., HACKETT, T. L., KNIGHT, D. A. & GRANVILLE, D. J. 2012. Granzyme B cleaves decorin, biglycan and soluble betaglycan, releasing active transforming growth factor-beta1. *PLoS One,* 7**,** e33163.

BONNANS, C., CHOU, J. & WERB, Z. 2014. Remodelling the extracellular matrix in development and disease. *Nat Rev Mol Cell Biol,* 15**,** 786-801.

BOURGUIGNON, C., LI, J. & PAPALOPULU, N. 1998. XBF-1, a winged helix transcription factor with dual activity, has a role in positioning neurogenesis in Xenopus competent ectoderm. *Development,* 125**,** 4889-900.

BOUWMEESTER, T., KIM, S., SASAI, Y., LU, B. & DE ROBERTIS, E. M. 1996. Cerberus is a head-inducing secreted factor expressed in the anterior endoderm of Spemann's organizer. *Nature,* 382**,** 595-601.

BRANDAN, E., RETAMAL, C., CABELLO-VERRUGIO, C. & MARZOLO, M. P. 2006. The low density lipoprotein receptor-related protein functions as an endocytic receptor for decorin. *J Biol Chem,* 281**,** 31562-71.

BRANNON, M. & KIMELMAN, D. 1996. Activation of Siamois by the Wnt pathway. *Dev Biol,* 180**,** 344-7.

BREDRUP, C., KNAPPSKOG, P. M., MAJEWSKI, J., RODAHL, E. & BOMAN, H. 2005. Congenital stromal dystrophy of the cornea caused by a mutation in the decorin gene. *Invest Ophthalmol Vis Sci,* 46**,** 420-6.

BROWN, E. L., WOOTEN, R. M., JOHNSON, B. J., IOZZO, R. V., SMITH, A., DOLAN, M. C., GUO, B. P., WEIS, J. J. & HOOK, M. 2001. Resistance to Lyme disease in decorin-deficient mice. *J Clin Invest,* 107**,** 845-52.

BROWN, K. E., KELLER, P. J., RAMIALISON, M., REMBOLD, M., STELZER, E. H., LOOSLI, F. & WITTBRODT, J. 2010. Nlcam modulates midline convergence during anterior neural plate morphogenesis. *Dev Biol,* 339**,** 14-25.

BRUN, R. B. 1981. The Movement of the Prospective Eye Vesicles from the Neural Plate into the Neural Fold in Andystoma mexjcanum and Xenopus Laevis. *Developmental Biology,* 88**,** 192-199.

BUGNER, V., AURHAMMER, T. & KUHL, M. 2011. Xenopus laevis insulin receptor substrate IRS-1 is important for eye development. *Dev Dyn,* 240**,** 1705-15.

BURMEISTER, M., NOVAK, J., LIANG, M. Y., BASU, S., PLODER, L., HAWES, N. L., VIDGEN, D., HOOVER, F., GOLDMAN, D., KALNINS, V. I., RODERICK, T. H., TAYLOR, B. A., HANKIN, M. H. & MCINNES, R. R. 1996. Ocular retardation mouse caused by Chx10 homeobox null allele: impaired retinal progenitor proliferation and bipolar cell differentiation. *Nat Genet,* 12**,** 376-84.

CANTY, E. G. & KADLER, K. E. 2002. Collagen fibril biosynthesis in tendon: a review and recent insights. *Comp Biochem Physiol A Mol Integr Physiol,* 133**,** 979-85.

CARRON, C., BOURDELAS, A., LI, H. Y., BOUCAUT, J. C. & SHI, D. L. 2005. Antagonistic interaction between IGF and Wnt/JNK signaling in convergent extension in Xenopus embryo. *Mech Dev,* 122**,** 1234-47.

CASAROSA, S., ANDREAZZOLI, M., SIMEONE, A. & BARSACCHI, G. 1997. Xrxl, a novel Xenopus homeobox gene expressed during eye
and pineal gland development. *Mechanisms of Development,* 61**,** 187-198.

CASTELLS, X., ACEBES, J. J., BOLUDA, S., MORENO-TORRES, A., PUJOL, J., JULIA-SAPE, M., CANDIOTA, A. P., ARINO, J., BARCELO, A. & ARUS, C. 2010. Development of a Predictor for Human Brain Tumors Based on Gene Expression Values Obtained from Two Types of Microarray Technologies. *OMICS A Journal of Integrative Biology,* 14**,** 157-164.

CAVODEASSI, F., CARREIRA-BARBOSA, F., YOUNG, R. M., CONCHA, M. L., ALLENDE, M. L., HOUART, C., TADA, M. & WILSON, S. W. 2005. Early stages of zebrafish eye formation require the coordinated activity of Wnt11, Fz5, and the Wnt/beta-catenin pathway. *Neuron,* 47**,** 43-56.

CHAUHAN, B. K., DISANZA, A., CHOI, S. Y., FABER, S. C., LOU, M., BEGGS, H. E., SCITA, G., ZHENG, Y. & LANG, R. A. 2009. Cdc42- and IRSp53-dependent contractile filopodia tether presumptive lens and retina to coordinate epithelial invagination. *Development,* 136**,** 3657-67.

CHAUHAN, B. K., LOU, M., ZHENG, Y. & LANG, R. A. 2011. Balanced Rac1and RhoA activities regulate cell shape and invagination morphogenesis in epthelia. *PNAS,* 108**,** 18289-18294.

CHEN, D. Y., STERN, S. A., GARCIA-OSTA, A., SAUNIER-REBORI, B., POLLONINI, G., BAMBAH-MUKKU, D., BLITZER, R. D. & ALBERINI, C. M. 2011. A critical role for IGF-II in memory consolidation and enhancement. *Nature,* 469**,** 491-7.

CHEN, L. & SANDERSON, R. D. 2009. Heparanase regulates levels of syndecan-1 in the nucleus. *PLoS One,* 4**,** e4947.

CHEN, S. & BIRK, D. E. 2011. Focus on molecules: decorin. *Exp Eye Res,* 92**,** 444-5.

CHEN, S. & BIRK, D. E. 2013. The regulatory roles of small leucine-rich proteoglycans in extracellular matrix assembly. *FEBS J,* 280**,** 2120-37.

CHEN, S., OLDBERG, A., CHAKRAVARTI, S. & BIRK, D. E. 2010. Fibromodulin regulates collagen fibrillogenesis during peripheral corneal development. *Dev Dyn,* 239**,** 844-54.

CHEN, X., WEISBERG, E., FRIDMACHER, V., WATANABE, M., NACO, G. & WHITMAN, M. 1997. Smad4 and FAST-1 in the assembly of activin-responsive factor. *Nature,* 389**,** 85-9.

CHEN, X. D., FISHER, L. W., ROBEY, P. G. & YOUNG, M. F. 2004. The small leucine-rich proteoglycan biglycan modulates BMP-4-induced osteoblast differentiation. *FASEB J,* 18**,** 948-58.

CHESIK, D., DE KEYSER, J., GLAZENBURG, L. & WILCZAK, N. 2006. Insulin-like growth factor binding proteins: regulation in chronic active plaques in multiple sclerosis and functional analysis of glial cells. *Eur J Neurosci,* 24**,** 1645-52.

CHHETRI, J., JACOBSON, G. & GUEVEN, N. 2014. Zebrafish--on the move towards ophthalmological research. *Eye (Lond),* 28**,** 367-80.

CHIANG, C., LITINGTUNG, Y., LEE, E., YOUNG, K. E., CORDEN, J. L., WESTPHAL, H. & BEACHY, P. A. 1996. Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. *Nature,* 383**,** 407-13.

CHO, K. W. 2012. Enhancers. *Wiley Interdiscip Rev Dev Biol,* 1**,** 469-78.

CHOW, R. L., ALTMANN, C. R., LANG, R. A. & HEMMATI-BRIVANLOU, A. 1999a. Pax6 induces ectopic eyes in a vertebrate. *Development,* 126**,** 4213-22.

CHOW, R. L., ALTMANN, C. R., LANG, R. A. & HEMMATI-BRIVANLOU, A. 1999b. Pax6 induces ectopic eyes in vertebrates. *Development,* 126**,** 4213-4222.

CHRISTIANSON, H. C. & BELTING, M. 2014. Heparan sulfate proteoglycan as a cell-surface endocytosis receptor. *Matrix Biol,* 35**,** 51-5.

COUCHMAN, J. R. 2010. Transmembrane signaling proteoglycans. *Annu Rev Cell Dev Biol,* 26**,** 89-114.

CUI, H., FREEMAN, C., JACOBSON, G. A. & SMALL, D. H. 2013. Proteoglycans in the central nervous system: role in development, neural repair, and Alzheimer's disease. *IUBMB Life,* 65**,** 108-20.

CUNNINGHAM, E. T. 2001. World blindness - no end in sight. *British Journal of Ophthalmology,* 85**,** 253-256.

CVEKL, A. & TAMM, E. R. 2004. Anterior eye development and ocular mesenchyme: new insights from mouse models and human diseases. *Bioessays,* 26**,** 374-86.

D'ERCOLE, A. J., YE, P., CALIKOGLU, A. S. & GUTIERREZ-OSPINA, G. 1996. The role of the insulin-like growth factors in the central nervous system. *Mol Neurobiol,* 13**,** 227-55.

DANIEL, A., DUMSTREI, K., LENGYEL, J. A. & HARTENSTEIN, V. 1999. The control of cell fate in the embryonic visual system by atonal, tailless and EGFR signaling. *Development,* 126**,** 2945-54.

DANNO, H., MICHIUE, T., HITACHI, K., YUKITA, A., ISHIURA, S. & ASASHIMA, M. 2008. Molecular links among the causative genes for ocular malformation: Otx2 and Sox2 coregulate Rax expression. *Proc Natl Acad Sci U S A,* 105**,** 5408-13.

DARWIN, C. R. 1859. *On the origin of species by means of natural selection, or the preservation of favoured races in the struggle for life.,* London, John Murray.

DAVENPORT, T. G. 2003. Mammary gland, limb and yolk sac defects in mice lacking Tbx3, the gene mutated in human ulnar mammary syndrome. *Development,* 130**,** 2263-2273.

DAVID, R. & WEDLICH, D. 2001. PCR-based RNA probes, a quick and sensitive method to improve whole mount embryo in situ hybridizations. *Biotechniques,* 30**,** 769-772.

DAVIES, J. E., TANG, X., DENNING, J. W., ARCHIBALD, S. J. & DAVIES, S. J. 2004. Decorin suppresses neurocan, brevican, phosphacan and NG2 expression and promotes axon growth across adult rat spinal cord injuries. *Eur J Neurosci,* 19**,** 1226-42.

DAVIS, R. J., TAVSANLI, B. C., DITTRICH, C., WALLDORF, U. & MARDON, G. 2003. Drosophila retinal homeobox (drx) is not required for establishment of the visual system, but is required for brain and clypeus development. *Developmental Biology,* 259**,** 272-287.

DE MARCO, N., TUSSELLINO, M., VITALE, A. & CAMPANELLA, C. 2011. Eukaryotic initiation factor 6 (eif6) overexpression affects eye development in Xenopus laevis. *Differentiation,* 82**,** 108-15.

DE PABLO, F., PEREZ-VILLAMIL, B., SERNA, J., GONZALEZ-GUERRERO, P. R., LOPEZ-CARRANZA, A., DE LA ROSA, E. J., ALEMANY, J. & CALDES, T. 1993. IGF-I and the IGF-I receptor in development of nonmammalian vertebrates. *Mol Reprod Dev,* 35**,** 427-32; discussion 432-3.

DECEMBRINI, S., ANDREAZZOLI, M., VIGNALI, R., BARSACCHI, G. & CREMISI, F. 2006. Timing the generation of distinct retinal cells by homeobox proteins. *PLoS Biol,* 4**,** e272.

DELLETT, M., HU, W., PAPADAKI, V. & OHNUMA, S. 2012. Small leucine rich proteoglycan family regulates multiple signalling pathways in neural development and maintenance. *Dev Growth Differ,* 54**,** 327-40.

DESNOYERS, L., ARNOTT, D. & PENNICA, D. 2001. WISP-1 binds to decorin and biglycan. *J Biol Chem,* 276**,** 47599-607.

DICICCO-BLOOM, E. & BLACK, I. B. 1988. Insulin growth factors regulate the mitotic cycle in cultured rat sympathetic neuroblasts. *Proc Natl Acad Sci U S A,* 85**,** 4066-70.

DOUAIHER, J., SUCCAR, J., LANCEROTTO, L., GURISH, M. F., ORGILL, D. P., HAMILTON, M. J., KRILIS, S. A. & STEVENS, R. L. 2014. Development of mast cells and importance of their tryptase and chymase serine proteases in inflammation and wound healing. *Adv Immunol,* 122**,** 211-52.

DUMONT, B., CASTRONOVO, V., PEULEN, O., BLETARD, N., CLEZARDIN, P., DELVENNE, P., DE PAUW, E. A., TURTOI, A. & BELLAHCENE, A. 2012. Differential proteomic analysis of a human breast tumor and its matched bone metastasis identifies cell membrane and extracellular proteins associated with bone metastasis. *J Proteome Res,* 11**,** 2247-60.

DUVAL, E., BIGOT, N., HERVIEU, M., KOU, I., LECLERCQ, S., GALERA, P., BOUMEDIENE, K. & BAUGE, C. 2011. Asporin expression is highly regulated in human chondrocytes. *Mol Med,* 17**,** 816-23.

EFSTRATIADIS, A. 1998. Genetics of mouse growth. *Int J Dev Biol,* 42**,** 955-76.

EGGENSCHWILER, J., LUDWIG, T., FISHER, P., LEIGHTON, P. A., TILGHMAN, S. & EFSTRATIADIS, A. 1997. Mouse mutant embryos overexpressing IGF-II exhibit phenotypic features
of the Beckwith–Wiedemann
and Simpson–Golabi–Behmel syndromes
. *Genes and Development,* 11**,** 3128-3142.

EIRAKU, M., TAKATA, N., ISHIBASHI, H., KAWADA, M., SAKAKURA, E., OKUDA, S., SEKIGUCHI, K., ADACHI, T. & SASAI, Y. 2011. Self-organizing optic-cup morphogenesis in three-dimensional culture. *Nature,* 472**,** 51-6.

EISEN, J. S. & SMITH, J. C. 2008. Controlling morpholino experiments: don't stop making antisense. *Development,* 135**,** 1735-43.

EIVERS, E., MCCARTHY, K., GLYNN, C., NOLAN, C. M. & BYRNES, L. 2004. Insulin-like growth factor (IGF) signalling is required for early dorso-anterior development of the zebrafish embryo. *Int J Dev Biol,* 48**,** 1131-40.

ELKAN, E. R. 1938. The Xenopus Pregnancy Test. *Br Med J,* 2**,** 1253-1274 2.

ENGLEKA, M. J. & KESSLER, D. S. 2001. Siamois cooperates with TGFbeta signals to induce the complete function of the Spemann-Mangold organizer. *Int J Dev Biol,* 45**,** 241-50.

ERCLIK, T., HARTENSTEIN, V., MCINNES, R. R. & LIPSHITZ, H. D. 2009. Eye evolution at high resolution: the neuron as a unit of homology. *Dev Biol,* 332**,** 70-9.

ESKOLA, P. J., LEMMELA, S., KJAER, P., SOLOVIEVA, S., MANNIKKO, M., TOMMERUP, N., LIND-THOMSEN, A., HUSGAFVEL-PURSIAINEN, K., CHEUNG, K. M., CHAN, D., SAMARTZIS, D. & KARPPINEN, J. 2012. Genetic association studies in lumbar disc degeneration: a systematic review. *PLoS One,* 7**,** e49995.

FAN, M. J. & SOKOL, S. Y. 1997. A role for Siamois in Spemann organizer formation. *Development,* 124**,** 2581-9.

FANTIN, V. R., LAVAN, B. E., WANG, Q., JENKINS, N. A., GILBERT, D. J., COPELAND, N. G., KELLER, S. R. & LIENHARD, G. E. 1999. Cloning, tissue expression, and chromosomal location of the mouse insulin receptor substrate 4 gene. *Endocrinology,* 140**,** 1329-37.

FERNALD, R. D. 2000. Evolution of eyes. *Curr Opin Neurobiol,* 10**,** 444-50.

FOLLI, F., BONFANTI, L., RENARD, E., KAHN, C. R. & MERIGHI, A. 1994. Insulin receptor substrate-1 (IRS-1) distribution in the rat central nervous system. *The Journal of Neuroscience,* 14**,** 6412-6422.

FUHRMANN, S. 2010. Eye morphogenesis and patterning of the optic vesicle. *Curr Top Dev Biol,* 93**,** 61-84.

GAGE, P. J., RHOADES, W., PRUCKA, S. K. & HJALT, T. 2005. Fate maps of neural crest and mesoderm in the mammalian eye. *Invest Ophthalmol Vis Sci,* 46**,** 4200-4208.

GALLAGHER, B. C., HAINSKI, A. M. & MOODY, S. A. 1991. Autonomous differentiation of dorsal axial structures from an animal cap cleavage stage biastomere in Xenopus. *Development,* 112**,** 1103-1114.

GAMMILL, L. S. & SIVE, H. 1997. Identification of otx2 target genes and restrictions in ectodermal competence during Xenopus cement gland formation. *Development,* 124**,** 471-481.

GANDIN, V., MILUZIO, A., BARBIERI, A. M., BEUGNET, A., KIYOKAWA, H., MARCHISIO, P. C. & BIFFO, S. 2008. Eukaryotic initiation factor 6 is rate-limiting in translation, growth and transformation. *Nature,* 455**,** 684-8.

GAO, L., WANG, X., WANG, X., ZHANG, L., QIANG, C., CHANG, S., REN, W., LI, S., YANG, Y., TONG, D., CHEN, C., LI, Z., SONG, T., ZHI, K. & HUANG, C. 2014. IGF-1R, a target of let-7b, mediates crosstalk between IRS- 2/Akt and MAPK pathways to promote proliferation of oral squamous cell carcinoma. *Oncotarget,* 5**,** 2526-2574.

GEHRING, W. & SEIMIYA, M. 2010. Eye evolution and the origin of Darwin's eye prototype. *Italian Journal of Zoology,* 77**,** 124-136.

GEHRING, W. J. 2004. Historical perspective on the development and evolution of eyes and photoreceptors. *Int J Dev Biol,* 48**,** 707-17.

GEHRING, W. J. 2014. The evolution of vision. *Wiley Interdiscip Rev Dev Biol,* 3**,** 1-40.

GENE TOOLS, L. L. C. 2016. *Why use Morpholinos* [Online]. Available: http://www.gene-tools.com/faq - wcm1 [Accessed 20 February 2016].

GENG, Y., MCQUILLAN, D. & ROUGHLEY, P. J. 2006. SLRP interaction can protect collagen fibrils from cleavage by collagenases. *Matrix Biol,* 25**,** 484-91.

GERHART, J., DANILCHIK, M., DONIACH, T., ROBERTS, S., ROWNING, B. & STEWART, R. 1989. Cortical rotation of the Xenopus egg: consequences for the anteroposterior pattern of embryonic dorsal development. *Development,* 107 Suppl**,** 37-51.

GERMAIN, S., HOWELL, M., ESSLEMONT, G. M. & HILL, C. S. 2000. Homeodomain and winged-helix transcription factors recruit activated Smads to distinct promoter elements via a common Smad interaction motif. *Genes Dev,* 14**,** 435-51.

GESTRI, G., CARL, M., APPOLLONI, I., WILSON, S. W., BARSACCHI, G. & ANDREAZZOLI, M. 2005. Six3 functions in anterior neural plate specification by promoting cell proliferation and inhibiting Bmp4 expression. *Development,* 132**,** 2401-13.

GHANBARI, H., SEO, H. C., FJOSE, A. & BRANDLI, A. W. 2001. Molecular cloning and embryonic expression of Xenopus Six homeobox genes. *Mech Dev,* 101**,** 271-7.

GILBERT, S. F. 2014. *Developmental Biology,* Sunderland, MA, USA, Andrew D. Sinauer.

GIORGI, P. P. & VAN DER LOOS, H. 1978. Axons from eyes grafted in Xenopus can grow into the spinal cord and reach the optic tectum. *Nature,* 275**,** 746-748.

GIRBAU, M., BASSAS, L., ALEMANY, J. & DE PABLO, F. 1989. In situ autoradiography and ligand-dependent tyrosine kinase activity reveal insulin receptors and insulin-like growth factor I receptors in prepancreatic chicken embryos. *Proc Natl Acad Sci U S A,* 86**,** 5868-72.

GLARDON, S., CALLAERTS, P., HALDER, G. & GEHRING, W. J. 1997. Conservation of Pax-6 in a lower chordate, the ascidian Phallusia mammillata. *Development,* 124**,** 817-25.

GLASS, A. S. & DAHM, R. 2004. The zebrafish as a model organism for eye development. *Ophthalmic Res,* 36**,** 4-24.

GLAVIC, A., GOMEZ-SKARMETA, J. L. & MAYOR, R. 2001. Xiro-1 controls mesoderm patterning by repressing bmp-4 expression in the Spemann organizer. *Dev Dyn,* 222**,** 368-76.

GOLDONI, S., HUMPHRIES, A., NYSTROM, A., SATTAR, S., OWENS, R. T., MCQUILLAN, D. J., IRETON, K. & IOZZO, R. V. 2009. Decorin is a novel antagonistic ligand of the Met receptor. *J Cell Biol,* 185**,** 743-54.

GONT, L. K., STEINBEISSER, H., BLUMBERG, B. & DE ROBERTIS, E. M. 1993. Tail formation as a continuation of gastrulation: the multiple cell populations of the Xenopus tailbud derive from the late blastopore lip. *Development,* 119**,** 991-1004.

GONZALEZ-CORDERO, A., WEST, E. L., PEARSON, R. A., DURAN, Y., CARVALHO, L. S., CHU, C. J., NAEEM, A., BLACKFORD, S. J., GEORGIADIS, A., LAKOWSKI, J., HUBANK, M., SMITH, A. J., BAINBRIDGE, J. W., SOWDEN, J. C. & ALI, R. R. 2013. Photoreceptor precursors derived from three-dimensional embryonic stem cell cultures integrate and mature within adult degenerate retina. *Nat Biotechnol,* 31**,** 741-7.

GUARDIOLA-DIAZ, H. M., ISHII, A. & BANSAL, R. 2012. Erk1/2 MAPK and mTOR signaling sequentially regulates progression through distinct stages of oligodendrocyte differentiation. *Glia,* 60**,** 476-86.

GULER, H. P., ZAPF, J., SCHMID, C. & FROESCH, E. R. 1989. Insulin-like growth factors I and II in healthy man. Estimations of half-lives and production rates. *Acta Endocrinol (Copenh),* 121**,** 753-8.

GURDON, J. B. & HOPWOOD, N. 2000. The introduction of Xenopus laevis into developmental biology: empire, pregnancy testing and ribosomal genes. *Int J Dev Biol,* 44**,** 43-50.

HALDER, G., CALLAERTS, P. & GEHRING, W. J. 1995a. Induction of ectopic eyes by targeted expression of the eyeless gene in Drosophila. *Science,* 267**,** 1788-92.

HALDER, G., CALLAERTS, P. & GEHRING, W. J. 1995b. New perspectives on eye evolution. *Curr Opin Genet Dev,* 5**,** 602-9.

HARLAND, R. 2000. Neural induction. *Curr Opin Genet Dev,* 10**,** 357-62.

HARLAND, R. M. & GRAINGER, R. M. 2011. Xenopus research: metamorphosed by genetics and genomics. *Trends Genet,* 27**,** 507-15.

HARRIS, W. A. 1986. Homing behaviour of axons in the embryonic vertebrate brain. *Nature,* 320**,** 266-9.

HARTLEY, K. O., HARDCASTLE, Z., FRIDAY, R. V., AMAYA, E. & PAPALOPULU, N. 2001. Transgenic Xenopus embryos reveal that anterior neural development requires continued suppression of BMP signaling after gastrulation. *Dev Biol,* 238**,** 168-84.

HAWKES, C., JHAMANDAS, J. H., HARRIS, K. H., FU, W., MACDONALD, R. G. & KAR, S. 2006. Single transmembrane domain insulin-like growth factor-II/mannose-6-phosphate receptor regulates central cholinergic function by activating a G-protein-sensitive, protein kinase C-dependent pathway. *J Neurosci,* 26**,** 585-96.

HE, M. L., WEN, L., CAMPBELL, C. C., WU, J. Y. & RAO, Y. 1999. Transcription repression by Xenopus ET and its human ortholog TBX3, a gene involved in ulnar-mammary syndrome. *Proc Natl Acad Sci U S A,* 96**,** 10212-10217.

HEMMATI-BRIVANLOU, A., FRANK, D., BOLCE, M. E., BROWN, B. D., SIVE, H. L. & HARLAND, R. M. 1990. Localization of specific mRNAs in Xenopus embryos by whole-mount in situ hybridization. *Development,* 110**,** 325-30.

HEMMATI-BRIVANLOU, A. & MELTON, D. 1997. Vertebrate Embryonic Cells Will Become Nerve Cells Unless Told Otherwise. *Cell,* 88**,** 13-17.

HEMMATI-BRIVANLOU, A. & MELTON, D. A. 1992. A truncated activin receptor inhibits mesoderm induction and formation of axial structures in Xenopus embryos. *Nature,* 359**,** 609-14.

HEMMATI-BRIVANLOU, A. & MELTON, D. A. 1994. Inhibition of activin receptor signaling promotes neuralization in Xenopus. *Cell,* 77**,** 273-81.

HEMMATI-BRIVANLOU, A. & THOMSEN, G. H. 1995. Ventral mesodermal patterning in Xenopus embryos: expression patterns and activities of BMP-2 and BMP-4. *Dev Genet,* 17**,** 78-89.

HENRY, J. J., WEVER, J. M., VERGARA, M. N. & FUKUI, L. 2008. Xenopus, an ideal vertebrate system for studies of eye development and regeneration. *In:* TSONIS, P. A. (ed.) *Animal Models in Eye Research*. Elsevier.

HENRY, S. P., TAKANOSU, M., BOYD, T. C., MAYNE, P. M., EBERSPAECHER, H., ZHOU, W., DE CROMBRUGGHE, B., HOOK, M. & MAYNE, R. 2001. Expression pattern and gene characterization of asporin. a newly discovered member of the leucine-rich repeat protein family. *J Biol Chem,* 276**,** 12212-21.

HIRSCH, N. & HARRIS, W. A. 1996. Xenopus Pax-6 and Retinal Development. *J Neurobiol,* 32**,** 45-61.

HODGKINSON, S. C., DAVIS, S. R., MOORE, L. G., HENDERSON, H. V. & GLUCKMAN, P. D. 1989. Metabolic clearance of insulin-like growth factor-II in sheep. *J Endocrinol,* 123**,** 461-8.

HSIEH, L. T., NASTASE, M. V., ZENG-BROUWERS, J., IOZZO, R. V. & SCHAEFER, L. 2014. Soluble biglycan as a biomarker of inflammatory renal diseases. *Int J Biochem Cell Biol,* 54**,** 223-35.

HUANG, S. & MOODY, S. A. 1993. The Retinal Fate of Xenopus Cleavage Stage Progenitors Is Dependent upon Blastomere Position and Competence: Studies of Normal and Regulated Clones. *The Journal of Neuroscience,* 13**,** 3193-3210.

HYNES, R. O. 2009. The extracellular matrix: not just pretty fibrils. *Science,* 326**,** 1216-9.

HYNES, R. O. & NABA, A. 2012. Overview of the matrisome--an inventory of extracellular matrix constituents and functions. *Cold Spring Harb Perspect Biol,* 4**,** a004903.

IKEDA, H., OSAKADA, F., WATANABE, K., MIZUSEKI, K., HARAGUCHI, T., MIYOSHI, H., KAMIYA, D., HONDA, Y., SASAI, N., YOSHIMURA, N., TAKAHASHI, M. & SASAI, Y. 2005. Generation of Rx+/Pax6+ neural retinal precursors from embryonic stem cells. *Proc Natl Acad Sci U S A,* 102**,** 11331-6.

IKEGAWA, S. 2008. Expression, regulation and function of asporin, a susceptibility gene in common bone and joint diseases. *Curr Med Chem,* 15**,** 724-8.

INKSON, C. A., ONO, M., BI, Y., KUZNETSOV, S. A., FISHER, L. W. & YOUNG, M. F. 2009. The potential functional interaction of biglycan and WISP-1 in controlling differentiation and proliferation of osteogenic cells. *Cells Tissues Organs,* 189**,** 153-7.

IOZZO, R. V. 1999. The Biology of the Small Leucine-rich Proteoglycan. *The Journal of Biological Chemistry,* 274**,** 18843-18846.

IOZZO, R. V. & SAN ANTONIO, J. D. 2001. Heparan sulfate proteoglycans: heavy hitters in the angiogenesis arena. *J Clin Invest,* 108**,** 349-55.

IOZZO, R. V. & SCHAEFER, L. 2010. Proteoglycans in health and disease: novel regulatory signaling mechanisms evoked by the small leucine-rich proteoglycans. *FEBS J,* 277**,** 3864-75.

IOZZO, R. V. & SCHAEFER, L. 2015. Proteoglycan form and function: A comprehensive nomenclature of proteoglycans. *Matrix Biol,* 42**,** 11-55.

JACOBSON, A. G. 1966. Inductive processes in embryonic development. *Science,* 152**,** 25-34.

JARVELAINEN, H., SAINIO, A., KOULU, M., WIGHT, T. N. & PENTTINEN, R. 2009. Extracellular matrix molecules: potential targets in pharmacotherapy. *Pharmacol Rev,* 61**,** 198-223.

JARVINEN, T. A. & PRINCE, S. 2015. Decorin: A Growth Factor Antagonist for Tumor Growth Inhibition. *Biomed Res Int,* 2015**,** 654765.

JOHNSON-FARLEY, N. N., PATEL, K., KIM, D. & COWEN, D. S. 2007. Interaction of FGF-2 with IGF-1 and BDNF in stimulating Akt, ERK, and neuronal survival in hippocampal cultures. *Brain Res,* 1154**,** 40-9.

JONASOVA, K. & KOZMIK, Z. 2008. Eye evolution: lens and cornea as an upgrade of animal visual system. *Semin Cell Dev Biol,* 19**,** 71-81.

JONES, J. I. & CLEMMONS, D. R. 1995. Insulin-like growth factors and their binding proteins: Biological actions. *Endocrine Reviews,* 16**,** 3-34.

KADLER, K. E., HILL, A. & CANTY-LAIRD, E. G. 2008. Collagen fibrillogenesis: fibronectin, integrins, and minor collagens as organizers and nucleators. *Curr Opin Cell Biol,* 20**,** 495-501.

KALAMAJSKI, S., ASPBERG, A., LINDBLOM, K., HEINEGARD, D. & OLDBERG, A. 2009. Asporin competes with decorin for collagen binding, binds calcium and promotes osteoblast collagen mineralization. *Biochem J,* 423**,** 53-9.

KALAMAJSKI, S. & OLDBERG, A. 2009. Homologous sequence in lumican and fibromodulin leucine-rich repeat 5-7 competes for collagen binding. *J Biol Chem,* 284**,** 534-9.

KANAKUBO, S., NOMURA, T., YAMAMURA, K., MIYAZAKI, J., TAMAI, M. & OSUMI, N. 2006. Abnormal migration and distribution of neural crest cells in Pax6 heterozygous mutant eye, a model for human eye diseases. *Genes Cells,* 11**,** 919-33.

KATZ, M. J. & LASEK, R. J. 1978. Eyes transplanted to tadpole tails send axons rostrally in two spinal-cord tracts. *Science,* 199**,** 202-4.

KEENE, D. R., SAN ANTONIO, J. D., MAYNE, R., MCQUILLAN, D. J., SARRIS, G., SANTORO, S. A. & IOZZO, R. V. 2000. Decorin binds near the C terminus of type I collagen. *J Biol Chem,* 275**,** 21801-4.

KELLER, R. E. 1976. Vital dye mapping of the gastrula and neurula of Xenopus laevis. II. Prospective areas and morphogenetic movements of the deep layer. *Dev Biol,* 51**,** 118-37.

KELLEY, R. L., ROESSLER, E., HENNEKAM, R. C., FELDMAN, G. L., KOSAKI, K., JONES, M. C., PALUMBOS, J. C. & MUENKE, M. 1996. Holoprosencephaly in RSH/Smith-Lemli-Opitz syndrome: does abnormal cholesterol metabolism affect the function of Sonic Hedgehog? *Am J Med Genet,* 66**,** 478-84.

KENYON, K. L., ZAGHLOUL, N. & MOODY, S. A. 2001. Transcription factors of the anterior neural plate alter cell movements of epidermal progenitors to specify a retinal fate. *Dev Biol,* 240**,** 77-91.

KESSLER, D. S. 1997. Siamois is required for formation of Spemann's organizer. *Proc Natl Acad Sci U S A,* 94**,** 13017-13022.

KIM, J. G., KANG, M. J., YOON, Y. K., KIM, H. P., PARK, J., SONG, S. H., HAN, S. W., PARK, J. W., KANG, G. H., KANG, K. W., OH DO, Y., IM, S. A., BANG, Y. J., YI, E. C. & KIM, T. Y. 2012. Heterodimerization of glycosylated insulin-like growth factor-1 receptors and insulin receptors in cancer cells sensitive to anti-IGF1R antibody. *PLoS One,* 7**,** e33322.

KIZAWA, H., KOU, I., IIDA, A., SUDO, A., MIYAMOTO, Y., FUKUDA, A., MABUCHI, A., KOTANI, A., KAWAKAMI, A., YAMAMOTO, S., UCHIDA, A., NAKAMURA, K., NOTOYA, K., NAKAMURA, Y. & IKEGAWA, S. 2005. An aspartic acid repeat polymorphism in asporin inhibits chondrogenesis and increases susceptibility to osteoarthritis. *Nat Genet,* 37**,** 138-44.

KNUDSON, C. B. & KNUDSON, W. 2001. Cartilage proteoglycans. *Semin Cell Dev Biol,* 12**,** 69-78.

KOHWI, M. & DOE, C. Q. 2013. Temporal fate specification and neural progenitor competence during development. *Nature Reviews Neuroscience,* 14**,** 823-838.

KOK, F. O., SHIN, M., NI, C. W., GUPTA, A., GROSSE, A. S., VAN IMPEL, A., KIRCHMAIER, B. C., PETERSON-MADURO, J., KOURKOULIS, G., MALE, I., DESANTIS, D. F., SHEPPARD-TINDELL, S., EBARASI, L., BETSHOLTZ, C., SCHULTE-MERKER, S., WOLFE, S. A. & LAWSON, N. D. 2015. Reverse genetic screening reveals poor correlation between morpholino-induced and mutant phenotypes in zebrafish. *Dev Cell,* 32**,** 97-108.

KOLB, M., MARGETTS, P. J., GALT, T., SIME, P. J., XING, Z., SCHMIDT, M. & GAULDIE, J. 2001. Transient transgene expression of decorin in the lung reduces the fibrotic response to bleomycin. *Am J Respir Crit Care Med,* 163**,** 770-7.

KONDOH, H., UCHIKAWA, M., YODA, H., TAKEDA, H., FURUTANI-SEIKI, M. & KARLSTROM, R. O. 2000. Zebrafish mutations in Gli-mediated hedgehog signaling leads to lens transdifferentiation from the adenohypophysis anlage. *Mechanisms of Development,* 96.

KOO, H. & GRAZIADEI, P. P. 1995. Eye primordium transplantation in Xenopus embryo. *Anat Embryol (Berl),* 191**,** 155-70.

KOU, I., NAKAJIMA, M. & IKEGAWA, S. 2007. Expression and regulation of the osteoarthritis-associated protein asporin. *J Biol Chem,* 282**,** 32193-9.

KOU, I., NAKAJIMA, M. & IKEGAWA, S. 2010. Binding characteristics of the osteoarthritis-associated protein asporin. *J Bone Miner Metab,* 28**,** 395-402.

KOZMIK, Z., DAUBE, M., FREI, E., NORMAN, B., KOS, L., DISHAW, L. J., NOLL, M. & PIATIGORSKY, J. 2003. Role of Pax genes in eye evolution: a cnidarian PaxB gene uniting Pax2 and Pax6 functions. *Dev Cell,* 5**,** 773-85.

KRESSE, H., LISZIO, C., SCHONHERR, E. & FISHER, L. W. 1997. Critical role of glutamate in a central leucine-rich repeat of decorin for interaction with type I collagen. *J Biol Chem,* 272**,** 18404-10.

KRESSE, H. & SCHONHERR, E. 2001. Proteoglycans of the extracellular matrix and growth control. *J Cell Physiol,* 189**,** 266-74.

KRISHNAN, P., HOCKING, A. M., SCHOLTZ, J. M., PACE, C. N., HOLIK, K. K. & MCQUILLAN, D. J. 1999. Distinct secondary structures of the leucine-rich repeat proteoglycans decorin and biglycan. Glycosylation-dependent conformational stability. *J Biol Chem,* 274**,** 10945-50.

KUBO, F. & NAKAGAWA, S. 2008. Wnt signaling in retinal stem cells and regeneration. *Dev Growth Differ,* 50**,** 245-51.

KUEMMERLE, J. F. & MURTHY, K. S. 2001. Coupling of the insulin-like growth factor-I receptor tyrosine kinase to Gi2 in human intestinal smooth muscle: Gbetagamma -dependent mitogen-activated protein kinase activation and growth. *J Biol Chem,* 276**,** 7187-94.

KURIYAMA, S., LUPO, G., OHTA, K., OHNUMA, S., HARRIS, W. A. & TANAKA, H. 2006. Tsukushi controls ectodermal patterning and neural crest specification in Xenopus by direct regulation of BMP4 and X-delta-1 activity. *Development,* 133**,** 75-88.

KURODA, H., WESSELY, O. & DE ROBERTIS, E. M. 2004. Neural induction in Xenopus: requirement for ectodermal and endomesodermal signals via Chordin, Noggin, beta-Catenin, and Cerberus. *PLoS Biol,* 2**,** E92.

KWAN, K. M. 2003. Xbra functions as a switch between cell migration and convergent extension in the Xenopus gastrula. *Development,* 130**,** 1961-1972.

LA TORRE, A., HOSHINO, A., CAVANAUGH, C., WARE, C. B. & REH, T. A. 2015. The GIPC1-Akt1 Pathway Is Required for the Specification of the Eye Field in Mouse Embryonic Stem Cells. *Stem Cells,* 33**,** 2674-85.

LAMB, T. D., COLLIN, S. P. & PUGH, E. N., JR. 2007. Evolution of the vertebrate eye: opsins, photoreceptors, retina and eye cup. *Nat Rev Neurosci,* 8**,** 960-76.

LAND, M. F. 2012. The evolution of lenses. *Ophthalmic Physiol Opt,* 32**,** 449-60.

LANGELAAN, M., DE BOER, M. R., VAN NISPEN, R. M., WOUTERS, B., MOLL, A. C. & VAN RENS, G. H. 2007. Impact of visual impairment on quality of life: a comparison with quality of life in the general population and with other chronic conditions. *Ophthalmic Epidemiol,* 14**,** 119-26.

LARABELL, C. A., TORRES, M., ROWNING, B. A., YOST, C., MILLER, J. R., WU, M., KIMELMAN, D. & MOON, R. T. 1997. Establishment of the Dorso-ventral Axis in Xenopus
Embryos Is Presaged by Early Asymmetries in b-Catenin
That Are Modulated by the Wnt Signaling Pathway. *J Cell Biol*.

LE GOFF, M. M. & BISHOP, P. N. 2007. Focus on molecules: opticin. *Exp Eye Res,* 85**,** 303-4.

LEE, D. C., HAMM, L. M. & MORITZ, O. L. 2013. Xenopus laevis tadpoles can regenerate neural retina lost after physical excision but cannot regenerate photoreceptors lost through targeted ablation. *Invest Ophthalmol Vis Sci,* 54**,** 1859-67.

LEE, E. H., PARK, H. J., JEONG, J. H., KIM, Y. J., CHA, D. W., KWON, D. K., LEE, S. H. & CHO, J. Y. 2011. The role of asporin in mineralization of human dental pulp stem cells. *J Cell Physiol,* 226**,** 1676-82.

LEE, H. S., BONG, Y. S., MOORE, K. B., SORIA, K., MOODY, S. A. & DAAR, I. O. 2006. Dishevelled mediates ephrinB1 signalling in the eye field through the planar cell polarity pathway. *Nat Cell Biol,* 8**,** 55-63.

LEE, H. S., MOOD, K., BATTU, G., JI, Y. J., SINGH, A. & DAAR, I. O. 2009. Fibroblast Growth Factor Receptor-induced Phosphorylation of EphrinB1 Modulates Its Interaction with Dishevelled. *Molecular Biology of the Cell,* 20**,** 124-133.

LEROITH, D., WERNER, H., BEITNER-JOHNSON, D. & ROBERTS JR., C. T. 1995. Molecular and cellular aspects of the insulin-like growth factor I receptor. *Endocrine Reviews,* 16**,** 143-163.

LEWIS, M. 2003. PRELP, collagen, and a theory of Hutchinson-Gilford progeria. *Ageing Res Rev,* 2**,** 95-105.

LI, H., TIERNEY, C., WEN, L., WU, J. Y. & RAO, Y. 1997. A single morphogenetic field gives rise to two retina primordia under the influence of the prechordal plate. *Development,* 124**,** 603-15.

LI, L., WANG, L., LI, T. T., LI, X., HUANG, X. Q., CHEN, X. W., LI, Z. L., LV, X. M., LIU, F. Y., LUO, Z. W., LIU, M., HU, X. H., HU, W. F., HUANG, Z. X., YI, M., LIU, S. J., LIU, Y. Z. & LI, D. W. C. 2013. ERK Signaling Pathway Regulates Embryonic Survival and Eye Development in Goldfish, Carassius auratus. *Current Molecular Medicine,* 13**,** 959-967.

LIGENSA, T., KRAUSS, S., DEMUTH, D., SCHUMACHER, R., CAMONIS, J., JAQUES, G. & WEIDNER, K. M. 2001. A PDZ domain protein interacts with the C-terminal tail of the insulin-like growth factor-1 receptor but not with the insulin receptor. *J Biol Chem,* 276**,** 33419-27.

LIN, H.-J., KUNG, Y.-J., LIN, Y.-J., SBEU, J. J. C., CHEN, B.-H., LAN, Y.-C., LAI, C.-H., HSU, Y.-A., WAN, L. & TSAI, F. J. 2010. Association of the Lumican Gene Functional 3'-UTR Polymorphism with High Myopia.

LIN, Y. C., BOONE, M., MEURIS, L., LEMMENS, I., VAN ROY, N., SOETE, A., REUMERS, J., MOISSE, M., PLAISANCE, S., DRMANAC, R., CHEN, J., SPELEMAN, F., LAMBRECHTS, D., VAN DE PEER, Y., TAVERNIER, J. & CALLEWAERT, N. 2014. Genome dynamics of the human embryonic kidney 293 lineage in response to cell biology manipulations. *Nat Commun,* 5**,** 4767.

LOGAN, A., BAIRD, A. & BERRY, M. 1999. Decorin Attenuates Gliotic Scar Formation in the Rat Cerebral Hemisphere. *Experimental Neurology,* 159**,** 504-510.

LOOSLI, F., KMITA-CUNISSE, M. & GEHRING, W. J. 1996. Isolation of a Pax-6 homolog from the ribbonworm Lineus sanguineus. *Proc Natl Acad Sci U S A,* 93**,** 2658-63.

LOPASHOV, G. V. & STROEVA, O. G. 1964. *Development of the eye; experimental studies.,* Jerusalem, Israel Program for Scientific Translations.

LORENZO, P., ASPBERG, A., ONNERFJORD, P., BAYLISS, M. T., NEAME, P. J. & HEINEGARD, D. 2001. Identification and characterization of asporin. a novel member of the leucine-rich repeat protein family closely related to decorin and biglycan. *J Biol Chem,* 276**,** 12201-11.

LOUVI, A., ACCILI, D. & EFSTRATIADIS, A. 1997. Growth-promoting interaction of IGF-II with the insulin receptor during mouse embryonic development. *Dev Biol,* 189**,** 33-48.

LUNARDI, A. & VIGNALI, R. 2006. Xenopus Xotx2 and Drosophila otd share similar activities in anterior patterning of the frog embryo. *Dev Genes Evol,* 216**,** 511-21.

LUPO, G., LIU, Y., QIU, R., CHANDRARATNA, R. A., BARSACCHI, G., HE, R. Q. & HARRIS, W. A. 2005. Dorsoventral patterning of the Xenopus eye: a collaboration of Retinoid, Hedgehog and FGF receptor signaling. *Development,* 132**,** 1737-48.

MA, Y., ZHANG, L. & HUANG, X. 2014. Genome modification by CRISPR/Cas9. *FEBS J,* 281**,** 5186-93.

MAJAVA, M., BISHOP, P. N., HAGG, P., SCOTT, P. G., RICE, A., INGLEHEARN, C., HAMMOND, C. J., SPECTOR, T. D., ALA-KOKKO, L. & MANNIKKO, M. 2007. Novel mutations in the small leucine-rich repeat protein/proteoglycan (SLRP) genes in high myopia. *Hum Mutat,* 28**,** 336-44.

MANGOLD, O. 1933. Über die Induktionsfahigkeit der verschiedenen Bezirke der Neurula von Urodelen. *Naturwissenschaften,* 21**,** 761-766.

MANN, F., HARRIS, W. A. & HOLT, C. E. 2004. New views on retinal axon development: a navigation guide. *Int J Dev Biol,* 48**,** 957-64.

MARIS, P., BLOMME, A., PALACIOS, A. P., COSTANZA, B., BELLAHCENE, A., BIANCHI, E., GOFFLOT, S., DRION, P., TROMBINO, G. E., DI VALENTIN, E., CUSUMANO, P. G., MAWEJA, S., JERUSALEM, G., DELVENNE, P., LIFRANGE, E., CASTRONOVO, V. & TURTOI, A. 2015. Asporin Is a Fibroblast-Derived TGF-beta1 Inhibitor and a Tumor Suppressor Associated with Good Prognosis in Breast Cancer. *PLoS Med,* 12**,** e1001871.

MARNEROS, A. G. & OLSEN, B. R. 2005. Physiological role of collagen XVIII and endostatin. *FASEB J,* 19**,** 716-28.

MARTINEZ-MORALES, J. R. & WITTBRODT, J. 2009. Shaping the vertebrate eye. *Curr Opin Genet Dev,* 19**,** 511-7.

MASHAYEKHI, F., MIRZAJANI, E., NAJI, M. & AZARI, M. 2010. Expression of insulin-like growth factor-1 and insulin-like growth factor binding proteins in the serum and cerebrospinal fluid of patients with Parkinson's disease. *J Clin Neurosci,* 17**,** 623-7.

MASSE, K., BHAMRA, S., EASON, R., DALE, N. & JONES, E. A. 2007. Purine-mediated signalling triggers eye development. *Nature,* 449**,** 1058-62.

MATHERS, P. H., GRINBERG, A., MAHON, K. A. & JAMRICH, M. 1997a. The Rx homeobox gene is essential for vertebrate eye development. *Nature,* 387**,** 603-607.

MATHERS, P. H., GRINBERG, A., MAHON, K. A. & JAMRICH, M. 1997b. The Rx homeobox gene is essential for vertebrate eye development. *Nature,* 387**,** 603-7.

MCCOLLUM, C. W., DUCHARME, N. A., BONDESSON, M. & GUSTAFSSON, J. A. 2011. Developmental toxicity screening in zebrafish. *Birth Defects Res C Embryo Today,* 93**,** 67-114.

MCEWAN, P. A., SCOTT, P. G., BISHOP, P. N. & BELLA, J. 2006. Structural correlations in the family of small leucine-rich repeat proteins and proteoglycans. *J Struct Biol,* 155**,** 294-305.

MCKINNON, T., CHAKRABORTY, C., GLEESON, L. M., CHIDIAC, P. & LALA, P. K. 2001. Stimulation of Human Extravillous Trophoblast Migration by IGF-II Is Mediated by IGF Type 2 Receptor Involving Inhibitory G Protein(s) and Phosphorylation of MAPK. *The Journal of Clinical Endocrinology & Metabolism,* 86**,** 3665-3674.

MECHAM, R. P. 2001. Overview of extracellular matrix. *Curr Protoc Cell Biol*.

MELCHING, L. I., FISHER, W. D., LEE, E. R., MORT, J. S. & ROUGHLEY, P. J. 2006. The cleavage of biglycan by aggrecanases. *Osteoarthritis Cartilage,* 14**,** 1147-54.

MELLOUGH, C. B., COLLIN, J., KHAZIM, M., WHITE, K., SERNAGOR, E., STEEL, D. H. & LAKO, M. 2015. IGF-1 Signaling Plays an Important Role in the Formation of Three-Dimensional Laminated Neural Retina and Other Ocular Structures From Human Embryonic Stem Cells. *Stem Cells,* 33**,** 2416-30.

MINOR, K., TANG, X., KAHRILAS, G., ARCHIBALD, S. J., DAVIES, J. E. & DAVIES, S. J. 2008. Decorin promotes robust axon growth on inhibitory CSPGs and myelin via a direct effect on neurons. *Neurobiol Dis,* 32**,** 88-95.

MOODY, S. A. 1987a. Fates of the blastomeres of the 16-cell stage Xenopus embryo. *Dev Biol,* 119**,** 560-78.

MOODY, S. A. 1987b. Fates of the blastomeres of the 32-cell-stage Xenopus embryo. *Dev Biol,* 122**,** 300-19.

MOORE, K. B., MOOD, K., DAAR, I. O. & MOODY, S. A. 2004. Morphogenetic Movements Underlying Eye Field Formation Require Interactions
between the FGF and ephrinB1 Signaling Pathways. *Developmental Cell,* 6**,** 55-67.

MORENO, M., MUNOZ, R., AROCA, F., LABARCA, M., BRANDAN, E. & LARRAIN, J. 2005a. Biglycan is a new extracellular component of the Chordin-BMP4 signaling pathway. *EMBO J,* 24**,** 1397-405.

MORENO, M., MUNOZ, R., AROCA, F., LABARCA, M., BRANDAN, E. & LARRAIN, J. 2005b. Biglycan is a new extracellular component of the Chordin-BMP4 signaling pathway. *The EMBO Journal,* 24.

MORRIS, S. A., ALMEIDA, A. D., TANAKA, H., OHTA, K. & OHNUMA, S. 2007. Tsukushi modulates Xnr2, FGF and BMP signaling: regulation of Xenopus germ layer formation. *PLoS One,* 2**,** e1004.

MOUW, J. K., OU, G. & WEAVER, V. M. 2014. Extracellular matrix assembly: a multiscale deconstruction. *Nat Rev Mol Cell Biol,* 15**,** 771-85.

MULLIGAN, K. A. & CHEYETTE, B. N. 2012. Wnt signaling in vertebrate neural development and function. *J Neuroimmune Pharmacol,* 7**,** 774-87.

NAKAJIMA, M., KIZAWA, H., SAITOH, M., KOU, I., MIYAZONO, K. & IKEGAWA, S. 2007. Mechanisms for asporin function and regulation in articular cartilage. *J Biol Chem,* 282**,** 32185-92.

NAKAMURA, O. & TAKASAKI, H. 1970. Further studies on the differentiation capacity of the dorsal marginal zone in the morula of Triturus pyrrhogaster. *Proc Jpn Acad,* 46**,** 700-705.

NAKANO, T., ANDO, S., TAKATA, N., KAWADA, M., MUGURUMA, K., SEKIGUCHI, K., SAITO, K., YONEMURA, S., EIRAKU, M. & SASAI, Y. 2012. Self-formation of optic cups and storable stratified neural retina from human ESCs. *Cell Stem Cell,* 10**,** 771-85.

NEAME, P. J., KAY, C. J., MCQUILLAN, D. J., BEALES, M. P. & HASSELL, J. R. 2000. Independent modulation of collagen fibrillogenesis by decorin and lumican. *Cell Mol Life Sci,* 57**,** 859-63.

NEILL, T., SCHAEFER, L. & IOZZO, R. V. 2012. Decorin: a guardian from the matrix. *Am J Pathol,* 181**,** 380-7.

NGUYEN, M. T. T. & ARNHEITER, H. 2000. Signaling and transcriptional regulation in early mammalian eye development: a link between FGF and MITF. *Development,* 127**,** 3581-3591.

NIETO, M. A., BRADLEY, L. C. & WILKINSON, D. G. 1991. Conserved segmental expression of Krox-20 in the vertebrate hindbrain and its relationship to lineage restriction. *Development,* Suppl 2**,** 59-62.

NIEUWKOOP, P. D. 1973. The "organisation center" of the amphibian embryo: Its origin, spatial organisation and morphogenetic action. *Adv Morphogenet,* 10**,** 1-39.

NIEUWKOOP, P. D. 1977. Origin and establishment of embryonic polar axes in amphibian development. *Curr Top Dev Biol,* 11**,** 115-32.

NIEUWKOOP, P. D. & FABER, J. 1967. *Normal Table of Xenopus laevis* Amsterdam, The Netherlands, North Holland Publishing Co.

NIEUWKOOP, P. D. & FABER, J. 1994. *Normal Table of Xenopus laevis (Daudin),* New York, Garland Publishing Inc.

NORNES, S., CLARKSON, M., MIKKOLA, I., PEDERSEN, M., BARDSLEY, A., MARTINEZ, J. P., KRAUSS, S. & JOHANSEN, T. 1998. Zebrafish contains two pax6 genes involved in eye development. *Mech Dev,* 77**,** 185-96.

NUMAN, S. & RUSSELL, D. S. 1999. Discrete expression of insulin receptor substrate-4 mRNA in adult rat brain. *Molecular Brain Research,* 72**,** 97-102.

O'KUSKY, J. & YE, P. 2012. Neurodevelopmental effects of insulin-like growth factor signaling. *Front Neuroendocrinol,* 33**,** 230-51.

OGINO, H., OCHI, H., REZA, H. M. & YASUDA, K. 2012. Transcription factors involved in lens development from the preplacodal ectoderm. *Dev Biol,* 363**,** 333-47.

OGINO, H. & YASUDA, K. 1998. Induction of lens differentiation by activation of a bZIP transcription factor, L-Maf. *Science,* 280**,** 115-8.

OHTA, K., ITO, A., KURIYAMA, S., LUPO, G., KOSAKA, M., OHNUMA, S., NAKAGAWA, S. & TANAKA, H. 2011. Tsukushi functions as a Wnt signaling inhibitor by competing with Wnt2b for binding to transmembrane protein Frizzled4. *PNAS,* 108**,** 14962-14967.

OHTA, K., ITO, A. & TANAKA, H. 2008. Neuronal stem/progenitor cells in the vertebrate eye. *Dev Growth Differ,* 50**,** 253-9.

OHTA, K., KURIYAMA, S., OKAFUJI, T., GEJIMA, R., OHNUMA, S. & TANAKA, H. 2006. Tsukushi cooperates with VG1 to induce primitive streak and Hensen's node formation in the chick embryo. *Development,* 133**,** 3777-86.

OHTA, K., LUPO, G., KURIYAMA, S., KEYNES, R., HOLT, C. E., HARRIS, W. A., TANAKA, H. & OHNUMA, S. 2004. Tsukushi functions as an organizer inducer by inhibition of BMP activity in cooperation with chordin. *Dev Cell,* 7**,** 347-58.

ORR, B., RIDDICK, A. C., STEWART, G. D., ANDERSON, R. A., FRANCO, O. E., HAYWARD, S. W. & THOMSON, A. A. 2012. Identification of stromally expressed molecules in the prostate by tag-profiling of cancer-associated fibroblasts, normal fibroblasts and fetal prostate. *Oncogene,* 31**,** 1130-42.

PAI, V. P., AW, S., SHOMRAT, T., LEMIRE, J. M. & LEVIN, M. 2012. Transmembrane voltage potential controls embryonic eye patterning in Xenopus laevis. *Development,* 139**,** 313-23.

PANNESE, M., POLO, C., ANDREAZZOLI, M., VIGNALI, R., KABLAR, B., BARSACCHI, G. & BONCINELLI, E. 1995. The Xenopus homologue of Otx2 is a maternal homeobox gene that demarcates and specifies anterior body regions. *Development,* 121**,** 707-720.

PARK, E. S., CHO, H. S., KWON, T. G., JANG, S. N., LEE, S. H., AN, C. H., SHIN, J. Y., KIM, J. Y. & CHO, J. Y. 2009. Proteomics Analysis of Human Dentin Reveals Distinct Protein Expression Profiles. *Journal of Proteome Research,* 8**,** 1338-1346.

PATTEN, I. & PLACZEK, M. 2000. The role of Sonic hedgehog in neural tube patterning. *Cell Mol Life Sci,* 57**,** 1695-708.

PELLEGATA, N. S., DIEGUEZ-LUCENA, J. L., JOENSUU, T., LAU, S., MONTGOMERY, K. T., KRAHE, R., KIVELA, T., KUCHERLAPATI, R., FORSIUS, H. & DE LA CHAPELLE, A. 2000. Mutations in KERA, encoding keratocan, cause cornea plana. *Nat Genet,* 25**,** 91-5.

PERA, E. M., WESSELY, O., LI, S. Y. & DE ROBERTIS, E. M. 2001. Neural and head induction by insulin-like growth factor signals. *Dev Cell,* 1**,** 655-65.

PERRON, M., KANEKAR, S., VETTER, M. L. & HARRIS, W. A. 1998. The genetic sequence of retinal development in the ciliary margin of the Xenopus eye. *Dev Biol,* 199**,** 185-200.

PETERSEN, C. P. & REDDIEN, P. W. 2009. Wnt signaling and the polarity of the primary body axis. *Cell,* 139**,** 1056-68.

PFAFFL, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res,* 29**,** e45.

PICCOLO, S., SASAI, Y., LU, B. & DE ROBERTIS, E. M. 1996. Dorsoventral patterning in Xenopus: inhibition of ventral signals by direct binding of chordin to BMP-4. *Cell,* 86**,** 589-98.

POLLET, N. & MAZABRAUD, A. 2006. Insights from Xenopus Genomes.

POPKEN, G. J., DECHERT-ZEGER, M., YE, P. & D'ERCOLE A, J. 2005. Brain development. *Advances in Experimental Medicine and Biology,* 567**,** 187-220.

PRINGLE, G. A. & DODD, C. M. 1990. Immunoelectron microscopic localization of the core protein of decorin near the d and e bands of tendon collagen fibrils by use of monoclonal antibodies. *Journal of Histochemistry and Cytochemistry,* 38**,** 1405-1411.

QUIRING, R., WALLDORF, U., KLOTER, U. & GEHRING, W. J. 1994. Homology of the eyeless gene of Drosophila to the Small eye gene in mice and Aniridia in humans. *Science,* 265**,** 785-9.

RANA, A. A., COLLART, C., GILCHRIST, M. J. & SMITH, J. C. 2006. Defining synphenotype groups in Xenopus tropicalis by use of antisense morpholino oligonucleotides. *PLoS Genet,* 2**,** e193.

RANKIN, S. A., KORMISH, J., KOFRON, M., JEGGA, A. & ZORN, A. M. 2011. A gene regulatory network controlling hhex transcription in the anterior endoderm of the organizer. *Dev Biol,* 351**,** 297-310.

RASMUSSEN, J. T., DEARDORFF, M. A., TAN, C., RAO, M. S., KLEIN, P. S. & VETTER, M. L. 2001. Regulation of eye development by frizzled signaling in Xenopus. *Proc Natl Acad Sci U S A,* 98**,** 3861-6.

RECANZONE, G. & HARRIS, W. A. 1985. Demonstration of neural induction using nuclear markers in Xenopus. *Wilhelm Roux Arch Dev Biol,* 194**,** 344-354.

REED, C. C., WATERHOUSE, A., KIRBY, S., KAY, P., OWENS, R. T., MCQUILLAN, D. J. & IOZZO, R. V. 2005. Decorin prevents metastatic spreading of breast cancer. *Oncogene,* 24**,** 1104-10.

REESE, B. E. 2011. Development of the retina and optic pathway. *Vision Res,* 51**,** 613-32.

REINBOTH, B., THOMAS, J., HANSSEN, E. & GIBSON, M. A. 2006. Beta ig-h3 interacts directly with biglycan and decorin, promotes collagen VI aggregation, and participates in ternary complexing with these macromolecules. *J Biol Chem,* 281**,** 7816-24.

RIBEIRO, I., KAWAKAMI, Y., BUSCHER, D., RAYA, A., RODRIGUEZ-LEON, J., MORITA, M., RODRIGUEZ ESTEBAN, C. & IZPISUA BELMONTE, J. C. 2007. Tbx2 and Tbx3 regulate the dynamics of cell proliferation during heart remodeling. *PLoS One,* 2**,** e398.

RICHARD-PARPAILLON, L., HELIGON, C., CHESNEL, F., BOUJARD, D. & PHILPOTT, A. 2002. The IGF pathway regulates head formation by inhibiting Wnt signaling in Xenopus. *Dev Biol,* 244**,** 407-17.

ROESSLER, E. & MUENKE, M. 2001. Midline and laterality defects: left and right meet in the middle. *Bioessays,* 23**,** 888-900.

ROMANELLI, R. J., LEBEAU, A. P., FULMER, C. G., LAZZARINO, D. A., HOCHBERG, A. & WOOD, T. L. 2007. Insulin-like growth factor type-I receptor internalization and recycling mediate the sustained phosphorylation of Akt. *J Biol Chem,* 282**,** 22513-24.

RORICK, A. M., MEI, W., LIETTE, N. L., PHIEL, C., EL-HODIRI, H. M. & YANG, J. 2007. PP2A:B56epsilon is required for eye induction and eye field separation. *Dev Biol,* 302**,** 477-93.

ROSEN, J. N., SWEENEY, M. F. & MABLY, J. D. 2009. Microinjection of zebrafish embryos to analyze gene function. *J Vis Exp*.

ROZARIO, T. & DESIMONE, D. W. 2010. The extracellular matrix in development and morphogenesis: a dynamic view. *Dev Biol,* 341**,** 126-40.

SAHA, M. S. & GRAINGER, R. M. 1992. A labile Period in the Determination of the Anterior-Posterior Axis during Early Neural Development in Xenopus. *Neuron,* 8**,** 1003-1014.

SANTRA, M., REED, C. C. & IOZZO, R. V. 2002. Decorin binds to a narrow region of the epidermal growth factor (EGF) receptor, partially overlapping but distinct from the EGF-binding epitope. *J Biol Chem,* 277**,** 35671-81.

SASAI, Y., LU, B., STEINBEISSER, H. & DE ROBERTIS, E. M. 1995. Regulation of neural induction by the Chd and Bmp-4 antagonistic patterning signals in Xenopus. *Nature,* 376**,** 333-6.

SASAI, Y., LU, B., STEINBEISSER, H., GEISSERT, D., GONT, L. K. & DE ROBERTIS, E. M. 1994. Xenopus chordin: a novel dorsalizing factor activated by organizer-specific homeobox genes. *Cell,* 79**,** 779-90.

SATOYOSHI, R., KURIYAMA, S., AIBA, N., YASHIRO, M. & TANAKA, M. 2015. Asporin activates coordinated invasion of scirrhous gastric cancer and cancer-associated fibroblasts. *Oncogene,* 34**,** 650-60.

SAVAGE, R. & PHILLIPS, C. R. 1989. Signals from the dorsal blastopore lip region during gastrulation bias the ectoderm toward a nonepidermal pathway of differentiation in Xenopus laevis. *Dev Biol,* 133**,** 157-68.

SCHAEFER, L. & IOZZO, R. V. 2008. Biological functions of the small leucine-rich proteoglycans: from genetics to signal transduction. *J Biol Chem,* 283**,** 21305-9.

SCHAEFER, L. & SCHAEFER, R. M. 2010. Proteoglycans: from structural compounds to signaling molecules. *Cell Tissue Res,* 339**,** 237-46.

SCHAEFER, L., TSALASTRA, W., BABELOVA, A., BALIOVA, M., MINNERUP, J., SOROKIN, L., GRONE, H. J., REINHARDT, D. P., PFEILSCHIFTER, J., IOZZO, R. V. & SCHAEFER, R. M. 2007. Decorin-mediated regulation of fibrillin-1 in the kidney involves the insulin-like growth factor-I receptor and Mammalian target of rapamycin. *Am J Pathol,* 170**,** 301-15.

SCHNEIDER, S., STEINBEISSER, H., WARGA, R. M. & HAUSEN, P. 1996. b-catenin translocation into nuclei demarcates the dorsalizing centers in frog and fish embryos. *Mechanisms of Development,* 57**,** 191-198.

SCHONHERR, E., SUNDERKOTTER, C., IOZZO, R. V. & SCHAEFER, L. 2005. Decorin, a novel player in the insulin-like growth factor system. *J Biol Chem,* 280**,** 15767-72.

SCHUBERT, M., BRAZIL, D. P., BURKS, D. J., KUSHNER, J. A., YE, J., FLINT, C. L., FARHANG-FALLAH, J., DIKKES, P., WAROT, X. M., RIO, C., CORFAS, G. & WHITE, M. F. 2003. Insulin receptor substrate-2 deficiency impairs brain growth and promotes tau phosphorylation. *J Neurosci,* 23**,** 7084-92.

SCHWARTZ, N. B. & DOMOWICZ, M. 2004. Proteoglycans in brain development. *Glycoconj J,* 21**,** 329-41.

SCIACCHITANO, S. & TAYLOR, S. I. 1997. Cloning, tissue expression, and chromosomal localization of the mouse IRS-3 gene. *Endocrinology,* 138**,** 4931-40.

SEDOHARA, A., KOMAZAKI, S. & ASASHIMA, M. 2003. In vitro induction and transplantation of eye during early Xenopus development. *Dev Growth Differ,* 45**,** 463-71.

SEHAT, B., TOFIGH, A., LIN, Y., TROCME, E., LILJEDAHL, U., LAGERGREN, J. & LARSSON, O. 2010. SUMOylation mediates the nuclear translocation and signaling of the IGF-1 receptor. *Sci Signal,* 3**,** ra10.

SEO, N. S., HOCKING, A. M., HOOK, M. & MCQUILLAN, D. J. 2005. Decorin core protein secretion is regulated by N-linked oligosaccharide and glycosaminoglycan additions. *J Biol Chem,* 280**,** 42774-84.

SILVA, A. C., FILIPE, M., KUERNER, K. M., STEINBEISSER, H. & BELO, J. A. 2003. Endogenous Cerberus activity is required for anterior head specification in Xenopus. *Development,* 130**,** 4943-53.

SINGLA, S., HU, C., MIZERACKI, A. & MEHTA, J. L. 2011. Decorin in atherosclerosis. *Ther Adv Cardiovasc Dis,* 5**,** 305-14.

SMITH, W. C. & HARLAND, R. M. 1992. Expression Cloning of noggin, a New Dorsalizing Factor Localized to the Spemann Organizer in Xenopus Embryos. *Cell,* 70**,** 829-840.

SMITHERS, L. E. & JONES, C. M. 2002. Xhex-expressing endodermal tissues are essential for anterior patterning in Xenopus. *Mech Dev,* 119**,** 191-200.

SNOW, A. D., MAR, H., NOCHLIN, D., KRESSE, H. & WIGHT, T. N. 1992. Peripheral Distribution of Dermatan Sulfate Proteoglycans (Decorin) in Amyloid-containing Plaques and Their Presence in Neurofibrillary Tangles of Alzheimer's Disease. *The Journal of Histochemistry and Cytochemistry,* 40**,** 105-113.

SOFEU FEUGAING, D. D., GOTTE, M. & VIOLA, M. 2013. More than matrix: the multifaceted role of decorin in cancer. *Eur J Cell Biol,* 92**,** 1-11.

SPEMANN, H. 1938. *Embryonic Development and Induction,* New Haven, Yale University Press.

SPEMANN, H. & MANGOLD, H. 1924. Induction of embryonic primordia by implantation of organizers from different species. *In:* WILLIER, H. & OPPENHEIMER, J. M. (eds.) *Foundations of Experimental Embryology*. New York: Hafner.

STAINIER, D. Y., KONTARAKIS, Z. & ROSSI, A. 2015. Making sense of anti-sense data. *Dev Cell,* 32**,** 7-8.

STERN, C. D. 2006. Neural induction: 10 years on since the 'default model'. *Curr Opin Cell Biol,* 18**,** 692-7.

STICHEL, C. C., KAPPLER, J., JUNGHANS, U., KOOPS, A., KRESSE, H. & MULLER, H. W. 1995. Differential expression of the small chondroitin/dermatan sulfate proteoglycans decorin and biglycan after injury of the adult rat brain. *Brain Res,* 704**,** 263-74.

SUGA, H., TSCHOPP, P., GRAZIUSSI, D. F., STIERWALD, M., SCHMID, V. & GEHRING, W. J. 2010. Flexible deployed Pax genes in eye development at the early evolution of animals demonstrated by studies on a hydrozoan jellyfish.

SVENSSON, L., HEINEGARD, D. & OLDBERG, A. 1995. Decorin-binding sites for collagen type I are mainly located in leucine-rich repeats 4-5. *The Journal of Biological Chemistry,* 270**,** 20712-20716.

TAKABATAKE, Y., TAKABATAKE, T., SASAGAWA, S. & TAKESHIMA, K. 2002. Conserved expression control and shared activity between cognate T-box genes Tbx2 and Tbx3 in connection with Sonic hedgehog signaling during Xenopus eye development. *Develop. Growth Differ.,* 44**,** 257-271.

TAKAI, A., INOMATA, H., ARAKAWA, A., YAKURA, R., MATSUO-TAKASAKI, M. & SASAI, Y. 2010. Anterior neural development requires Del1, a matrix-associated protein that attenuates canonical Wnt signaling via the Ror2 pathway. *Development,* 137**,** 3293-302.

TANIHARA, H., INATANI, M., KOGA, T., YANO, T. & KIMURA, A. 2002. Proteoglycans in the eye. *Cornea,* 21**,** S62-9.

TASHEVA, E. S., KLOCKE, B. & CONRAD, G. W. 2004. Analysis of transcriptional regulation of the small leucine rich proteoglycans. *Mol Vis,* 10**,** 758-72.

TERADA, K., KITAYAMA, A., KANAMOTO, T., UENO, N. & FURUKAWA, T. 2006. Nucleosome regulator Xhmgb3 is required for cell proliferation of the eye and brain as a downstream target of Xenopus rax/Rx1. *Dev Biol,* 291**,** 398-412.

TETREAULT, N., CHAMPAGNE, M. P. & BERNIER, G. 2009. The LIM homeobox transcription factor Lhx2 is required to specify the retina field and synergistically cooperates with Pax6 for Six6 trans-activation. *Dev Biol,* 327**,** 541-50.

THOMAS, P. & SMART, T. G. 2005. HEK293 cell line: a vehicle for the expression of recombinant proteins. *J Pharmacol Toxicol Methods,* 51**,** 187-200.

TIAN, W., ZHENG, S., JIANG, X. Z., WU, C. A., WANG, N. & ZHAO, D. H. 2013. Asporin, a candidate protein for treatment of disc degenerative disease *Chinese Medical Journal,* 126**,** 369-372.

TOMAREV, S. I., CALLAERTS, P., KOS, L., ZINOVIEVA, R., HALDER, G., GEHRING, W. & PIATIGORSKY, J. 1997. Squid Pax-6 and eye development. *Proc Natl Acad Sci U S A,* 94**,** 2421-6.

TOMOEDA, M., YAMADA, S., SHIRAI, H., OZAWA, Y., YANAGITA, M. & MURAKAMI, S. 2008. PLAP-1/asporin inhibits activation of BMP receptor via its leucine-rich repeat motif. *Biochem Biophys Res Commun,* 371**,** 191-6.

TORRES, B., OROZCO, G., GARCIA-LOZANO, J. R., OLIVER, J., FERNANDEZ, O., GONZALEZ-GAY, M. A., BALSA, A., GARCIA, A., PASCUAL-SALCEDO, D., LOPEZ-NEVOT, M. A., NUNEZ-ROLDAN, A., MARTIN, J. & GONZALEZ-ESCRIBANO, M. F. 2007. Asporin repeat polymorphism in rheumatoid arthritis. *Ann Rheum Dis,* 66**,** 118-20.

TORRES-ALEMAN, I., NAFTOLIN, F. & ROBBINS, R. J. 1990. Trophic effects of insulin-like growth factor-I on fetal rat hypothalmic cells in culture. *Neuroscience,* 35**,** 601-608.

TOZER, S., LE DREAU, G., MARTI, E. & BRISCOE, J. 2013. Temporal control of BMP signalling determines neuronal subtype identity in the dorsal neural tube. *Development,* 140**,** 1467-74.

TURTOI, A., MUSMECI, D., WANG, Y., DUMONT, B., SOMJA, J., BEVILACQUA, G., DE PAUW, E., DELVENNE, P. & CASTRONOVO, V. 2011. Identification of novel accessible

proteins bearing diagnostic and therapeutic potential in human pancreatic ductal adenocarcinoma. *J Proteome Res,* 10**,** 4302-13.

TUSSELLINO, M., DE MARCO, N., CAMPANELLA, C. & CAROTENUTO, R. 2012. Involvement of the eukaryotic initiation factor 6 and kermit2/gipc2 in Xenopus laevis pronephros formation. *Int J Dev Biol,* 56**,** 357-62.

UNO, Y., NISHIDA, C., TAKAGI, C., UENO, N. & MATSUDA, Y. 2013. Homoeologous chromosomes of Xenopus laevis are highly conserved after whole-genome duplication. *Heredity (Edinb),* 111**,** 430-6.

VAN BUUL-OFFERS, S. C., DE HAAN, K., REIJNEN-GRESNIGT, M. G., MEINSMA, D., JANSEN, M., OEI, S. L., BONTE, E. J., SUSSENBACH, J. S. & VAN DEN BRANDE, J. L. 1995. Overexpression of human insulin-like growth factor-II in transgenic mice causes increased growth of the thymus. *J Endocrinol,* 144**,** 491-502.

VAN DE WATER, S., VAN DE WETERING, M., JOORE, J., ESSELING, J., BINK, R., CLEVERS, H. & ZIVKOVIC, D. 2001. Ectopic Wnt signal determines the eyeless phenotype of zebrafish masterblind mutant. *Development,* 128**,** 3877-88.

VERGARA, M. N. & DEL RIO-TSONIS, K. 2009. Retinal regeneration in the Xenopus laevis tadpole: a new model system. *Mol Vis,* 15**,** 1000-13.

VICZIAN, A. S., SOLESSIO, E. C., LYOU, Y. & ZUBER, M. E. 2009. Generation of functional eyes from pluripotent cells. *PLoS Biol,* 7**,** e1000174.

VOGEL-HOPKER, A., MOMOSE, T., ROHRER, H., YASUDA, K., ISHIHARA, L. & RAPAPORT, D. H. 2000. Multiple functions of ®broblast growth factor-8 (FGF-8) in chick eye development. *Mechanisms of Development,* 94**,** 25-36.

WANG, B., LI, G. X., ZHANG, S. G., WANG, Q., WEN, Y. G., TANG, H. M., ZHOU, C. Z., XING, A. Y., FAN, J. W., YAN, D. W., QIU, G. Q., YU, Z. H. & PENG, Z. H. 2011. Biglycan expression correlates with aggressiveness and poor prognosis of gastric cancer. *Exp Biol Med (Maywood),* 236**,** 1247-53.

WANG, F., SHI, Z., CUI, Y., GUO, X., SHI, Y. B. & CHEN, Y. 2015. Targeted gene disruption in Xenopus laevis using CRISPR/Cas9. *Cell Biosci,* 5**,** 15.

WELLS, G. P. 1978. Lancelot Thomas Hogben. *Biographical Memoirs of Fellows of the Royal Society,* 24**,** 183-221.

WILDA, M., BACHNER, D., JUST, W., GEERKENS, C., KRAUS, P., VOGEL, W. & HAMEISTER, H. 2000. A comparison of the expression pattern of five genes of the family of small leucine-rich proteoglycans during mouse development. *J Bone Miner Res,* 15**,** 2187-96.

WILKINSON-BERKA, J. L. 2004. Diabetes and retinal vascular disorders: role of the renin–angiotensin system. *Expert Reviews in Molecular Medicine,* 6**,** 1-18.

WONG, K., PENG, Y., KUNG, H. F. & HE, M. L. 2002. Retina dorsal/ventral patterning by Xenopus TBX3. *Biochem Biophys Res Commun,* 290**,** 737-42.

WU, H. Y., PERRON, M. & HOLLEMANN, T. 2009. The role of Xenopus Rx-L in photoreceptor cell determination. *Dev Biol,* 327**,** 352-65.

WU, J., O'DONNELL, M., GITLER, A. D. & KLEIN, P. S. 2006. Kermit 2/XGIPC, an IGF1 receptor interacting protein, is required for IGF signaling in Xenopus eye development. *Development,* 133**,** 3651-60.

XENBASE. 2016. *Xenopus Fate Maps - Xenbase* [Online]. Available: http://www.xenbase.org/anatomy/static/xenbasefate.jsp [Accessed 20 Feb 2016].

XU, S., CHENG, F., LIANG, J., WU, W. & ZHANG, J. 2012. Maternal xNorrin, a canonical Wnt signaling agonist and TGF-beta antagonist, controls early neuroectoderm specification in Xenopus. *PLoS Biol,* 10**,** e1001286.

YAMADA, M., TANABE, K., WADA, K., SHIMOKE, K., ISHIKAWA, Y., IKEUCHI, T., KOIZUMI, S. & HATANAKA, H. 2001. Differences in survival-promoting effects and intracellular signaling properties of BDNF and IGF-1 in cultured cerebral cortical neurons. *J Neurochem,* 78**,** 940-51.

YAMADA, S., TOMOEDA, M., OZAWA, Y., YONEDA, S., TERASHIMA, Y., IKEZAWA, K., IKEGAWA, S., SAITO, M., TOYOSAWA, S. & MURAKAMI, S. 2007. PLAP-1/asporin, a novel negative regulator of periodontal ligament mineralization. *J Biol Chem,* 282**,** 23070-80.

YAMAGUCHI, Y., MANN, D. M. & RUOSLAHTI, E. 1990. Negative regulation of transforming growth factor-b by the proteoglycan decorin. *Nature,* 346**,** 281-284.

YAMAMOTO, Y., BYERLY, M. S., JACKMAN, W. R. & JEFFERY, W. R. 2009. Pleiotropic functions of embryonic sonic hedgehog expression link jaw and taste bud amplification with eye loss during cavefish evolution. *Dev Biol,* 330**,** 200-11.

YANG, X. J. 2004. Roles of cell-extrinsic growth factors in vertebrate eye pattern formation and retinogenesis. *Semin Cell Dev Biol,* 15**,** 91-103.

YANG-SNYDER, J., MILLER, J. R., BROWN, J. D., LAI, C. J. & MOON, R. T. 1996. A frizzled homolog functions in a vertebrate Wnt signaling pathway. *Curr Biol,* 6**,** 1302-6.

YE, P., HU, Q., LIU, H., YAN, Y. & D'ERCOLE A, J. 2010. beta-catenin mediates insulin-like growth factor-I actions to promote cyclin D1 mRNA expression, cell proliferation and survival in oligodendroglial cultures. *Glia,* 58**,** 1031-41.

YE, P., LI, L. L., RICHARDS, R. G., DIAUGUSTINE, R. P. & D'ERCOLE, A. J. 2002a. Myelination Is Altered in Insulin-Like Growth Factor-I Null Mutant Mice. *The Journal of Neuroscience,* 22.

YE, P. Y., LI, L. L., LUND, P. K. & D'ERCOLE, A. J. 2002b. Deficient expression of insulin receptor substrate-1 (IRS-1) fails to block insulin-like growth factor-I (IGF-I) stimulation of brain growth and myelination. *Developmental Brain Research,* 136**,** 111-121.

ZACKENFELS, K., OPPENHEIM, R. W. & ROHRER, H. 1995. Evidence for an Important Role of IGF-I and IGF-II for the Early Development
of Chick Sympathetic Neurons. *Neuron,* 14**,** 731-741.

ZAGRIS, N., GILIPATHI, K., SOULINTZI, N. & KONSTANTOPOULOS, K. 2011. Decorin developmental expression and function in the early avian embryo. *Int J Dev Biol,* 55**,** 633-9.

ZHANG, J., MOATS-STAATS, B. M., YE, P. & D'ERCOLE, A. J. 2007. Expression of insulin-like growth factor system genes during the early postnatal neurogenesis in the mouse hippocampus. *J Neurosci Res,* 85**,** 1618-27.

ZHANG, X., ABREU, J. G., YOKOTA, C., MACDONALD, B. T., SINGH, S., COBURN, K. L., CHEONG, S. M., ZHANG, M. M., YE, Q. Z., HANG, H. C., STEEN, H. & HE, X. 2012. Tiki1 is required for head formation via Wnt cleavage-oxidation and inactivation. *Cell,* 149**,** 1565-77.

ZHOU, S., FLAMIER, A., ABDOUH, M., TETREAULT, N., BARABINO, A., WADHWA, S. & BERNIER, G. 2015. Differentiation of human embryonic stem cells into cone photoreceptors through simultaneous inhibition of BMP, TGFbeta and Wnt signaling. *Development,* 142**,** 3294-306.

ZHOU, X., HOLLEMANN, T., PIELER, T. & GRUSS, P. 2000. Cloning and expression of xSix3, the Xenopus homologue of murine Six3. *Mechanisms of Development,* 91**,** 327-330.

ZHU, F., FRIEDMAN, M. S., LUO, W., WOOLF, P. & HANKENSON, K. D. 2012. The transcription factor osterix (SP7) regulates BMP6-induced human osteoblast differentiation. *J Cell Physiol,* 227**,** 2677-85.

ZIMMERMAN, L. B., DE JESUS-ESCOBAR, J. M. & HARLAND, R. M. 1996. The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4. *Cell,* 86**,** 599-606.

ZOELLER, J. J., PIMTONG, W., CORBY, H., GOLDONI, S., IOZZO, A. E., OWENS, R. T., HO, S. Y. & IOZZO, R. V. 2009. A central role for decorin during vertebrate convergent extension. *J Biol Chem,* 284**,** 11728-37.

ZUBER, M. E. 2010. Eye field specification in Xenopus laevis. *Curr Top Dev Biol,* 93**,** 29-60.

ZUBER, M. E., GESTRI, G., VICZIAN, A. S., BARSACCHI, G. & HARRIS, W. A. 2003. Specification of the vertebrate eye by a network of eye field transcription factors. *Development,* 130**,** 5155-67.

ZUBER, M. E., PERRON, M., PHILPOTT, A., BANG, A. & HARRIS, W. A. 1999. Giant eyes in Xenopus laevis by overexpression of XOptx2. *Cell,* 98**,** 341-52.

ZYGAR, C. A., COOK, T. L. & GRAINGER, R. M., JR. 1998. Gene activation during early stages of lens induction in Xenopus. *Development,* 125**,** 3509-19.