The Small Leucine-rich Repeat Secreted Protein Asporin Induces Eyes in *Xenopus* Embryos through the IGF Signalling Pathway

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Essential roles of asporin in eye development

ABSTRACT

Small leucine-rich repeat proteoglycan (SLRP) family proteins play important roles in a number of biological events. Here we demonstrate that the SLRP family member, asporin (ASPN), plays a critical role in the early stages of eye development in *Xenopus* embryos.

During 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 a morpholino-oligonucleotide (*ASPN-MO*) 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 (IGFR) and is essential for activating the IGF-receptor mediated intracellular signalling pathway. Moreover, 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.

INTRODUCTION

The visual system is conserved among a broad range of species from invertebrates to vertebrates, and is an important afferent component of the central nervous system transducing external stimuli from the periphery to the central part of the body (Kumar, 2001). The sensory organ of the visual system, the eye, comprises a number of different cell types including neurons and glial cells whose development is orchestrated in a complicated yet precise manner (Kohwi and Doe, 2013; Reese, 2011). How these cells differentiate and organize themselves has been a longstanding research interest, not least from a clinical point of view, since disorganization of the eye structure and loss of vision can have a severe impact on quality of life. Unsurprisingly, a lot of research effort has been invested to generate retinal tissues in test tubes, mainly for transplantation purposes (Gonzalez-Cordero et al., 2013). In this sense, the development of retinal cells has been a highly interesting and popular subject from both scientific and clinical points of view.

Like other tissues derived from the neuroectoderm, the development of the eye consists of a number of regulatory steps. At least in amphibians, the development is triggered at the gastrula stages when the naïve ectodermal cells are exposed to the neural inducers chordin, noggin and follistatin (Harland, 2000; Munoz-Sanjuan and Brivanlou, 2002; Ozair et al., 2013). These secreted factors are BMP antagonists, and induce a set of transcription factors including ET, Pax6 and Rx in the broad area of the presumptive forebrain region (Zuber et al., 2003). These transcription factors are required for the development of the whole forebrain (Klimova and Kozmik, 2014; Lagutin et al., 2003; Mathers et al., 1997). They subsequently drive the downstream transcription network formed by Six3, Lhx2 and Optx2/Six6, and the expression starts to be confined to a specific area in the diencephalon during the neurulation process, which is later termed eye-field (Zuber, 2010; Zuber et al., 2003). As such, the expression of these eye-field specific transcription factors is dynamic and the expression patterns change over time. While a number of secreted factors, including FGF and Wnt, are involved during this specification (Ikeda et al., 2005), more secreted factors must be involved in the fine-tuning of the eye-field specific transcription factors' expression patterns.

In addition to the neural inducers, the signals mediated by RTKs (receptor-tyrosine kinases), mainly induced by IGFs (insulin growth factors) and FGFs (fibroblast growth factors), have also been shown to take important roles in the eye development as well as the head formation. The RTK signals activate ERK (extracellular regulated kinases), and prime the degradation of Smad1 in concert with GSK3β (Fuentealba et al., 2007; Kuroda et al., 2005; Pera et al., 2003). Therefore, the two signalling systems of anti-BMP and RTK signals cooperate with each other and enable the embryos to acquire competence and differentiate into the eyes.

In vitro generation of retinal precursor cells is now technically feasible. In frogs, combining dorsal mesoderm and animal cap explants induces a fairly organized eye structure (Sedohara et al., 2003).

In addition, animal cap cells which overexpress a cocktail of eye-field transcription factors acquire a competence for retinal cell fate and differentiate into a functional eye when transplanted back into an embryo (Viczian et al., 2009). In mouse embryonic stem (ES) cells, timed treatment with different cytokines results in the induction of eye-field specific transcription factors (Ikeda et al., 2005). Furthermore, three-dimensional formation of the retinal cell precursors is also achievable under a specific differentiation condition (Eiraku et al., 2011; Gonzalez-Cordero et al., 2013). In contrast to these outstanding accomplishments in manipulating eye development, the molecular basis of gene regulation has not fully been elucidated.

The family of small leucine-rich repeat proteoglycan (SLRP) proteins comprises a number of extracellular matrix proteoglycans that have been shown to be involved in a number of biological events including development, growth and cancer in recent years (Dellett et al., 2012; Edwards, 2012; Schaefer and Schaefer, 2010). SLRPs feature leucine-rich repeat domains in the middle of their protein core, and can be categorised into five subclasses depending on their amino-acid sequences (Dellett et al., 2012). While most proteoglycans form large heterologous complexes of 300-400 kDa, SLRPs are thought to mostly act as monomers or possibly dimers of approximately 40kDa (Goldoni et al., 2004; McEwan et al., 2006; Scott et al., 2004).

Once thought to be solely involved in collagen fibril organization, SLRPs are now well known for their ability to modulate a number of intracellular signalling pathways (Chen and Birk, 2013; Wilda et al., 2000). This activity is exerted through interactions with specific extracellular signalling molecules and/or their receptor proteins. In *Xenopus* embryogenesis, SLRPs have been shown to play important roles in a number of developmental processes, such as germ layer specification, pattern formation and morphogenesis (Dellett et al., 2012; Kuriyama et al., 2006; Moreno et al., 2005; Munoz et al., 2006). For instance, biglycan binds to BMP4 and changes the interaction between BMP4 and chordin (Moreno et al., 2005). Another SLRP, Tsukushi (TSK), also binds to BMP, Wnt, Notch and inhibits their respective signalling pathways (Kuriyama et al., 2006; Morris et al., 2007; Ohta et al., 2004). These findings exemplify the diverse functions of SLRPs and their ability to coordinate multiple signals in a context-dependent manner.

During a systematic screening of the functions of SLRPs by means of mRNA injection in *Xenopus* embryos, we found that ASPN (ASPN; also known as PLAP-1; periodontal ligament-associated protein-1) had a strong activity to induce ectopic eyes. ASPN was originally isolated from mice (Henry et al., 2001; Lorenzo et al., 2001), where it is expressed in cartilage and bone at the mid-gestation periods. ASPN inhibits the TGF-β signalling pathway, and an aspartic acid repeat polymorphism of the ASPN protein has been linked to osteoarthritis in humans (Kizawa et al., 2005). However, the activity of ASPN in the early stages of embryogenesis is still elusive, and the striking eye phenotype in *Xenopus* prompted us to analyse the function of ASPN in detail. We found that ASPN is an essential gene for eye induction that works by potentiating the IGF signalling pathway. Moreover, we found that ASPN interacts with a number of major signalling molecules and modulates their activities. Our results

suggest that ASPN acts as a modulator for a number of signal molecules, and thereby contributes to specify the eye-forming region.

RESULTS

Structure and expression of *Xenopus* asporin

During the systematic investigation of SLRP functions (Supplementary Table S1), we found one of the clones demonstrating strong ectopic eye formation activity upon forced expression (as described below), and became interested in its detailed molecular function. This clone encoded a polypeptide sequence similar to human ASPN belonging to the class 1 SLRP (Dellett et al., 2012), and drawing a phylogenic tree evidenced the clone contained a *Xenopus* orthologue of ASPN (Fig. 1A) (Henry et al., 2001; Lorenzo et al., 2001).

Sequence alignment 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 (Fig. 1B, 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 1 SLRPs ASPN, biglycan and decorin, where the second cysteine is replaced by arginine in *Xenopus* (Fig. 1B) (Dellett et al., 2012). The characteristic stretch of 8 leucine-rich repeats was also found to be conserved among vertebrate species.

To address the expression profile of ASPN during embryogenesis, we performed RT-PCR from different stages of embryos. The expression was already apparent in unfertilized eggs and the whole expression level gradually increased during embryogenesis (Fig. 1C).

We next performed whole-mount *in situ* hybridization to map its expression pattern throughout development. During neurula stages, *ASPN* was rather ubiquitously expressed, with a little more abundance at the neural plate (Fig. 1D), including the presumptive eye field, as shown by *Pax6* (Fig. 1E). The expression became more evident around the presumptive eye field at the tailbud stage (Fig. 1F). At stage 35, strong expression can be observed in the whole head region (Fig. 1G).

In order to supplement our *in situ* data, we quantified the expression of ASPN in different areas of the embryos. We prepared explants of animal cap, dorsal marginal zone (DMZ) and ventral marginal zone (VMZ) and assayed for ASPN by qRT-PCR. Animal cap explants for embryos pre-injected with *chordin* (*Chd*) to mimic the forebrain (Fig. 1H(ii)) (Sasai et al., 1995) or *Chd* plus *Wnt8* to mimic more posterior neural domains (Fig. 1H(iii)) (Takai et al., 2010), showed enhanced ASPN expression compared to animal caps from uninjected control embryos (Fig. 1H(i)). ASPN expression in explants from DMZ (Fig. 1H(iv)) and VMZ (Fig. 1H(v)), mimicking dorsal and ventral mesoderm respectively, showed low ASPN expression similar to that seen in the control animal cap. *Otx2* (Chow et al., 1999) and *Krox20* expression (Nieto et al., 1991) (Supplementary Fig. S1) confirmed the character of the explants.

Together, these observations revealed that *ASPN* is expressed during early embryogenesis, and prompted us to investigate its embryonic functions, especially in neural development.

Overexpression of ASPN induces ectopic eyes

During the initial screen of the SLRPs' activities by overexpression, we found a striking ectopic eye-like structure upon microinjection of mRNA encoding ASPN (Fig. 2A,B), and we decided to analyse this structure in detail.

We microinjected *ASPN* mRNA into dorsal animal blastomeres, and examined the phenotype at stage 42 (tadpole stage; Fig. 2C,C',C'',F for control injection). 1 ng of mRNA induced enlarged eyes (Fig. 2D,D',D'',G, I), while higher doses induced ectopic pigmented eye-like structures (Fig. 2E,E',E'',H ,I). In order to characterise the induced pigmented structure, we performed Hematoxylin and Eosin staining on the sectioned tadpoles. The pigmented epithelium was thicker in the enlarged eyes on the injected side, and a separate retinal layer structure was occasionally found (Fig. 2G). Additionally, in the case of the embryos that had the ectopic pigmented structure, the pigment was never found inside the ectopic structure and the induced structure had an epithelial character (Fig. 2H). Notably, we did not find any expansion of the cement gland, or the anterior-most structure, suggesting that ASPN function is not entirely the same as IGF signalling (Pera et al., 2001), and it also differs from the effects of cerberus (Fig. 2C'',D'',E'') (Bouwmeester et al., 1996). Based on these observations, we supposed that this condensed pigment structure formed ectopically was a pigmented epithelium of the retina, and the retinal structure was induced by the forced expression of ASPN.

In order to verify this hypothesis, we performed immunohistochemistry with antibodies specific to the eye cell types. As a result, β-Crystallin (lens, Fig. 2J,K) Glutamine Synthetase (Müller glia, Fig. 2L,M) and Hu-C/Hu-D (Retinal ganglion and amacrine cells, Fig. 2N,O) expression was found in the ectopically-induced tissue.

SLRP family members often exhibit overlapping functions, as is for example the case with Tsukushi and biglycan for the induction of the organiser (Moreno et al., 2005; Ohta et al., 2004). To examine whether other SLRP members induce a similar eye phenotype to ASPN, we overexpressed lumican, decorin, epiphycan and chondroadherin in the *Xenopus* embryos and analysed the resulting eye phenotype (Fig. 2P, Supplementary Fig. S2A-C for representative images). Both lumican and epiphycan induced a weak expansion of the normal eye, while the other SLRPs had no effect on the embryos' eyes.

Together these data suggest that ASPN specifically induces an eye structure containing retina, retinal pigmented epithelium (RPE) and lens.

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ASPN induces Eye-field Specific Transcription factors (EFTFs) both *in vivo* and *in vitro*.

In order to address the earlier effects of ASPN overexpression, we analysed gene expression patterns by whole-mount in situ hybridisation.

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. The expression of the EFTFs *Rx1* (Mathers et al., 1997) (Fig. 3A,B; 90%, n=11) and *Pax6* (Chow et al., 1999) (Fig. 3C,D; 100%, n=11) was clearly expanded or appeared ectopically in the ASPN overexpressing side of the embryo, while the *Otx2* (Chow et al., 1999) expression pattern remained unchanged (Fig. 3E,F; 100%, n=11) (Blitz and Cho, 1995). In contrast, the telenchephalon marker *FoxG1* (*XBF1*; telencephalon; (Bourguignon et al., 1998)) (Fig. 3G,H; 50%; n=20), *En-2* (the midbrain-hindbrain junction; (Hemmati-Brivanlou et al., 1990)) (Fig. 3I,J; 81.5%, n=27) and *Krox20* (hindbrain/rhombomere3 and 5; (Nieto et al., 1991)) (Fig. 3K,L; 100%, n=12) were down regulated. These observations suggest that ASPN specifically encourages retinal development *in vivo*.

We next investigated the function of ASPN *in vitro*. For this purpose, we prepared animal cap explants from embryos injected with the *ASPN* mRNA, and analysed their gene expression when the sibling whole embryos reached stage 22. We found ASPN increased *Sox2* and *NCAM* (general neural), *XAG1* (cement gland), *FoxG1* (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) was not induced by ASPN (Fig. 3M), suggesting that ASPN can induce eye development on its own.

Taken together, these data show that ASPN has an ability to induce eye formation both *in vivo* and *in vitro*.

ASPN is essential for the eye development

To better understand ASPN's role in early embryogenesis and eye development, we performed loss-of-function analysis by using morpholino oligonucleotides (MO) of *ASPN* (Supplementary Fig. S3A).

The *ASPN* morpholino (*ASPN-MO1*) was injected into the dorsal animal blastomere of the embryos and the phenotype was analysed. At the tadpole stage, the injected side exhibited defective eye phenotypes such as small or abnormal eyes (Fig. 4B,D), while the embryo injected with the *control-MO* remained intact (Fig. 4A,D). In addition, the eye defects induced by *ASPN-MO1* could be rescued by coinjection of *ASPN* mRNA containing only the coding region (ASPN$_{CDR}$), further verifying the specificity of *ASPN-MO1* (Fig. 4C,D). We also injected another set of *ASPN* morpholinos, termed *ASPN-MO2*, and found a similar phenotype (Supplementary Fig. S3B-E), which further confirmed the validity of the morpholinos.

We next attempted to describe the phenotype induced by *ASPN-MO* on the molecular level, and performed *in situ* hybridisation with probes for the eye and other regional genes. *ASPN-MO* significantly affected the expression of the early eye-field transcription factors *Rx* (80%, n=10; Fig. 4F) and *Pax6* (89%, n=11; Fig. 4J), while *Otx2*, *En2* and *Krox20* were unaffected at neurula stages (n=20 each; Fig. 4N,R,V). 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; Fig. 4H) and *Optx2*/*Six6* (56%, n=16; Fig. 4L) (Zuber et al., 2003), while other regional markers remained unaffected (n=10 each; Fig. 4P,T,X). In all cases, the *control-MO* injected embryos were completely unaffected and showed normal expression patterns (n=10 each; Fig. 4E,G,I,K,M,O,Q,S,U,W). These results suggest that ASPN is required for eye-field specification and eye development.

Finally, we attempted to unveil the relationship between the neural inducer chordin and ASPN. For this purpose we prepared animal caps injected with *chordin* (Sasai et al., 1994) and found an elevation in forebrain gene expression (Fig. 4Y). In contrast, when *ASPN-MO* was combined with *chordin* mRNA, the expression was severely down-regulated (Fig. 4Y), suggesting that ASPN acts downstream of the neural inducer *chordin*.

In summary, ASPN is required for eye development, especially during the initial stages of the whole developmental process.

ASPN induces eye development mainly via IGF receptor mediated signalling pathway

The ability of ASPN to produce an ectopic eye was reminiscent of that of IGF (Pera et al., 2001; Richard-Parpaillon et al., 2002). In addition, a previous study has shown that some SLRPs bind to the IGF receptor (Schaefer and Iozzo, 2008). These facts prompted us to investigate how ASPN is associated with the IGF signalling pathway.

We first asked whether ASPN activates the same signalling pathway as IGF. Since IGF has been shown to induce phosphorylation of ERK and AKT both in cultured cells and in the animal cap (Rorick et al., 2007; Wu et al., 2006), we examined whether ASPN activates the same intracellular signalling molecules. For this purpose, we prepared conditioned media of secreted ASPN and IGF2 from HEK293 cells, and applied these media onto another set of HEK293 cells that had been cultured separately. The cells treated either with ASPN or IGF2 activated the phosphorylation of AKT and ERK within 20 minutes of the treatment (Fig. 5A, lanes 2,3), suggesting that ASPN and IGF share the same downstream intracellular signalling pathways.

Next we asked if ASPN forms a complex with the IGF1 receptor, and performed an immuno-precipitation assay. We co-transfected HEK293 cells with plasmids encoding ASPN and IGF1-receptor

(IGF1R) and analysed cell lysates 24 hours post-transfection. We found that ASPN does indeed establish a complex with IGF1R (Fig. 5B).

To confirm that the ASPN signal is transduced via IGF1R, we injected the dominant-negative version of the IGF1 receptor (dnIGFR) (Pera et al., 2001) together with ASPN mRNA and observed the phenotype of the eyes at the tadpole stage (Fig. 5C for control, n=20). In contrast to the ectopic eye formation following the single injection of ASPN mRNA (Fig. 5D, 12%, n=112), the combined injection of ASPN and dnIGFR significantly decreased the size of the eyes (Fig. 5E, 22.6%, n=62).

To further establish the relationship between ASPN and IGF, we conversely perturbed the function of ASPN with *ASPN-MO*. As reported by the previous study, IGF2 injection caused enlarged eyes (Fig. 5F, 90%, n=22) (Pera et al., 2001). This enlargement was however blocked by co-injection of *ASPN-MO* (Fig. 5G, 91%, n=23). Thus, eye development requires both ASPN and IGF signals.

We further attempted to confirm the necessity of both ASPN and IGF at the molecular level, and conducted an animal cap assay. For this purpose, we co-injected *ASPN* and *dnIGFR* or *IGF2* and *ASPN-MO* and assayed the expression of *Pax6* and *Rx2a*. We found that the expression of both genes was down-regulated by the inhibiting constructs (Fig. 5H,I), suggesting that both ASPN and IGF are required for the early steps of eye development.

We also attempted to identify the interacting point of ASPN and IGF signals. We prepared animal caps injected with *dnIGFR* or *ASPN-MO* and treated them with conditioned media containing secreted ASPN or IGF2 respectively. The phosphorylation of ERK, which was activated in the control explants, was inhibited by the injection of *dnIGFR* or *ASPN-MO* (Supplementary Fig. S4). These data further suggest that both signals of ASPN and IGF interact with each other at the initial steps of their signalling pathways, but not with a secondary effect interfering with the transcription of other genes.

Together, these data demonstrate that ASPN induces eye development by regulating the IGF signalling pathway through a physical association with the IGF1-receptor.

ASPN interacts and antagonises Nodal, BMP and Wnt molecules

Eye induction is regulated by a number of signalling molecules (Ikeda et al., 2005). Since it has been shown that SLRP family members interact with and inhibit the function of a number of signalling compounds in a context-dependent manner (Dellett et al., 2012), we investigated how ASPN affects some of the important signalling pathways, such as the Nodal/Activin, BMP and Wnt pathways.

First, we asked how ASPN influences these other signalling pathways. For this purpose, we injected reporter constructs of either the Activin-Response Element (ARE; for Nodal/Activin), BMP-Response Element (BRE; for BMP signals) or TOPFLASH (for Wnt), together with mRNAs of Activin (for ARE),

BMP4 (for BRE) or Wnt8 (for TOPFLASH) in the embryos, and confirmed all reporter activities were elevated at early gastrula stage. By contrast, when *ASPN* was co-injected with either of these signalling molecules, their activity was reduced significantly (Fig. 6A).

In order to further confirm that ASPN has indeed the ability to inhibit these signals, we performed expression analyses, 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 found to be down-regulated in the ASPN injected animal caps (Fig. 6B). Consistently, the injection of *ASPN* mRNA at the equator region at the 4-cell stage resulted in a reduction of *Xbra* expression (Fig. 6C,D) at the gastrula stage, suggesting that mesoderm determination was severely influenced by ASPN. Actually, a shortened body axis phenotype was exhibited at the tadpole stage (Supplementary Fig. S6).

Next, we investigated ASPN's inhibition of the BMP signal more closely. We injected either *chordin*, which is a well-known BMP inhibitor (Sasai et al., 1995), or *ASPN* mRNA and analysed the expression of general neural markers at the early neurula stage. We found *Sox2* and *NCAM* to be expressed at stage 14 in both conditions, which is consistent with the idea that ASPN inhibits the BMP signalling pathway (Fig. 6E).

We further investigated the involvement of ASPN in the Wnt signalling pathway. Again, we injected *Wnt8* mRNA either on its own or together with *ASPN* mRNA and used qRT-PCR to analyse the expression of *Xnr3*, which is one of the target genes of the Wnt signalling pathway (Yang-Snyder et al., 1996). We found the expression of *Xnr3* was induced when only *Wnt8* was injected, however *Xnr3* levels were reduced by co-injection with ASPN (Fig. 6F).

In summary, these analyses revealed that ASPN is a multiple inhibitor for Nodal, BMP and Wnt signals.

We further attempted to find out how ASPN exerts its inhibitory effect on the aforementioned signalling molecules. As ASPN is a secreted factor, we hypothesised that ASPN forms complexes with the other signalling molecules. To verify this, we performed a binding assay: tagged versions of expression constructs encoding Xnr-1, BMP4 or Wnt8 were transfected into HEK293 culture cells together with a tagged version of ASPN, and a co-immunoprecipitation analysis was performed. Our results show that ASPN does indeed form complexes with all three tested molecules BMP4 (Fig. 6G), Xnr-1 (Fig. 6H) and Wnt8 (Fig. 6I), suggesting that ASPN interacts with these molecules in the extracellular space and thereby impedes their activities.

Together these data suggest that ASPN interacts with major signalling molecules that antagonise the eye formation in the extracellular space and blocks those activities (Fig. 6J).

DISCUSSION

ASPN is a unique small Leucine-rich repeat proteoglycan involved in eye development

SLRPs have been shown to play significant roles in a number of biological events including development, growth and the prevention of tumours (Brezillon et al., 2009; Dellett et al., 2012; Dupuis and Kern, 2014; Iozzo and Schaefer, 2010; Nikitovic et al., 2008; Shimizu-Hirota et al., 2004). Based on the amino acid sequence, ASPN is categorised with decorin and biglycan as a class I SLRP (Fig. 1A). Although all three members share Leucine-rich repeats (LRRs), ASPN differs from decorin and biglycan in the number and spacing of cysteine residues at its N- and C-terminals (Henry et al., 2001; Lorenzo et al., 2001). 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 prevents them from binding to their respective receptors (Nakajima et al., 2007; Tomoeda et al., 2008; Yamada et al., 2007), which is in good agreement with our findings (Fig. 6). On the other hand, 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 (Chen et al., 2004; Moreno et al., 2005). So whilst ASPN, decorin and biglycan share similarities in terms of structure and amino acid sequences (Fig. 1A), the biochemical characteristics of these three proteins differ from each other. This functional diversity is probably caused by differences in binding partners and/or the affinities of the interactions.

This diversity is reflected in the embryonic activities of each protein. ASPN induces a strong eye phenotype, which could not be found when injecting the other class I SLRPs in *Xenopus* embryos (Fig. 1A, 2I,P, Supplementary Fig. S2A-C) (Kalamajski et al., 2009; Kizawa et al., 2005). In addition, SLRP members of other classes did not elicit an eye phenotype when they were overexpressed – the exceptions being the type 2 SLRP lumican and type 3 SLRP epiphycan, which occasionally induced a subtle eye phenotype (Fig. 2P, Supplementary Fig. S2B) (Kuriyama et al., 2006). This is consistent with the fact that the levels of ERK and AKT activation by lumican and decorin are different from each other (Supplementary Fig. S2D). Therefore, each SLRP seems to have its own unique functions and is not redundant with each other.

ASPN was first identified as a TGF-β modulating molecule and was recognized as a causal gene for osteoarthritis (Henry et al., 2001; Kizawa et al., 2005; Lorenzo et al., 2001). In the mouse, ASPN is strongly expressed in the developing cartilage and its related tissues from the mid gestation period. In the eye, ASPN expression was found in the sclera from stage 15.5, while significant expression has not been reported at earlier stages (Henry et al., 2001). As mouse models with disrupted *ASPN* gene expression have not yet been reported, it is currently uncertain if ASPN is involved in the early stages of eye development in mice. Therefore, the functions of ASPN in different organisms are of interest for future studies.

ASPN antagonises BMP, Nodal and Wnt molecules and makes the IGF-mediated signal prominent.

Our experiments have demonstrated that ASPN interacts with IGF and IGF1R, and activates the downstream signalling pathways (Fig. 5A,B, Supplementary Fig. S4, S5A). In addition, both IGF and ASPN are required for the activation of these pathways (Supplementary Fig. S4). Furthermore, it is interesting to note that *ASPN* expression is induced by the neural inducer chordin, but not by IGF (Supplementary Fig. S1), suggesting that there are two independent regulatory pathways of chordin/ASPN and IGF for the induction of eye development. Taken together, one possible model is that the specification of the presumptive eye region relies on time and space; when and where the two molecules' signals intersect with each other.

However, according to the previous and present *in situ* hybridisation analyses, IGFs and ASPN are expressed rather ubiquitously (Fig. 1D) (Pera et al., 2001). Detection of the IGFs, ASPN and activated forms of ERK and AKT proteins at *in vivo* levels will therefore enable us to demonstrate the relevance between signalling molecule distribution and eye formation in a more precise manner.

Other IGF-related proteins may also be involved in the specification of the eye region. For instance, IGF-binding proteins (IGFBPs) have been shown to modulate IGF signals (Pera et al., 2001; Pollak, 2009). In addition, the two mediator proteins IRS-1 and Kermit-2 bind to the IGF1R and play essential roles in eye development (Bugner et al., 2011; Wu et al., 2006). IRS-1 and Kermit-2 are localised intracellularly and may determine the mode of responsiveness to the extracellular signals of ASPN and IGF. Therefore, both the inducing activities and cellular responses seem to contribute to the spatio-temporal specification of the eye region.

Our experiments have further suggested that ASPN interacts with many other regulatory molecules including BMP, Wnt and Nodal (Fig. 6G-I) in addition to IGF and IGF1R (Fig. 5A,B, Supplementary Fig. S5A), but has no, or very little if any, affinity to bind other receptor proteins (Supplementary Fig. S5B-D). This means that ASPN is not just an extracellular matrix protein that randomly binds to proteins, but rather an active modulator for other signal molecules. Furthermore, ASPN can apparently regulate bound proteins in different ways. BMP, Nodal and Wnt are opposed by ASPN while in contrast the IGF signal is encouraged. This finding may account for the differences in the phenotypes by the overexpression of IGF and ASPN; ASPN exhibits a strong phenotype specifically in the eye (Fig. 2), while IGF induces the whole head structure including cement grand (Pera et al., 2001).

It is also possible that ASPN binds additional, currently unidentified molecules. Actually, the expression of FoxG1 was decreased upon ASPN overexpression in the whole embryos (Fig. 3H), while the same overexpression instead up-regulated FoxG1 expression in the simpler system of the animal caps (Fig. 3M), and this takes place probably due to the differences of the proteins ASPN may

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bind to. The question of how ASPN interacts with many proteins is an intriguing research subject and deserves detailed quantitative (e.g. measuring dissociation constants) and systematic (e.g. high-throughput searching for interacting proteins) analyses.

There have been a number of intracellular molecules isolated as the "eye-maker" factors (Rorick et al., 2007; Xu et al., 2012; Yang et al., 2003). Nevertheless, one advantage in identifying ASPN is that it is an extracellular protein and is easily applied to differentiating cells. We envisage that ASPN will be useful in improving the efficiency of eye production from embryonic stem cells. The increase in efficiency will be useful not only for clinical applications but also for the development of drug screening systems, and will consequently reduce the number of animals used to explore new therapeutic methods for retinal diseases. In conclusion, the discovery of ASPN raises possibilities of novel scientific and clinical applications.

MATERIALS AND METHODS

Isolation of *ASPN*

IMAGE (Integrated Molecular Analysis of Genomes and their Expression; http://www.imageconsortium.org) clones were purchased from Source Biosciences and the synthesised mRNA of each clone was injected into *Xenopus* embryos. The clone #6931202, encoding *Xenopus asporin* (NCBI Gene ID 495030), demonstrated a strong activity and we started further analyses. The very similar gene *asporin-b*, which exists probably because of the pseudotetraploidity of *Xenopus laevis*, was isolated in this study and has been registered in GenBank (accession number LC056842). Unless mentioned, all injection experiments were carried out with the mRNA of *asporin-a* (*ASPN-a*). The other genes tried during this screening were presented in Supplementary Table S1.

Embryonic manipulation, *in situ* hybridisation and immunohistochemistry

All animals in this study were subject to local and national ethical approval and guidance (University College London Ethical Committee, Cambridge University Ethical Committee and the Animals (Scientific Procedures) Act 1986 (UK Home Office)). Frogs were purchased from Nasco (Salida, California) and primed one week before use with 50 IU of PMSG (Chorulon, Intervet) and then injected with hCG (Chorulon, Intervet) on the previous evening (300-400 U) and *in vitro* fertilization was performed. Injection of mRNAs was performed with a fine glass capillary with a pressure injector (Harvard Apparatus). Staging of the embryos was done according to the normal table by Nieuwkoop and Faber (Nieuwkoop and Faber, 1967).

Embryos were harvested at indicated stages, fixed with MEMFA (0.1 M MOPS (3-(N-morpholino) propanesulfonic acid) (pH 7.4), 2 mM EGTA (ethylene glycol tetraacetic acid), 1 mM MgSO$_4$, 3.7% formaldehyde) for 1 hour and stained with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) if necessary. Antisense DIG (Digoxigenin) RNA probes were synthesized with RNA polymerase (Roche). *In situ* hybridisation was performed as described elsewhere (Harland, 1991). Hybridisation buffer contained 1.3xSSC (saline-sodium citrate) pH 5.0, 1 mg/ml Torula RNA (SIGMA), 0.2% Tween 20 detergent (SIGMA), 0.5% CHAPS detergent (SIGMA), 100 μg/ml Heparine sodium salt, and 50% formamide, and the hybridisation was performed at 60°C. Signals were developed by BM-Purple (Roche).

Three morpholino antisense oligonucleotides against ASPN were designed around the translation initiation site of the ASPN gene with the sequences of ATTCCTT<u>CAT</u>GGTGTTGTTTCAGAG for *ASPN-MO1*, TATAATTCTGCGGATCATAGATAAA for *ASPNa-MO2* and TCTTCTTAACTGTAAATCCACCTGA for *ASPNb-MO2* (the underlined sequence is complementary to the translation initiation site; Gene Tools). *ASPNa-MO2* and *ASPNb-MO2* were injected together (termed *ASPN-MO2*) to ensure both alleles of ASPN genes were knocked down. The control oligonucleotide (*control-MO*; CCTCTTACCTCAGTTACAATTTATA) was used as a specificity control.

For the rescue experiment, the mRNA of ASPN$_{CDR}$ was used, which only contains the coding region of ASPN and is therefore unlikely to bind to either morpholino oligonucleotides.

For immunohistochemistry of sectioned eyes (Fig. 2), embryos fixed with 4% paraformaldehyde were incubated in 30% sucrose buffered with PBS (phosphate-buffered saline) overnight. Embryos were then embedded in OCT (Optimal Cutting Temperature) compound (Tissue-Tek) and sectioned with 10 μm increments (Leica). The antibodies used in this study were: Glutamine Synthetase (Millipore; #MAB302), Calbindin D-28K (SIGMA; #C-2724), HuC/HuD (LifeTechnologies; #A21271), β-Crystallin (Abcam; #ab90379). Goat anti-mouse or anti-rabbit Alexa Fluor 488® (Abcam) were used as secondary antibodies.

Animal cap, semi-quantitative and quantitative RT-PCR

20 animal caps in each condition were prepared at stage 10 and were cultured in Steinberg's solution (58 mM NaCl, 0.67 mM KCl, 0.34 mM Ca(NO$_3$)$_2$, 0.83 mM MgSO$_4$, 4.6 mM Tris-Cl (pH 7.4)) (Sive et al., 2000) (doi:10.1101/pdb.rec11904 Cold Spring Harb Protoc 2009) until indicated time points and RNA was extracted with an RNeasy RNA extraction kit (QIAGEN). Complementary DNAs (cDNAs) were synthesized with the reverse transcription by Superscript II with random hexamers (LifeTechnologies). Semi-quantitative RT-PCR was performed with the Platinum Taq DNA polymerase (LifeTechnologies). Primers sequences were referred to previous reports (Mizuseki et al., 1998; Shimizu et al., 2013) and the De Robertis laboratory webpage (http://www.hhmi.ucla.edu/derobertis/protocol_page/Pdfs/Frog%20protocols/Primers%20for%20RT-PCR.pdf). Quantitative RT-PCR (qRT-PCR) was performed with the 7900 HT Fast Real-Time PCR machine (Applied Biosystems) with the SYBR Green detection system (Applied Biosystems). Each gene expression level was normalized with that of ODC (ornithine decarboxylase). Primer sequences for qRT-PCR are available in Supplementary Table S2.

Transfection, Immunoprecipitation and Western Blotting

Human embryonic kidney HEK293 cells (ATCC number CRL-1753) were maintained with DMEM (Dulbecco's Modified Eagle's medium) (Gibco) supplemented with 10% Fetal Bovine Serum (Gibco) and antibiotics (Penicillin and Streptomycin) (LifeTechnologies). For preparing the conditioned media, HEK293 cells were transfected with the expression vectors that carry the indicated genes and were incubated for three days in Opti-MEM (Life Technologies). The cells that had separately been acclimatised to the serum-free condition were treated with the conditioned media.

Cell extracts were prepared in TN Buffer (150 mM NaCl, 5 mM KCl, 0.5% NP-40 detergent, 10 mM Tris-Cl (pH7.8)) with protease inhibitor cocktail (Roche). For the analysis of phosphorylated proteins, 5 mM NaF and 1 mM Na$_3$VO$_4$ were supplemented to inhibit dephosphorylation. Immunoprecipitation was performed by using Protein G sepharose (GE healthcare) with the indicated antibodies. After overnight incubation, beads were washed 3 times with the TN Buffer and analysed by western blotting as described previously (Wang et al., 2013). The antibodies to phosphorylated ERK (#9101), ERK

(#9102), phosphorylated AKT (Ser473; #4060), AKT (#4691), myc (#2276), IGF1 Receptor (#9750) were from Cell Signaling Technology; FLAG (#F3165) and HA (#6908) from Sigma. HRP (Horseradish peroxisome)-conjugated mouse or rabbit IgG (GE healthcare) were used as the secondary antibodies and signals were detected with ECL Western Blotting Detection Reagents (GE healthcare).

Reporter assay

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) was used as a normalization control, and luciferase assays were performed by a dual-luciferase assay system (Promega).

FIGURE LEGENDS

Figure 1 Structure and expression of *ASPN*. (A) A phylogenic tree of SLRPs. (B) Comparison of the amino acid sequences of ASPN in different species. The amino-terminal aspartic-acid rich domain and leucine-rich domain are circled with red and light-blue rectangles, respectively. 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). (C) Semi-quantitative RT-PCR of *ASPN* and *Histone4*. Whole embryos from various stages were analysed by RT-PCR. (D-G) Spatial expression of *ASPN* in *Xenopus* embryos at neurula (stage 18; anterior view; D), compared to Pax6 expression at neurula (stage 18; anterior view; E) and ASPN expression in early tail-bud (stage 22; lateral view; F), tadpole (stage 36; G) was analysed by *in situ* hybridisation. The neural plate border (D) and the presumptive eye region (F) are indicated by yellow arrowheads. (H) Expression levels of *ASPN* in various types of explants, as assayed by qRT-PCR. Animal caps (control (i) or injected with mRNAs of *Chd* (ii) and *Chd* + *Wnt8* (iii)) and dorsal marginal zone (DMZ; iv) and ventral marginal zone (VMZ; v) were prepared at stage 10.5 and assayed at stage 18.

Figure 2 ASPN induces eye-like structure. (A,B) Ectopic eye-like structure that appeared following overexpression of *ASPN* mRNA (B). (C-H) Injection of *ASPN* mRNA induces an eye-like structure. In contrast to the embryos injected with 3 ng of control (β-Galactosidase) mRNA (C,C',C'',F), either 1 ng (D,D',D'',G) or 3 ng (E,E',E'',H) of *ASPN* mRNA at 4-cell stage induced ectopic pigmented structures (arrows) at stage 42. The whole structure was imaged (C-E, C'-E' and C''-E''), or Hematoxylin and Eosin staining was performed with sectioned samples (F-H). (I) Quantification of the phenotypes. The phenotypes were divided into four categories; embryos with normal eyes (black), with enlarged eyes (green; like (D,D', D'')), with ectopic eyes (red; like (E,E', E'')) and short axis (white). (J-O) The pigmented structure found in the ASPN-injected embryos contains eye specific components. Immunohistochemistry performed on sections of the eye-like structure induced by ASPN. Embryonic eyes (J,L,N) and pigmented structure induced by injection of ASPN (K,M,O) were analysed at stage 42 with β-Crystallin (J,K), Glutamine Synthetase (L,M) and HuD/Hu-C antibodies (N,O). (P) The phenotypes found following the injection of SLRP family members. SLRP family members were injected at 3 ng into a dorsal animal blastomere at 4 cell stage and the phenotype categorised at stage 42.

Figure 3 ASPN induces forebrain marker genes both *in vivo* and *in vitro*. (A-L) Forebrain marker genes were increased at the expense of posterior markers in vivo. The tracer β-Galactosidase (A-L; light blue product) was injected without (A,C,E,G,I,K) or with *ASPN* mRNA (B,D,F,H,J,L) and embryos were analysed by *in situ* hybridisation with either *Rx* (A,B), *Pax6* (C,D), *Otx2* (E,F), *FoxG1* (G,H), *En2* (I,J) or *Krox20* (K,L) probes at stage 18. Affected areas are pointed by arrowheads. (M) Control (lane 2) or *ASPN*-injected (lane 3) animal cap explants were analysed with semi-quantitative RT-PCR. Whole embryos (lane 1) were used as a positive control for the PCR.

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Figure 4 ASPN is required for eye development. (A-C) Representative images from the injection of *control-MO* (A), *ASPN-MO1* (B) 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$_{CDR}$*, or 20 ng *ASPN-MO1* and 3 ng *ASPN$_{CDR}$* and the phenotypes analysed at stage 41. (E-X) The expression of marker genes caused by *ASPN-MO1*. Either *control-MO* (E,G,I,K,M,O,Q,S,U) or *ASPN-MO* (F,H,J,L,N,P,R,T,V,X) was injected together with *β-Galactosidase* mRNA as a tracer (light blue product) and embryos were analysed at stage 17 (E,F,I,J,M,N,Q,R,U,V) or at 22 (G,H,K,L,O,P,S,T,W,X) by *in situ* hybridisation with the probes of *Rx* (E,F), *Six3* (G,H), *Pax6* (I,J), *Optx2/Six6* (K,L), *Otx2* (N-Q), *En2* (R-U) and *Krox20* (V-Y). (Y) 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* (iii; red bars) -injected embryos were prepared and the animal caps were analysed at stage 22 by qRT-PCR (*p<0.01; Student's t-test).

Figure 5 Cooperation of ASPN and IGF is essential for the eye development. (A) ASPN activates ERK and AKT. Conditioned media expressing (lane 1), ASPN (lane 2) or IGF2 (lane 3) were prepared and applied to HEK 293 cells for 20 min. Western blotting analysis was performed with antibodies for phosphorylated ERK, ERK, phosphorylated AKT and AKT. (B) ASPN physically interacts with IGF1R. HEK 293 cells were transfected with expression vectors carrying IGF1R (lanes 1,2) and ASPN (lane 2) and coimmunoprecipitation analysis was performed with the IGF1R antibody and detected with the Myc antibody. IB; immunoblotting, IP; immunoprecipitation. (C-I) The embryonic eye formation requires both signals of ASPN and IGF. (C-G) Embryos were injected with 3 ng *β-Galactosidase* mRNA (control: C), 1 ng *ASPN* mRNA (D), 1 ng *ASPN* + 3ng *dnIGFR* mRNAs (E), 1 ng *IGF2* mRNA + 20 ng *control-MO* (F) or 1 ng *IGF2* mRNA + 10 ng *ASPN-MO* (G) at the dorsal blastomere at 4-cell stage and phenotypes were evaluated at stage 42. Affected areas are indicated with yellow arrowheads. (H,I) The same combination of mRNAs and morpholinos were injected. Animal caps were prepared and analysed at stage 22 for *Pax6* and *Rx2a* expression with qRT-PCR. (*p<0.01; Student's t-test).

Figure 6 ASPN inhibits multiple signal molecules. (A) ASPN blocks endogenous Activin, BMP and Wnt signals, as examined by Luciferase assays. Either ARE-luc, BRE-luc or TOPFLASH reporter constructs were injected with 1ng *β-Galactosidase* (lane 1), 100 pg *Xnr1* mRNA (for ARE), 100 pg *BMP4* mRNA (for BRE) or 100 pg *Wnt8* mRNA (lane 2), 100 pg *Xnr1* + 1ng *ASPN* mRNAs (for ARE), 100 pg *BMP4* + 1ng *ASPN* mRNAs (for BRE) or 100 pg *Wnt8* + 1ng *ASPN* mRNAs (lane 3) and were assayed at stage 12. (B) ASPN inhibits the Nodal signalling pathway. Animal caps injected with control (lane 1,2) or *ASPN* mRNA (lane 3) were prepared at stage 9 and cultured with control medium (lane 1) or medium containing human Nodal protein (lane 3) until stage 10.5. *Mix.2* expression was analysed by qRT-PCR. (C,D) *Xbra* expression was inhibited by ASPN, as analysed by in situ hybridisation. The *β-Galactosidase* mRNA (light blue product) was injected without (C) or with *ASPN* (D) mRNAs into one blastomere at the equator region of 4-cell stage embryos and embryos were cultured until stage 10.5. Affected areas are pointed with arrowheads. (E) ASPN has neural inducing activity. Animal caps

injected with 500 pg *Chd* (lane 3) or 1ng *ASPN* (lane 4) mRNAs were analysed at stage 14 by semi-quantitative PCR. (F) ASPN inhibits the Wnt signaling pathway. The animal caps injected with *Wnt8* and *ASPN* mRNAs were prepared and the expression of *Xnr3* was analysed at stage 10.5. (G-I) ASPN forms complexes with Xnr1 (G), BMP (H) and Wnt (I) proteins. In order to avoid the artificial interactions in the same cells, each expression construct was separately transfected into HEK293 cells and cells were combined on the following day as indicated. The cell lysates were collected after two additional days and immunoprecipitation (IP) was performed with the HA antibody and western blotting (IB) was performed with the FLAG (G) or myc (H,I) antibodies. (*$p<0.01$; **$p<0.05$; Student's t-test).

SUPPLENTARY MATERIAL

Figure S1 Expression of *ASPN* and other related genes in various explants. Expression levels of *ASPN* (A), *Otx2* (B) and *Krox20* (C) in various types of explants, 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 stage 10.5 and assayed at stage 18. Note that the data indicated with (†) are identical to those in Fig.1H.

Figure S2 Characterisation of Lumican and Decorin. (A-C) Representative images of the embryos injected with 3 ng *ASPN* (A), 3 ng *Lumican* (B) and 3 ng *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 as in Fig. 5A.

Figure S3 Designation of morpholino oligonucleotides against *ASPN* and the phenotypes caused by the *ASPN-MO2*. (A) 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 nucleotide sequences (black characters) around the start codon (circled) of *Xenopus* ASPN and the sequences of *ASPN-MO1* (red) *ASPNa-MO2* (blue) and *ASPNb-MO2* (purple) are shown. (B-D) Representative images from the injection of 20 ng *control-MO* (B), 20 ng *ASPN-MO2* (C) and 20ng *ASPN-MO2* together with 1 ng of the coding region of *ASPN* (*ASPN$_{CDR}$*) mRNA (D). (E) Quantification of the phenotypes. For the rescue experiment embryos were injected with either 20 ng *ASPN-MO2* and 1 ng *ASPN$_{CDR}$*, or 20 ng *ASPN-MO2* and 3 ng *ASPN$_{CDR}$* and the phenotypes analysed at stage 41.

Figure S4 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), 3ng *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 phosphor-ERK or ERK antibodies.

Figure S5 Interactions between ASPN and other molecules. The expression plasmids encoding ASPN-HA, 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 as in Fig. 6G-I. The cell extracts were analysed by coimmunoprecipitation assays.

Figure S6 The phenotypes caused by the ventral injection of *ASPN* mRNA. 3 ng of *ASPN* mRNA was injected at the equator regions of one of the blastomeres at 4-cell stage and the phenotype observed at stage 42. In contrast to the control embryos (A), the injected embryos exhibited shortened bodies (B).

Supplementary Table S1 The SLRPs used for the screening
Supplementary Table S2 The primer sequences for semi-quantitative PCR and qRT-PCR

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