Zinc fingers 1, 2, 5 and 6 of transcriptional regulator, PRDM4, are required for its nuclear localisation.

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Running title: Nuclear localisation of PRDM4

Abstract:

PRDM4 is a member of the PRDM family of transcriptional regulators which control various aspects of cellular differentiation and proliferation. PRDM proteins exert their biological functions both in the cytosol and the nucleus of cells. All PRDM proteins are characterised by the presence of two distinct structural motifs, the PR/SET domain and the zinc finger (ZF) motifs. We previously observed that deletion of all six zinc fingers found in PRDM4 leads to its accumulation in the cytosol, whereas overexpressed full length PRDM4 is found predominantly in the nucleus. Here, we investigated the requirements for single zinc fingers in the nuclear localisation of PRDM4. We demonstrate that ZF's 1, 2, 5 and 6 contribute to the accumulation of PRDM4 in the nucleus. Their effect is additive as deleting either ZF1-2 or ZF 5-6 redistributes PRDM4 protein from being almost exclusively nuclear to cytosolic and nuclear. We investigated the potential mechanism of nuclear shuttling of PRDM4 via the importin α/β-mediated pathway and find that PRDM4 nuclear targeting is independent of α/β-mediated nuclear import.

Key words: PRDM4, zinc finger, nuclear transport, neural stem cells, PC12 cells, confocal microscopy

Abbreviations: PRDM4 (positive regulatory domain I-binding factor 4), PC12 cells – pheochromocytoma 12 cell line, MTase – methyltransferase, HMTase – histone methyltransferase, NLS – nuclear localisation signal.

Introduction

The PRDM family of proteins are transcriptional regulators which control a variety of developmental processes in multiple cell lineages [1-4]. PRDM proteins exert their biological function either by directly modifying chromatin or by recruiting third party chromatin modifiers to control gene transcription [1-9]. Gene ablation experiments have identified the requirement for PRDM1 in the specification of germ cells, forelimb patterning, placental morphogenesis and postnatal reprogramming of intestinal enterocytes [8, 10, 11]. PRDM16 is critical for directing brown fat cell identity as well as haematopoiesis and cardiac development [12, 13]. PRDM3 and 16 maintain mammalian heterochromatin integrity [14], while PRDM9 controls meiotic recombination [15-20]. Deregulated expression of many PRDM proteins is implicated in different types of cancers [1] and mutations in some are linked to congenital disorders [21-23] highlighting the importance of these proteins in normal development and disease.

All PRDM proteins are characterised by the presence of highly conserved structural domains, the so-called PR/SET (Positive regulatory/Suppressor of variegation 3-9, Enhancer of zeste, Trithorax) domain, zinc finger (ZF) domains, with some having a variety of other structural motifs, e.g., zinc knuckle (ZK) domain, proline-rich repressor domain, KRAB repressor domain to name a few [1, 3]. The PR/SET domains share 20-30% identity to the SET domains of histone lysine methyltransferases (MTases), which modify chromatin structure by mediating methylation of histone lysine residues. Several PRDM proteins have been shown to possess histone MTase (HMTase) activity [4], while most recruit chromatin modifiers, such as histone deacetylases and lysine or arginine HMTases to control gene expression [5, 24, 25]. In order to exert their chromatin-mediated transcriptional control, PRDM proteins must enter the nucleus.

Many of the PRDM proteins shuttle between the nucleus and the cytosol of the cells mediating their biological actions in different subcellular compartments. PRDM3 and 16 act

as HMTases predominantly in the cytosol catalysing H3K9 monomethylation, which is converted to the H3K9 trimethylation by a different HMTase, Suv39h, in the nucleus [14]. PRDM1/Blimp1 recruits the chromatin modifier, protein arginine methyltransferase, PRMT5, in the nucleus of primordial germ cells (PGCs) early in development, and both translocate to the cytosol of PGCs at a later stage [5]. We previously showed that PRDM4 recruits PRMT5 in PC12 cells and the activity of this complex is upregulated in the cytosol by the differentiation inducing Nerve growth factor (NGF), but not by the proliferation sustaining Epidermal growth factor (EGF) [26]. Together, these observations highlight the importance of controlled subcellular localisation of PRDM proteins for their biological function and underscore the significance of understanding which of the domains of these proteins regulate their subcellular localisation.

Nuclear import of proteins is mediated by a family of specialised transport receptors which move macromolecules between the cytosol and the nucleus [27, 28]. Such directional transport depends on the nuclear localisation signals (NLSs), sequences within the proteins which direct them into the nucleus [27]. The canonical NLSs are characterised by a short stretch of basic amino acids and are imported into the nucleus by the heterodimer of importin α/β [28, 29]. Another well characterised NLS is the so-called PY-NLS, subdivided into two subclasses, hPY-NLS (hydrophobic) and bPY-NLS (basic), defined by their NH-terminal motifs enriched either in hydrophobic or basic residues [30]. This type of NLS is imported into the nucleus via the karyopherin-β2 (Kapβ2)/transportin proteins [30].

PRDM4 contains neither the classical basic NLS nor the PY-NLS consensus sequence. Generally, the C2H2 type ZFs of PRDM proteins mediate both their DNA binding specificity and their nuclear import [10, 24, 31, 32]. PRDM4 contains six ZFs at its COOH-terminus and we and others previously showed that nuclear localisation of PRDM4 requires the presence of its ZFs [24, 31]. Deletion of all six ZFs of PRDM4 results in exclusively cytosolic localisation of the protein in a variety of cells lines and primary Schwann cells and mouse

embryonic stem cells (ESCs). However, it is not clear whether all ZFs are necessary for the nuclear localisation of PRDM4. Here, we dissect the requirement of single ZFs in the control of nuclear localisation of PRDM4 using PC12 cells and primary mouse embryonic neural stem cells (NSCs). We find that ZFs 1, 2, 5 and 6 are required for efficient nuclear accumulation of PRDM4 and appear to cooperate in this process. ZF3-4 and ZK are dispensable for the nuclear targeting of PRDM4. Further, we investigate the potential mechanism of PRDM4 import into the nucleus by using peptide inhibitors of importin α/β-mediated nuclear transport, bimax 1 and 2 [33] and demonstrate that PRDM4 nuclear localisation is independent of the importin α/β pathway.

Material and Methods

Plasmids

Deletion mutants of PRDM4 were generated using InFusion®HD Cloning kit (Clontech Laboratories, Inc. A Takara Bio Company) according to the manufacturer's instructions, subcloned into pCMV-Myc vector (Clontech laboratories) and sequenced. The following primers were used to generate various deletions of PRDM4 protein: mut dZF1 5' aaaatcacacttgtgcttcctttcctt 3'; fwd dZF1 5' cacaagtgtgatttttgtagcaaggct 3'; mutdZF2 5' caaagtacacctgtagggcttcatgcc 3'; fwd dZF2 5' tacaggtgtactttgtgtgacaagtct 3'; mut dZF3 5' cagaagaattacaggggtgagaagaat 3'; fwd dZF3 5' gtaatcacacttgagattcttctcacc 3'; mut dZF4 5' cacatggtcatccaccagatcaagtgt 3'; fwd dZF4 5' gaacagtttatcacacttcggacacttgatctg 3'; mut dZF5 5' tttctcacagacatactggcgttcttgtgtgtg 3'; fwd dZF5 5' tatgtctgtgagaaatgcacaaaggct 3'; mut dZF6 5' ggcgggctccttgcagtctcgtcttccctcgtg 3'; fwd dZF6 5' tgcaaggagcccgcctccagct 3'; mut dZF1-2 5' gaaaggaagtggtgctacaggtgtactttg 3'; fwd dZF1-2 5' gcaccacttcctttccttgctgtggcttg 3'; mut dZF3-4 5' cagaagaattacaggcagatcaagtgtccg 3'; fwd dZF3-4 5' gaacagtttatcacacttcggacacttgatctg 3'; mut dZF5-6 5' atcaagtgtccgaagaaggagcccgcctcca 3'; fwd dZF5-6 5' cttcggacacttgatctggcgttcttgtgtgtgga 3'; mut dZF3-6 5' cagaagaattacaggaaggagcccgcctccagctcc 3'; fwd dZF3-6 5'

cctgtaattcttctgacctgtatgtattttgaggtgtgtccgt 3'; mut dZK 5' atggaagactccaattcaaacagagcaagactatcccctcccg 3'; fwd dZK 5' agaacaagctgcttcgggagggatagtcttgctct 3'. Protein expression was verified by transiently transfecting 293HEK cells with the cDNAs encoding various deletions of PRDM4, lysing the cells 48 hours post-transfection and detecting overexpressed proteins by Western blotting. pCMV-GRX1-bimax1 and pCMV-GRX1-bimax2 plasmids overexpress bimax 1 and bimax 2 peptides fused to a Flag-tag.

Cell culture and transfections

293HEK cells were cultured in DMEM with glutamax® (Invitrogen) supplemented with 10% fetal calf serum (FCS) (Invitrogen). PC12 cells were cultured in DMEM with glutamax® supplemented with 5% FCS and 10% horse serum (HS) on poly-D-lysine (PDL)-coated glass chamber slides and transfected using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Cells were fixed and processed for imaging 48 hours post-transfection. All experiments were performed at least three times in triplicate each and ca 100 transfected cells were scored/experiment.

Primary neural stem cell cultures

Primary NSCs were isolated from CD1 mouse E13.5 cerebral cortices and maintained according to published procedures [25]. Briefly, cortices from embryonic day 13.5 (E13.5) were dissected in EBSS (Invitrogen), the meninges were removed and NSCs were dissociated with Trypsin for 45 minutes at 37^{o}C. NSCs were then further separated into single cell suspension using MACS pre-separation filters (Milteney Biotech), counted and plated at 2x10^{5}cells/13-mm glass coverslip coated with poly-D-lysine (PDL). We routinely monitored the ability of NSCs to generate neurons, astrocytes and oligodendrocytes by immunolabelling the cultures with lineage-specific antibodies. NSC were transfected with Lipofectamine® LTX reagent (Invitrogen) according to the manufacturer's instructions.

Immunofluorescence Confocal Microscopy

Transfected cells were fixed in 4% (w/v) paraformaldehyde (PFA) and permeabilized with cold methanol for 2–3 min at −20°C following fixation or with 0.1% (v/v) Triton-X-100 in blocking buffer (10% normal goat serum in PBS). The following antibodies were used: anti-FLAG M2 (Sigma, F1804, Clone M2, 1:1000), anti-Myc-Tag (Millipore, 05-724, Lot 2387824 1:1000; Cell Signaling, #2272, 1:1000), anti-actin (1:1000, ab11003, [AC-40], Abcam). The following secondary antibodies were used: goat anti-mouse Alexa 488 or 568, goat anti-rabbit Alexa 488 or 568 (Invitrogen, 1:1000), together with Hoechst to visualise DNA. Coverslips were mounted in DAKO mounting medium. Fluorescent images were taken with Leica Microsystems CMS confocal.

Results

Deletion of ZF1-2 and ZF5-6 impairs nuclear localisation of PRDM4

We first confirmed our previous findings that deletion of all six ZFs (dZF1-6) of PRDM4 leads to its accumulation in the cytosol using PC12 cells and primary mouse NSCs. PC12 cells or NSCs were transfected with Myc-tagged full length PRDM4 (PRDM4FL) or dZF1-6 encoding plasmids. Protein expression was confirmed by Western blotting (FigS. 1A and B) and the subcellular distribution of the proteins was assessed immunocytochemically 48 hours post-transfection. As previously observed [24, 31, 34], PRDM4FL and dZF1-6 mutant were found almost exclusively in the nucleus or the cytosol, respectively, of both PC12 cells and NSCs (FigS. 2 A,B,C).

To dissect out the requirement for various ZFs in nuclear localisation of PRDM4, we generated three mutants where ZFs 1-2, 3-4 or 5-6 were deleted simultaneously (FigS1A,B). Transfection of these mutants into PC12 cells revealed that proteins lacking dZF1-2 and dZF5-6 are distributed in both the cytosol and the nucleus suggesting that they are required for efficient nuclear accumulation of PRDM4 (Fig.1A). Deletion of ZF3-4 did not change the subcellular distribution of PRDM4, which localised predominantly to the nucleus, similar to PRDM4FL (Fig. 1A). Interestingly, deletion of ZF3-6 led to a nearly exclusive cytosolic

accumulation of PRDM4 (Fig. 1A) suggesting that ZFs 3-4 collaborate with ZFs 5 and 6 in assisting efficient nuclear localisation or that the presence of ZFs 3-4 may influence PRDM4 export from the nucleus. Again, the validity of these observations was confirmed by using primary NSCs where a similar distribution of the above deletion mutants was observed (Fig. 1B). We conclude that ZFs 1, 2, 5 and 6 are necessary for efficient nuclear localisation of PRDM4.

Contribution of single ZFs to nuclear localisation of PRDM4

In order to understand the contribution of single ZFs to the nuclear targeting of PRDM4, we generated a series of deletion mutants where each ZF was deleted on its own (FigS1A,B). We also tested the contribution of the zinc knuckle (ZK) to the nuclear accumulation of PRDM4. Transfection of single ZF/ZK deletion mutants revealed that deleting of ZFs 1, 2, 5, and 6 singly does not have such a pronounced effect on the efficiency of nuclear localisation of PRDM4 as the double deletions, dZF1-2 and dZF5-6, suggesting that both combinations are necessary to mediate either nuclear import or nuclear retention of PRDM4. While practically no dZF1-2 and dZF5-6 proteins were found exclusively in the nucleus (Fig. 1A), considerable fraction of single deletion mutants were found in the nucleus, with the remainder localised to both cytosolic and nuclear fractions (Fig. 2A). Deletion of ZF5 alone had the most pronounced effect on changes in subcellular localisation of PRDM4, with ca 50% of the protein found in the nucleus and the other 50% being distributed in both compartments (Fig. 2A).

Deletion of ZFs 3 and 4 as well as the ZK did not change the subcellular distribution of PRDM4 protein, with most of these mutants still localised to the nucleus, similar to PRDM4FL protein (Fig. 2A). Transfection and immunostaining of primary mouse NSCs with single dZF and a dZK mutant cDNAs revealed the same subcellular distribution of the proteins as observed in PC12 cells (Fig. 2B). We conclude that combinations of ZFs, e.g.,

ZF 1-2 and ZF 5-6, act cooperatively to control PRDM4 nuclear localisation with ZF5 being most effective on its own in influencing the subcellular distribution of PRDM4.

PRDM4 nuclear import is independent of importin α/β-mediated transport mechanism

To address the possible mechanism responsible for PRDM4 nuclear transport, we utilised potent peptide inhibitors of a well characterised pathway which mediates nuclear import. Bimax1 and 2 peptides inhibit importin α/β-mediated nuclear transport [33] of proteins with classical NLSs, that are either monopartite or bipartite (single or two cluster(s) of basic amino acid residues, respectively) [33].

To investigate the effect of specific inhibitors of nuclear import on the subcellular localisation of PRDM4FL we co-transfected PC12 cells with PRDM4FL and pCMV-GRX1-bimax1- or bimax2-expressing plasmids. Transfected cells were fixed and immunolabelled with anti-Myc-tag antibodies to detect PRDM4 or anti-Flag-tag antibodies to detect bimax peptides 48 hours post-transfection. We observed no effect on the subcellular localisation of PRDM4FL after co-transfection with either Bimax 1 or Bimax 2 peptide (Fig. 3) consistent with the lack of a classical NLS in PRDM4 sequence, while an endogenous substrate with a classical NLS showed aberrant subcellular distribution (data not shown). Co-transfection of PRDM4 with pCMV-GRX1 vector did not change the subcellular distribution of PRDM4FL (data not shown). We conclude that PRDM4 nuclear transport is independent of the importin α/β-mediated mechanism.

Discussion

We investigated the contribution of different ZFs and the ZK motif of PRDM4 in the regulation of its nuclear localisation. We demonstrate that four out of six of the ZFs present in PRMD4 proteins are necessary for its efficient nuclear targeting. These are ZFs 1, 2, 5 and 6, while ZFs 3 and 4 or ZK appear dispensable for the nuclear localisation of PRDM4. Moreover, pairs of ZFs, e.g., ZF5-6 and ZF1-2 appear to cooperate in mediating efficient nuclear accumulation of PRDM4. Interestingly, deletion of ZFs 1-2 and 5-6 does not entirely

abolish nuclear import of PRDM4, but appears to make it less efficient, such that the protein is found in both nuclear and cytosolic compartments. However, ZFs 3 and 4 appear to play a role in mediating efficient transport into the nucleus since deleting ZF3-6 effectively abolishes nuclear localisation of PRDM4, which remains confined to the cytosol. It is possible that ZFs 3 and 4 assist ZFs 1-2 and ZFs 5-6 in more efficient transport into the nucleus perhaps by generating a more stable structure of these domains required for their interactions with import-mediating proteins. It is conceivable that ZFs 1-2 and 5-6 may need to be brought in closer proximity of each other for effective nuclear import and retention. This would be achieved by deleting ZF3-4 and is consistent with our observations that such a deletion indeed results in predominantly nuclear localisation of PRDM4 as observed for the full length protein. The results of our deletion analysis are summarised in Table 1.

In addressing the mechanism of PRDM4 nuclear transport, we found that inhibiting importin α/β-mediated nuclear transport did not affect PRDM4 localisation consistent with the absence of the classical NLS in PRDM4 sequence. These observations suggest that other member(s) of nuclear transport receptors of the Karyopherin-β/importin family [27], may be responsible for PRDM4 nuclear import. For example, another ZF-containing transcription factor, Snail, is transported into the nucleus via three different Kapβ receptors, Kapβ1/Impβ, importin 7 and Kapβ2/transportin [35, 36]. Such a scenario is possible for PRDM4 as well. PRDM4 has similarly spaced basic amino acids, lysine/arginine, in its ZFs 2, 4 and 5, as was shown for Snail and suggested for other proteins with ZFs of Krüppel type [35]. ZF6 of PRDM4 does not entirely comply with the consensus C2H2 ZF sequence as the spacing between the C2 and H2 groups is not conserved. However, it does have two lysine residues in positions described as necessary for efficient importin β-mediated transport of Snail and may therefore also contribute to PRDM4 nuclear import as suggested by our single ZF deletion studies. It is also conceivable that the ZFs of PRDM4 help its nuclear localisation and/or retention by binding to DNA as well, since they have been shown to bind to specific DNA sequences [31]. In summary, we define ZFs 1, 2, 5 and 6 of PRDM4 as necessary for

regulating efficient nuclear localisation of the protein and show that its nuclear import is independent of importin α/β-mediated transport. It will be important to address the roles of other importins in mediating nuclear transport of PRDM4 in future work. Further, it will be important to investigate the contribution of basic amino acid residues found in the ZFs of PRDM4 as well as the requirement for binding to DNA for the efficiency of its nuclear import in future studies.

Conflict of Interests

The authors declare that they have no conflicts of interest with the contents of this article.

Author Contributions

AC conceived, performed some of the experiments, analysed the data and wrote the manuscript. HT and CG[a] performed the experiments and analysed the data. CG[b] helped with some of the Western blot analysis experiments. WDR obtained funding with AC, provided space and advised on the manuscript.

Ethics statement

All animal experiments were approved by the University College London local ethical committee and conformed to the UK Animals (Scientific Procedures) Act 1986. Project license number PPL 70-7614.

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Figure Legends

Fig. 1. ZFs 1-2 and 5-6 are necessary for efficient nuclear transport of PRDM4. Indicated constructs driving the expression of PRDM4 deletion mutants were transiently transfected into PC12 cells (A) or mouse NSCs (B). The subcellular localisation of proteins was visualised by confocal microscopy 48 hours post-transfection. Quantification of subcellular localisation of PRDM4 deletion mutants in PC12 cells is presented. Data are from one of three independent experiments performed in triplicate each. PRDM4 (green), actin (red), DNA (blue).

Fig. 2. Deletion of single ZFs or ZK has minor effect on nuclear transport of PRDM4. Indicated constructs driving the expression of PRDM4 deletion mutants were transiently transfected into PC12 cells (A) or mouse NSCs (B). The subcellular localisation of proteins was visualised by confocal microscopy 48 hours post-transfection. Quantification of subcellular localisation of PRDM4 deletion mutants in PC12 cells is presented. Data are from one of three independent experiments performed in triplicate each. PRDM4 (green), actin (red), DNA (blue).

Fig. 3. PRDM4 nuclear transport is independent of importin α/β pathway. Constructs driving the expression of PRDM4FL fusion protein and either bimax 1 or 2 were co-transfected into PC12 cells and subcellular localisation of PRDM4 protein was analysed by confocal microscopy. Quantification of subcellular localisation of PRDM4 deletion mutants in PC12 cells is presented. Data are from one of three independent experiments performed in triplicate each. PRDM4 (green), bimax1/2-/Flag-tag (red), DNA (blue).

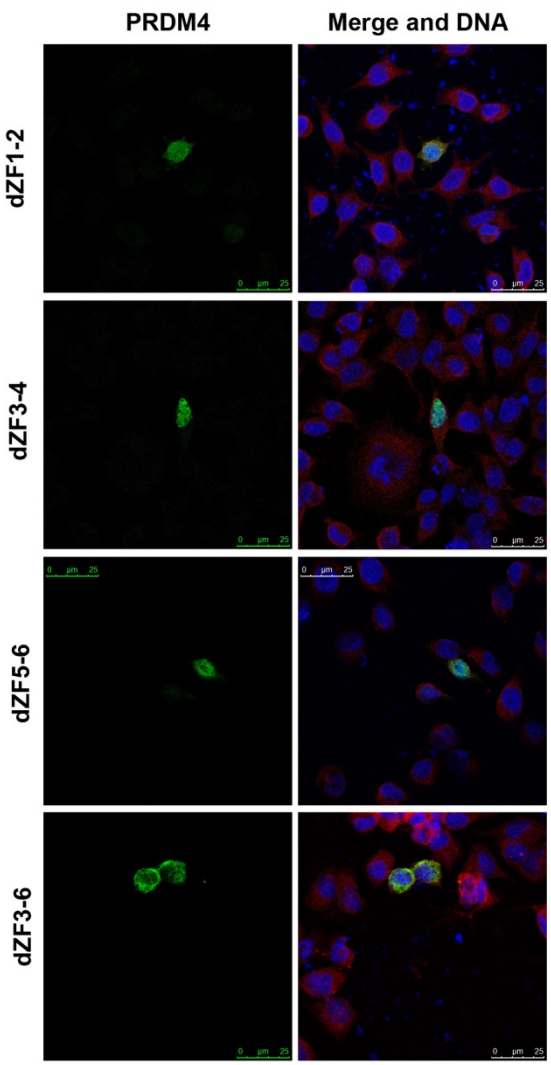
Table 1. Summary of the subcellular distribution of various PRDM4 deletion mutants in PC12 cells.

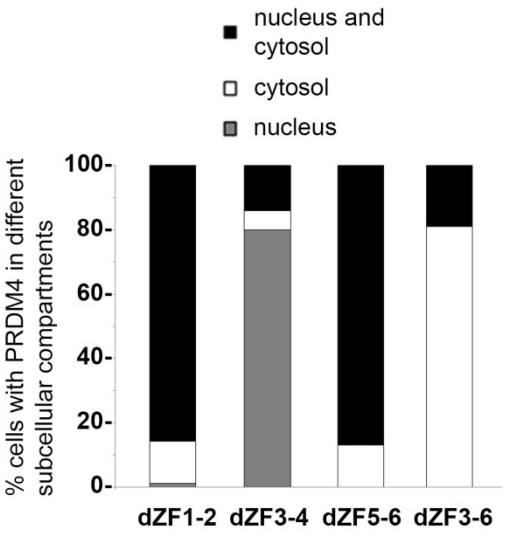
Deletion Mutant	Nuclear	Nuclear and cytosolic	Cytosolic
FL	++++	+	-
dZF1	+++	++	-
dZF2	+++	++	-
dZF3	++++	+	+
dZF4	++++	+	+
dZF5	++	++	-
dZF6	+++	+	-
dZF1-2	+	++++	++
dZF3-4	++++	++	+
dZF5-6	-	++++	+
dZF3-6	-	++	++++
dZF1-6	-	++	++++
dZK	++++	+	-

++++ very strong enrichment, +++ strong enrichment, ++ moderate enrichment, + weak enrichment, - absent in the specified subcellular compartment.

A

Fig. 1


PRDM4
Merge and DNA
dZF1-2
dZF3-4
dZF5-6
dZF3-6


nucleus and cytosol
cytosol
nucleus
% cells with PRDM4 in different subcellular compartments
100
80
60
40
20
0
dZF1-2
dZF3-4
dZF5-6
dZF3-6

B

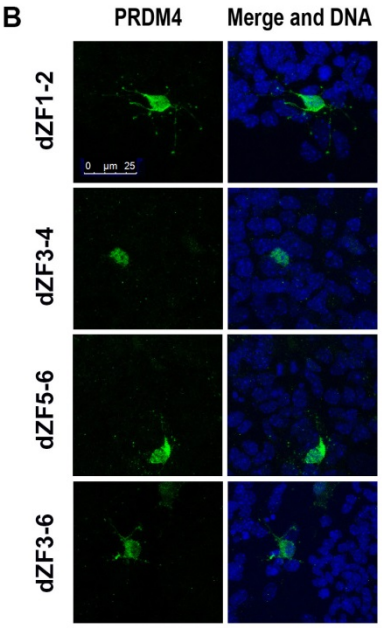

PRDM4
Merge and DNA
dZF1-2
dZF3-4
dZF5-6
dZF3-6

Fig. 2

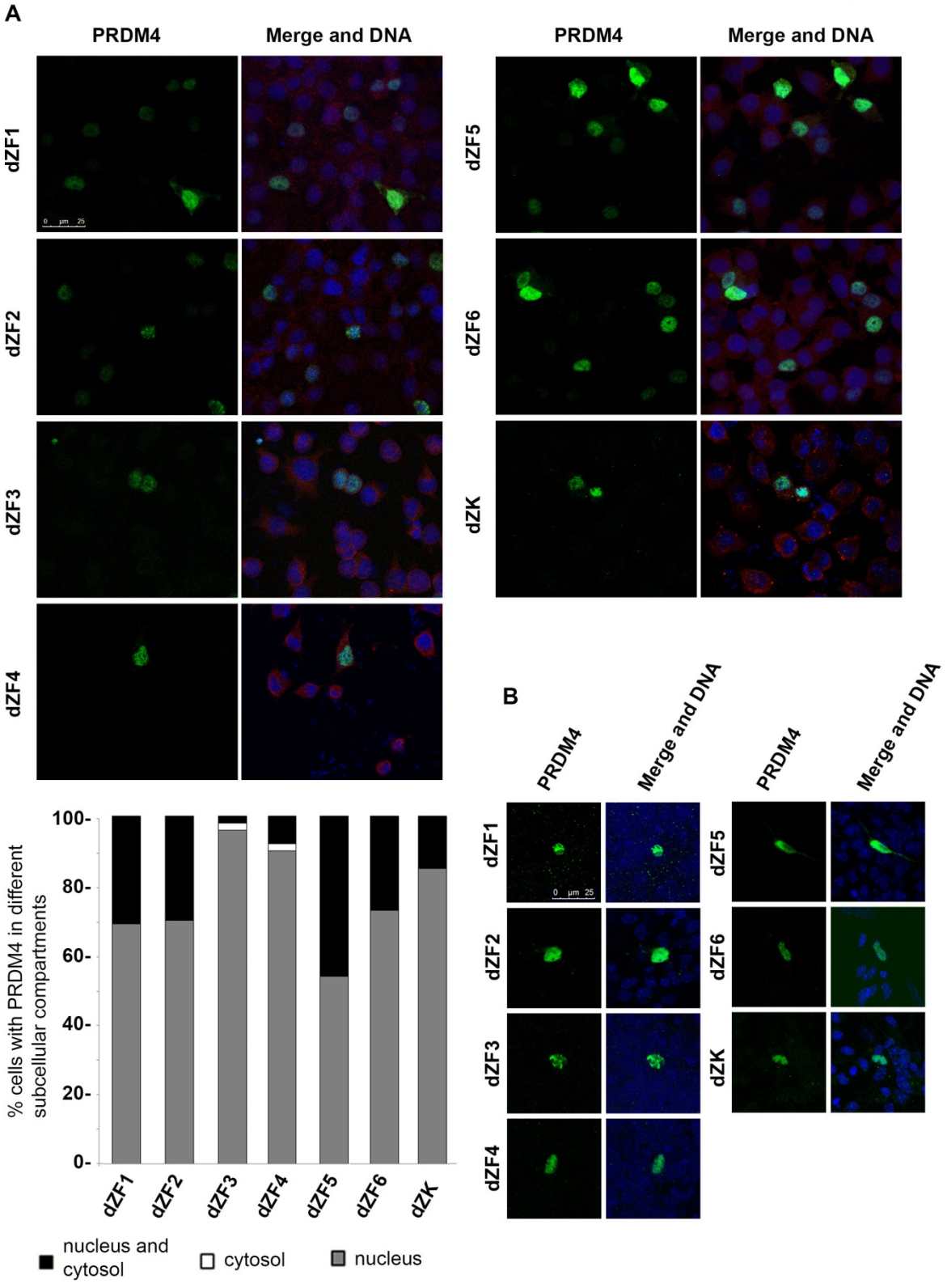
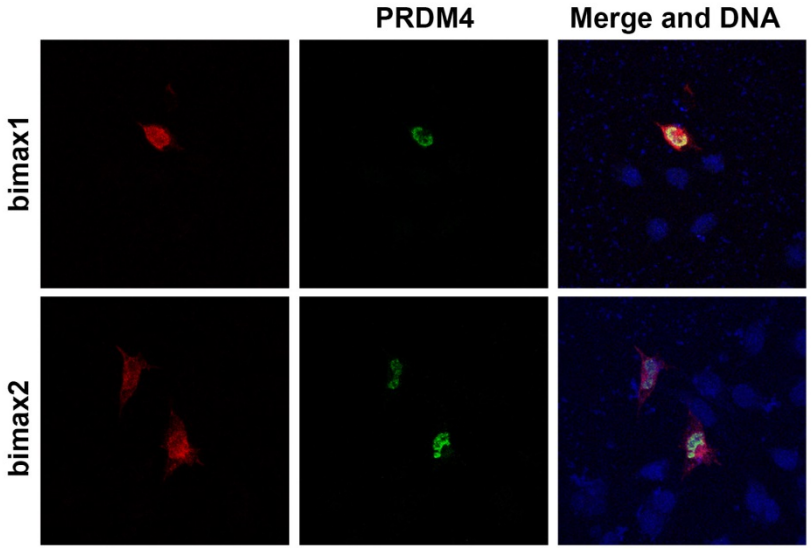
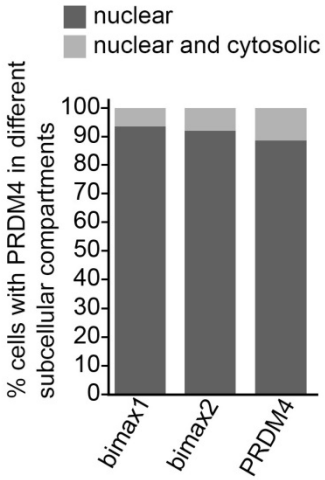

A
PRDM4
Merge and DNA
PRDM4
Merge and DNA
dZF1
dZF2
dZF3
dZF4
dZF5
dZF6
dZK
0 µm 25
B
PRDM4
Merge and DNA
PRDM4
Merge and DNA
dZF1
dZF2
dZF3
dZF4
dZF5
dZF6
dZK
0 µm 25
% cells with PRDM4 in different subcellular compartments
100-
80-
60-
40-
20-
0-
dZF1
dZF2
dZF3
dZF4
dZF5
dZF6
dZK
nucleus and cytosol
cytosol
nucleus

Fig. 3


PRDM4
Merge and DNA
bimax1
bimax2


nuclear
nuclear and cytosolic
% cells with PRDM4 in different subcellular compartments
100
90
80
70
60
50
40
30
20
10
0
bimax1
bimax2
PRDM4