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The genetics and the molecular functions of the PREP1 homeodomain transcription factor

DIVYA PURUSHOTHAMAN and FRANCESCO BLASI*

IFOM (Foundation FIRC Institute of Molecular Oncology), Milano, Italy

ABSTRACT Prep1 (pKnox1) is a homeodomain transcription factor of the TALE superclass whose members can act as co-factors of Hox. Prep1 is essential for embryogenesis, but in the adult it also acts as a tumor suppressor. We describe and analyze here the available mutant mice, their phenotypes and a few discordant cases. Moreover we specify the basic rules underlying the binding of Prep1 and its TALE partners to DNA, and their plasticity during embryonic development. We finally review recent data on Prep1 which indicate a very basic cellular function at the level of DNA replication and DNA damage.

KEY WORDS: *Prep1, pKnox1, tumor, tumor suppressor, epiblast*

Introduction

Prep1 (PKnox1) is a homeodomain transcription factor and a cofactor of Hox proteins belonging to the TALE (Three Amino acids Loop Extension) super class of the Homeobox proteins (reviewed in (Moens and Selleri, 2006). The TALE superclass comprises two families: PBC (Pbx1-4) and Meinox. The Meinox family consists of two sub families; Meis (Meis1-3) and Prep (Prep1-2). All TALE proteins share a very similar homeodomain sequence characterized by the presence of a three amino acids loop extension. Additionally, the Meinox family contains a conserved domain through which they interact with the PBC domains of Pbx proteins. A range of studies have shown that co-operative binding with TALE super class proteins, greatly increases the DNA binding specificity of Hox factors (Mann and Affolter, 1998; Mann and Chan, 1996; Mann *et al.*, 2009). Like in all homeobox proteins, DNA binding of Prep1 is brought about and strengthened by its hetero-dimerization with other homeodomain partners, members of the PBC family (Pbx1-4). In addition, Prep-Pbx can also form ternary complexes with HoxB1 and bind to sequences which are essential for the expression of several Hox genes *in vitro* and *in vivo* (Ferretti *et al.*, 2005; Ferretti *et al.*, 2000). Chipseq data in mouse embryos show that Prep1 and Pbx1 binding sites overlap with each other and also with some of the Hox binding sites, thus participating in the regulation of Hox gene expression (Penkov *et al.*, 2013). A role for Prep1 in HoxB and HoxA gene expression has been shown in Zebrafish (Deflorian *et al.*, 2004). However, more recent studies suggest that TALE proteins also have Hox independent functions (Laurent *et al.*, 2007; Moens and Selleri, 2006).

Biochemical and genetic properties of Prep1, as well as its role in cancer have been reviewed recently (Blasi *et al.*, 2017; Longobardi *et al.*, 2014). Here we will review the role of Prep1 in development and organogenesis as demonstrated by various mutants, mostly in mice. Further, we will also review the basic rules of TALE binding to DNA and their physiological significance. Finally, we will mention recent findings which reveal a basic cellular function for the Prep1 protein.

Mutants and down-regulation phenotypes

Multiple *Prep1*-deficient mutants have been isolated (table 1). *Prep1*$^{i/i}$ is a hypomorphic mutation, caused by an insertion of a beta-galactosidase gene containing a splice site at the 3' end that

Abbreviations used in this paper: BrdU, bromodeoxyuridine; ChIP-Seq, chromatin immunoprecipitation followed by DNA sequencing; Cre, cyclization recombinase of E. coli virus P1; DDR, DNA damage repair; DECA, decameric Prep1 consensus sequence; DN, double negative thymocytes (for CD4 and CD8 surface markers); DP, double positive thymocytes (for CD4 and CD8 surface markers); ES, embryonic stem; FL, fetal liver; HEXA, hexameric Prep1 consensus sequence; HSPC, hematopoietic stem progenitor cell; LAD, lamin associated domain; MEF, mouse embryo fibroblast; Meis, myeloid ecotropic viral integration site ; OCTA, octameric Prep1 consensus sequence; pKnox1, Pbx/Knotted 1 box homeobox; Pbx, Pre-B-cell leukemia homeobox; Prep1, Pbx-regulating protein-1; SP, single positive thymocytes (for CD4 or CD8 surface markers); TALE, three amino acids loop extension; WT, wild type.

***Address correspondence to:** Francesco Blasi. IFOM, via Adamello 16, 20139 Milan, Italy. E-mail: francesco.blasi@ifom.eu - https://orcid.org/0000-0001-9406-1784

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splices out the normal Prep1 mRNA (Ferretti *et al*., 2006). Since this splice site is not 100% efficient, *Prep1$^{i/i}$* embryos still produce a small amount of normal Prep1 mRNA and protein. The presence of residual Prep1 allows the embryo to survive until e17. Seventy five percent of the homozygous embryos from a heterozygous *Prep1$^{+/i}$* x *Prep1$^{+/i}$* cross die at about e17.5 while 25% are born and give rise to *Prep1$^{i/i}$* mice that live an almost normal-length life (Ferretti *et al*., 2006). Instead in Prep1 null mice (*Prep1$^{-/-}$*), exons 7 and 8 corresponding to the amino-terminal region of the homeodomain are deleted. No truncated protein was observed although the mRNA corresponding to the deleted gene was visible. No mice carrying the *Prep1$^{-/-}$* mutation was born, and no embryo older than e7.5 (Fernandez-Diaz *et al*., 2010). A trans heterozygous mutant mice (*Prep1$^{i/-}$*) was developed by crossing *Prep1$^{i/i}$* with *Prep1$^{-/+}$* mice and should have an intermediate Prep1 expression levels (not measured). The embryos survived till e12.5-14.5 (Rowan *et al*., 2010). A conditional *Prep1* knockout mice was produced by Cre-mediated deletion of exon 8 (Carbe *et al*., 2012). Deletion of Prep1 using ubiquitously expressed Ella-Cre yielded no homozygous Prep1$^{-/-}$ genotype at e12.5 (Carbe *et al*., 2012) agreeing with the observation that complete deletion of Prep1 is early embryonic lethal (Fernandez-Diaz *et al*., 2010). This mutation, however, generates a 59kDa truncated Prep1 band in whole embryo, though the effects, if any of this truncated form was not tested (Carbe *et al*., 2012). An additional *Prep1* conditional deletion of exon 3 was also generated with no expression of truncated products (Yoshioka *et al*., 2015). These mice showed some defects in adult haematopoiesis.

As shown in Table 1, studies in *D. rerio* and *X. laevis* also have given useful information. In zebrafish, the most evident phenotype of *Prep1.1* morpholino down-regulation is the apoptosis in the nervous system, analogous to that in the mouse (see below). Moreover, the data also show an important role on the segmentation and patterning of the hindbrain with loss of several hindbrain markers, including the anteriorly expressed *Hox* genes. This affects the migration of facial nerve motor neurons and the lack of reticulospinal neurons. Finally, the head of the *Prep1.1* morphants lacked completely all pharyngeal cartilages because of the inability of neural crest cells to differentiate into chondroblasts (De Florian *et al*., 2004).

Also in *X. laevis* Prep1 is involved in development. A recent study on the effect of YAP down-regulation on retinal stem cell population has uncovered several key phenotypes reminiscent of Prep1 down-regulation like the reduced size or absence of the eye, changes in cell cycle status, changes in the temporal regulation of DNA replication and subsequent DNA damage and apoptosis (Cabochette *et al*., 2015). Interestingly, PREP1 is identified as an interacting partner of YAP in the cytoplasm of retinal stem cells. The interaction was also confirmed by immunoprecipitation of tagged proteins. Further, knockdown of PREP1 rescues the phenotype with significant reduction in eye size and lower numbers of cells in mid/late S phase. Finally, knockdown of Prep1 rescues the phenotypes observed in YAP over-expression suggesting a synergistic interaction between the two proteins. However, details of this interactions are still outstanding.

Embryonic and fetal lethality

The difference in timing of the embryonic lethality of *Prep1-/-* null embryos (Fernandez-Diaz *et al*., 2010) v. the later death of the hypomorphic *Prep1$^{i/i}$* embryos must reside in the 3-10% residual expression of the protein in the latter mice (Ferretti *et al*., 2006). Trans heterozygous *Prep1$^{i/-}$* mutants which should have an intermediate expression level of (i.e. about 1-3% of control), on the other hand survive till e12.5 (Rowan *et al*., 2010). Therefore, the absence of Prep1 leads to death at the epiblast stage; its expression at one twentieth of the normal Prep1 level (*Prep1$^{i/-}$*) allows to prolong the embryonic life span from e7.5 to e12.5; expression at one tenth of the normal level (*Prep1$^{i/i}$*) prolongs life for ten more days almost until the end of gestation. Further, *Prep1$^{+/-}$* embryos develop normally (Fernandez-Diaz *et al*., 2010) indicating that half the normal expression of Prep1 is enough to ensure normal embryonic development. These data show that during embryogenesis, minor difference in Prep1 levels can profoundly affect development. Why 25% of the *Prep1$^{i/i}$* embryos are born and live normal length life is unknown (Ferretti *et al*., 2006) but it is possible that a slightly higher Prep1 expression makes the difference.

TABLE 1

PHENOTYPES OF DIFFERENT MUTANTS IN THE *PREP1 (PKNOX1)* GENE

Genotype	Time of embryonic death	Phenotype
Mouse: *Prep1$^{i/i}$*	e17.5	Angiogenesis, erythropoiesis, oculogensis (Ferretti, et.al., 2006) T-cell development in adult (Penkov et.al., 2005) B-cell development in the embryo (Di Rosa P et.al., 2007) Extended Apoptosis (Micali et.al., 2009) Tumorigenesis (Longobardi et.al., 2010) Chromosome aberrations (Iotti et.al., 2011) Hematopoietic stem cell cycling (Modica et.al., 2014)
Mouse: *Prep1$^{-/-}$* Deletion of exon 7 and 8	E6.5	Embryos fail to gastrulate. Epiblasts cells undergo p53 dependent apoptosis (Fernandez-Diaz et.al., 2010). The same phenotype is visible also in heterozygotes in the Atm$^{-/-}$ background.
Mouse: *Prep1$^{i/-}$*	E12.5-14.5	Eye development (Rowan et.al., 2010)
Mouse:*Prep1$^{D/D}$* (Ella-Cre *Prep1$^{f/f}$*)	<e12.5	Apa2-Cre;*Prep1$^{f/f}$* and Le-Cre;*Prep1f^{f}*: Healthy and fertile mice. No eye phenotype (Carbe et.al., 2012)
Mouse: *Prep1*^{DSM} (*Prep1$^{f/f\ Cre/+}$*) Muscle specific conditional	viable	Increased mitochondrial enzymatic activity and better endurance; higher maximal oxidative capacity (Kanzleiter et.al., 2014)
Mouse: *Prep1* CKO Conditional deletion of exon 3	viable	Tie-2 Cre;*Prep1f^{f}*: defects in thymocyte development, Increased HSPC cycling (Yoshioka et.al., 2015). Rosa26-CreER^{T2}; *PKnox1$^{f/f}$* and TNAP-Cre; *PKnox1f^{f}*: Defective spermatogenesis (Kawai et.al., 2018)
D. rerio: Morpholino down-regulation of *Prep1.1*	Late embryonic lethal	Extended apoptosis mostly located in embryonic neural tissues. Defects in hindbrain segmentation, patterning and cranial nerves formation, loss of expression of anterior *Hox* genes in hindbrain; loss of pharyngeal cartilages due to defective chondroblast differentiation (Deflorian et.al., 2004)
X. laevis: Morpholino down-regulation of *Prep1*	Retinal stem cells	Reduced size of (or absent) eye, DNA damage and apoptosis (Cabochette *et al*., 2015).

Interpreting phenotypes

Within the TALE family, each sub-family, Pbx, Meis and Prep, has an identical DNA binding domain. Therefore, all proteins belonging to one group can bind the same target sequence. Hence, the interpretation of the KO mice phenotype is in fact complicated when isoforms or other TALE family members are expressed, which is the rule rather than the exception. Under these circumstances, the phenotype of the different *Prep1* KO mice may also be interfered by the expression of *Prep2* (*pKnox2*) (Fognani *et al*., 2002; Imoto *et al*., 2001). It should be noted, however, that the expression of this gene is different from *Prep1*; since however its homeodomain is identical (Haller *et al*., 2004; Haller *et al*., 2002) it might well, when expressed, bind the same Prep1 targets. Moreover, the existence of several alternative splicing forms (Haller *et al*., 2004) suggests a multiplicity of functions. Therefore, substitution of Prep1 with Prep2 might differentially affect different aspects of the *Prep1* phenotypes. All these speculations need to be verified.

Pbx1 and Pbx2 can bind mostly the same genes (Penkov *et al*., 2013). Indeed, the genes bound by Pbx2 in the thymus where Pbx1 is largely absent, are found among the Pbx1 genes bound in the mouse embryo. In situations where multiple Pbx proteins are expressed, it is possible that the sites bound by different Pbx proteins are dictated by the relative concentration of the individual proteins. These data explain why Pbx1 and Pbx2 can at least partially complement each other (Capellini *et al*., 2006; Selleri *et al*., 2004). Further, Prep1 and Meis1 proteins display competitive binding to Pbx proteins *in vivo* (Dardaei *et al*., 2014), affecting the stability of the individual proteins (Ferretti *et al*., 2006; Laurent *et al*., 2015), raising the possibility of increased Meis-Pbx complexes in Prep1 knockouts. The observed phenotypes, therefore, should be re-examined on the basis of these considerations, to test whether they are due to the absence of the deleted-gene product or also due to the occupation of the same sites by other partially complementing transcription factors.

This type of considerations also raises other interesting questions. For example, if Prep1 acted uniquely through a dimer with Pbx1, one would expect that the *Prep1* and *Pbx1* KO embryos should die at a very similar embryonic age. However, the single *Pbx1* KO embryos die much later than the *Prep1* KO while the double *Pbx1-Pbx2* KO embryos die earlier than *Pbx1* KO. Should one delete all three, or even four, *Pbx* genes to observe a *Prep1*-like phenotype, i.e. death at the epiblast stage? A deeper analysis would clarify these issues and uncover mistaken interpretations.

Organ specific differentiation-related phenotypes

Hematopoiesis

Data on hematopoiesis from *Prep1*$^{i/i}$ mice (germline mutation) and *Prep1*CKO (somatic conditional) suggests that the intrinsic effect of Prep1 on hematopoiesis is controversial and possibly minimal. *Prep1*$^{i/i}$ mice show reduced numbers of erythropoietic cells (Ferretti *et al*., 2006) and common myeloid progenitors (Di Rosa *et al*., 2007) in the embryo. Further, a block in B-cell differentiation in both embryo (Di Rosa *et al*., 2007) and adult (Iotti *et al*., 2012) was reported in *Prep1*$^{i/i}$ mice and tamoxifen-inducible Rosa26-CreER-mediated *Prep1* knockout mice respectively. However, a Tie-2-Cre mediated hematopoietic and endothelial cell specific deletion of *Prep1* (*Prep1*CKO), did not display any defect in the maintenance or differentiation of hematopoietic cells in fetal livers nor any difference in early B-cell lineage or immature and mature B-cells (Yoshioka *et al*., 2015). Similarly, fetal livers from *Prep1*$^{i/i}$ mice revealed the presence of an increased pool of Lin$^-$Sca1$^+$c-Kit$^+$ progenitor cells at the expense of highly replicative stem cell pool which exhaust rapidly their replicative potential (Modica *et al*., 2014). However, FL from *Prep1*CKO mice did not have a significantly higher pool of Lin$^-$Sca1$^+$c-Kit$^+$ cells (Yoshioka *et al*., 2015) indicating that the relative expression of other TALE proteins or differences due to germline vs somatic expression level can drastically modify the phenotype. Possibly the defects observed in *Prep1*$^{i/i}$ embryos could depend on fetal liver cells different from those originating from the tie-2-Cre lineage that provide niche function for the HSC. Another possible explanation for the discrepancy between the two reports might be the level of Prep1 interactors, ie Pbx and Meis proteins. While the expression of Pbx and Meis in Tie-2 Cre Prep1 CKO is not known, *Prep1*$^{i/i}$ mice and *Prep1*$^{-/-}$ ES cells show generally reduced levels of Pbx and Meis protein expression (Ferretti *et al*., 2006; Laurent *et al*., 2015). Since Pbx1 and Meis1 have been shown to be important in hematopoiesis (Azcoitia *et al*., 2005; DiMartino *et al*., 2001), it is conceivable that hematopoietic defects observed in *Prep1*$^{i/i}$ mice could be a compound effect of these interactors.

Nevertheless, Prep1 seems to have significant effect during T-cell development in thymus. In the adult *Prep1*$^{i/i}$ mice, even though a higher numbers of DN (double negative) thymocytes were present, due to increased apoptosis at the positive selection checkpoint of double positive (DP) thymocytes in the thymus, significantly lower SP (single positive) thymocytes were detected (Penkov *et al*., 2005). Consequently, an absolute decrease in the numbers of CD4$^+$ and CD8$^+$ peripheral T-cells in spleen, blood and lymphnodes was seen in *Prep1*$^{i/i}$ mice. Similar results were also obtained in a transgenic mice with a thymus specific expression of the N-terminal fragment of Pbx1 which causes cytosolic sequestration of Prep1, which makes it unable to carry out its transcriptional activities (Penkov *et al*., 2008). Again, this induced a concomitant decrease of Pbx2 in these mice, suggesting that Prep1 acts in combination with its binding partners. Data from *Prep1*CKO also reports a significant decrease in single positive (SP) thymocyte even though the reduced thymic output did not result in reduced peripheral T-cell numbers as reported for *Prep1*$^{i/i}$ mice (Yoshioka *et al*., 2015). Differences in the deletion strategy (germline vs somatic), differences in the mice background and/or differences in the levels of Pbx and/or Meis proteins could explain the changes in homeostatic expansion of peripheral T-cells.

Oculogenesis

Prep1 also regulates eye development. Reduction in the lens size, neural retina abnormalities and encasement of eyes deep within the head were observed with varying penetrance in *Prep1*$^{i/i}$ mice (Ferretti *et al*., 2006). The study also showed that the level of Pax 6, an essential factor for oculogenesis was reduced drastically in the iris, ciliar body, corneal epithelium and lens epithelium. Further, in WT mice, Prep1 co-localized with Pax 6 in these tissues. Using trans-heterozygotes *Prep1*$^{-/i}$ mice with a further reduction in Prep1 protein levels, Rowan et.al., demonstrated a more severe phenotype with complete absence of the lens. In these mice lens epithelial cells, lens fiber cells or lens precursor cells were not formed. *Prep1*$^{i/-}$ mice also did not initiate Foxe3 expression (Rowan *et al*., 2010). However, direct involvement of Prep1 in the regulation

of Pax 6 is difficult to ascertain, given that Meis1 has been shown to regulate Pax 6 levels during vertebrate lens morphogenesis (Zhang *et al*., 2002). Though no data is available for mouse, Pbx proteins have been shown to regulate eye development in zebrafish, *Xenopus* and planaria (Chen *et al*., 2013; French *et al*., 2007; Morgan *et al*., 2004). Reduction in Pax 6 levels in the absence of Prep1 thus could be due to the concomitant decrease in Pbx and Meis proteins (Ferretti *et al*., 2006). This is the more likely scenario given that a more recent study using conditional knockout mice made by cre-mediated deletion of exon 8 of Prep1 gene failed to reproduce the eye phenotype. Both Ap2a-Cre- (expressed in the presumptive lens ectoderm at E8) and Le-Cre- (expressed in the lens and pancreas at E9.5) -driven knockout of Prep1 during lens development failed to show any lens defects in mutant mice and Pax6 was expressed at normal levels (Carbe *et al*., 2012). However, the levels of other TALE proteins in these mice was not measured. Further, the study also raises the possibility that lens extrinsic factors contribute to the phenotype since previous studies used germline mutants. However, another source of variability may be the presence of truncated Prep1 protein which lack the homeodomain in the conditional mutant (Carbe *et al*., 2012). A functional homeodomain-less splice form of Meis1 and its orthologue Homothorax is present in mouse and *Drosophila* (Noro *et al*., 2006). Truncated forms of Meis have also been identified in human colorectal cancer tissues (Crist *et al*., 2011). Therefore, it is necessary to rule out the possibility of a partially functional truncated Prep1 in order to understand the role of Prep1 in oculogenesis.

Adipogenesis

Prep1 appears to have a regulatory role in adipogenesis as well (Maroni *et al*., 2017). Both *Prep1*$^{i/i}$ mesenchymal stromal cells from bone marrow and the 3T3L1 cell line down-regulated for Prep1 by an shRNA, reveal a pre-adipocytic stage in the absence of specific hormonal stimuli and a faster and more efficient adipogenesis after specific stimulation. Upon Prep1 down-regulation some of the key adipogenic factors are affected in the same direction as if they had been treated with the hormonal inducing cocktail: PPARγ, CCAAT enhancer binding protein α (cEBPα) among others, are induced while Klf5, Pref1 and cEBPδ are repressed. Moreover, phosphorylation of Irs1 and pAkt is greatly and rapidly increased by Prep1 down-regulation in the absence of the hormonal cocktail. The most important effect is the expansion of the DNA-binding landscape of c/EBPβ with a major pre-differentiation increase in DNA binding sites, which occurs without a concurrent increase or activation of the protein (Maroni *et al*., 2017). Therefore, possibly in 3T3L1 cells and in mesenchymal stromal cells Prep1 acts by repressing adipogenesis and its absence may modify the state of the genome making it more amenable to the induction of adipogenesis. While the mechanism is still not known, one attractive possibility is that Prep1 regulates adipogenesis by modifying the genomic accessibility by chromatin modifications.

Spermatogenesis

In mouse the highest expression of Prep1 mRNA is observed in the testes (Ferretti *et al*., 1999). The role of Prep1 in regulating spermatogenesis has been addressed recently in a Prep1 conditional knockout (Kawai *et al*., 2018). Prep1 expression is temporally regulated; first detectable at p6, increases with age and reaches a plateau with the first wave of spermatogenesis at p35. Both Rosa26 Cre-ERT2;*Prep1*$^{fl/fl}$ and germ cell specific TNAP-Cre; Prep1$^{fl/fl}$ mice showed smaller testes with atrophic seminiferous tubules containing very few spermatocytes and accumulation of TUNEL^{+} apoptotic cells (Kawai *et al*., 2018). In the absence of Prep1, spermatogenesis failed to proceed beyond the c-Kit^{+} spermatogonial differentiation stage and subsequent stages including meiosis were absent. Absence of PCNA in Prep1-CKO c-Kit^{+} spermatogonial cells suggests that defects in DNA replication could explain the arrest of spermatogenesis (Kawai *et al*., 2018).

Cell-level phenotypes

Prep1 and oxidative phosphorylation: Analysis of skeletal muscle-specific *Prep1* knockout revealed a significant increase in respiratory chain subunits like the succinate dehydrogenase subunit (Sdha) from complex 2, cytochrome c1 subunit (Cyc1) from complex 3, and ATP synthase subunit (Atp5k) from complex 5, at both mRNA and protein levels (Kanzleiter *et al*., 2014). Though increased mitochondrial DNA and increased activity of mitochondrial enzyme citrate synthase was detected in the muscle cells of *Prep1*-ablated mice, the mitochondrial volume fraction or ultra-structure was unchanged. Prep1 could be acting directly by stabilizing Mybbp1a (Diaz *et al*., 2007), as previously shown in *Prep1*$^{i/i}$ muscles (Oriente *et al*., 2008), that in turn can repress PGC1-α, a well-known regulator of muscle oxidative capacity. Additionally, Prep1 can also act directly through its binding at promoter regions of 16 mitochondrial proteins (Kanzleiter *et al*., 2014). Though Prep1 ablated skeletal muscles show increased PGC1-α which can drive mitochondrial biogenesis, surprisingly no change in mitochondrial volume fraction was observed in these cells.

Prep1, cell proliferation and DNA damage: An important phenotype of the loss of the *Prep1* gene across different species is apoptosis. In zebrafish, acridine orange staining revealed widespread cell death in *prep1.1* morphants. This effect was specific for prep1.1 since neither control nor *pbx4* (equivalent to *pbx1* in zebrafish) morphants showed any apoptosis. Likewise, TUNEL staining showed intense DNA fragmentation in the brain of *prep1.1* morphants at the 22 somites stages, in particular in the hindbrain (De Florian *et al*., 2004). A similar effect has been observed also in the *Prep1*$^{i/i}$ mice. Sections of E9.5 and E11.5 *Prep1*$^{i/i}$ mice were positive in TUNEL analysis in the nervous system, the apoptosis being evident mostly in the nervous system, particularly in the hindbrain and in several intersomitic regions. The trend towards apoptosis was confirmed by biochemical studies on hypomorphic MEFs that identified genotoxic stress as a major cause of apoptosis in *Prep1*$^{i/i}$ MEFs (Micali *et al*., 2009). The tendency to apoptosis suggests the presence of DNA damage, which is an important clue to understand another function of Prep1, that of tumor suppression, since DNA damage is a major cause of cancer. Furthermore, *Prep1*$^{-/-}$ mice epiblasts undergo p53 dependent apoptosis (Fernandez-Diaz *et al*., 2010). This is exacerbated in the absence of Atm kinase which is essential for DNA repair, suggesting that the DNA damage accumulation is the trigger for apoptosis. Accumulation of DNA damage and errors in DNA damage repair machinery is the major cause of cancer (Khanna and Jackson, 2001). Indeed, Prep1$^{i/i}$ mice which escape embryonic lethality and survive to adulthood develop tumors of various origins indicating that *Prep1* is a tumor suppressor gene. Furthermore, this role of Prep1 is supported by the absence of Prep1 in a large percent of human cancers (Longobardi *et al*., 2010). Further experiments in *Prep1*$^{i/i}$ MEFs revealed gross chromosomal

anomalies and high level of DNA damage, whereas the DNA Damage Response (DDR) machinery appeared to be functional. This was observed also in human Prep1 down-regulated fibroblasts (Iotti *et al*., 2011). The presence of an intact DDR machinery suggest that the DNA damage accumulation in Prep1-deficient cells could be due to anomalies in DNA replication.

A recent study has analyzed DNA replication in Prep1 down regulated Hela cells and shows that in the absence of Prep1, the time spent by the cells in the early S phase is decreased while that in the late S phase is increased. This change is accompanied by an increase in the number of unscheduled origin firings (Palmigiano *et al*., 2018). Sequencing of BrdU-incorporated DNA by Repliseq revealed that 25% of the DNA was replicated earlier in Prep1-down regulated than in control cells. In fact, PREP1 down-regulation did not affect the replication timing of the entire DNA, but only of a fraction of the genome with very special properties. In the nucleus, the timing of replication is regulated by the attachment of 40% of the DNA to the nuclear lamina. These so called Lamin-Associated Domains (LAD) (Guelen *et al*., 2008) depend on a complex interaction of DNA with Lamins, major components of the nuclear lamina. The DNA bound to LADs is gene-poor, late-replicated and mostly silent (Guelen *et al*., 2008). In PREP1 down-regulated Hela cells about one quarter of the genome, mostly represented by the LADs and including most of the late-replicating genome, is replicated earlier in the S phase (Palmigiano *et al*., 2018). While the mechanism is not yet established, the data exclude the possibility that the shifted replication is related to the direct binding of PREP1 to DNA. Fig. 1 shows a proposed model for the effect of Prep1 on DNA replication. Since the data show a corresponding decrease of the Lamin B1 protein, it is possible that the effect of Prep1 is funneled via the control of the level of the Lamin proteins.

DNA target sequence and *Prep1* developmental functions

ChIP-seq analysis of the mixed cell populations E10.5 embryo trunk has identified three specific consensus DNA-binding sites for TALE transcription factors, DECA, OCTA and HEXA, corresponding to previously observed targets of TALE proteins. The DECA sequence TGANTGACAG, previously known as a Prep1 and a Pbx1 half-site combination (Knoepfler *et al*., 1997), is indeed bound *in vivo* by both Prep1 and Pbx1, hence likely by the Prep-Pbx dimers and includes the highest affinity sites. In a large number of these sites, DECA is followed 7-10 nucleotides downstream by the CCAAT sequence, known to bind NF-Y (Penkov *et al*., 2013).

Meis1, on the other hand, preferentially binds to the TGATTTAT (OCTA) type sequence, corresponding to the Hox-Pbx DNA-binding site (Penkov *et al*., 2013). Also these peaks are likely bound by the Pbx1-Meis1 dimers. Finally, Prep1, Pbx1 and Meis1 also bind the HEXA motif TGACAG, although Prep1 less frequently than the others (Penkov *et al*., 2013).

In this and many other ChIP-seq studies, Prep1 binds more frequently promoters than enhancers, while Meis1 does the reverse (Blasi *et al.,* 2017). Gene Ontology analysis of the binding sites in the E10.5 embryo shows that the genes bound by Meis1 are highly enriched in the embryonic development categories whereas those bound by Prep1 are enriched in genes more related to basic cell functions (Penkov *et al*., 2013). However, it is clear that Prep1 sites differ from cell to cell with only a conserved core set of genes (Blasi *et al*., 2017; Laurent *et al*., 2015; Dardaei *et al*., 2015; Palmigiano *et al*., 2018). Finally, the binding specificity is also affected by the concentration of the proteins *in vivo*. Indeed, when the intracellular concentration of Prep1 is artificially increased, the number of bound genes increases proportionally and new binding variant consensus sequences. In this case Prep1 tends to bind much more to enhancers than promoters, indicating a change in the transcription regulation mechanisms (Dardaei *et al*., 2015).

In mouse ES cells, that share the same binding rules with all other cells, Prep1 target genes are more enriched in embryonic development genes. In particular the developmentally important signaling pathways like *Wnt*, *Fgf* and *Hh* are among Prep1 binding sites (Laurent *et al*., 2015).

In addition to the essential functions at post-gastrulation and adult stages, TALE proteins clearly are also essential before gastrulation. The most important example is the phenotype of Prep1 null embryos that die before gastrulation. At this stage, Prep1 and Pbx are present as maternal transcripts (Fernandez *et al*., 2010) which will become ubiquitously expressed in later embryos. An essential Pbx function at this stage has not been directly demonstrated but is likely to be obscured by complementation of other family members. Indeed, at early stages Prep1 and Pbx are bound at regulatory elements even before the expression of cooperating transcription factors factors (Berkes *et al*., 2004).

An important report by Sagerstrøm group while confirming in zebrafish embryos all of the above DNA-binding rules for TALE factors, shows that the DNA-binding of maternal v. somatic TALE factors is somewhat different and has important functional implications. In fact, the pre-gastrulation stage separates the DNA-binding landscapes of maternal v. somatic TALE factors. At blastula maternal Prep1 and Pbx1 bind the combined DECA…CCAAT sequence at promoter sites, adjacent to sites occupied by NF-Y pioneer factors; this binding is required for the transition into active chromatin of

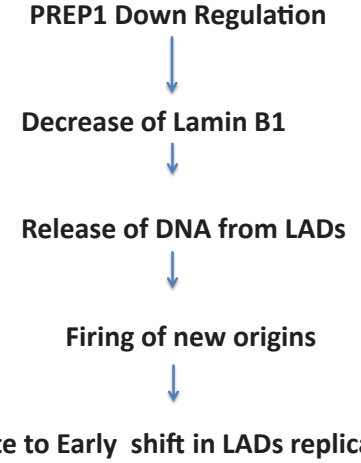

Fig. 1. Possible mechanism explaining the effect of PREP1 down-regulation on DNA replication timing in HeLa cells. *Down-regulation of PREP1 in HeLa has profound effects (Palmigiano* et al*., 2018), among which a decrease in Lamin B1, a major component of the nuclear lamina. In the fraction of DNA (about 40%) associated with Lamins and forming the Lamin Associated Domains (LADs), DNA replication occurs late in the S phase and most genes are silenced. It is likely that the decrease of Lamins caused by PREP1 down-regulation, induces a release of DNA which therefore becomes free to be replicated ahead of time through the firing of new origins.*

a gene network that controls anterior embryonic development. At later stages, the binding landscape of these factors expands to include the OCTA and HEXA sites, mostly present in enhancers also associated with the same network. These changes appear to be associated to modifications of chromatin accessibility (Ladam *et al*., 2018).

Is *Prep1* a developmental gene?

While hypomorphic *Prep1*$^{i/i}$ or conditional knockouts show significant defects in embryonic development some of which have been reviewed here, a germline knockout of Prep1 dies before gastrulation (Fernandez-Diaz *et al*., 2010). The pre-gastrulation death does not appear to be due to the loss of a developmental function. In fact, at the epiblast stage, the expression of *Oct4* and *Nanog*, essential for the multi-potency of the epiblast, is not affected in *Prep1*$^{-/-}$ embryos which express no Prep1 mRNA, and the cells die because they undergo Tp53-dependent and Atm-exacerbated apoptosis. In this case, epiblast cells die because they accumulate DNA damage and undergo apoptosis, and the absence of these cells blocks development.

On the other hand, *Prep1*$^{-/-}$ ES cells, although able to propagate themselves in culture, undergo apoptosis in response to differentiation cues (Fernandez-Diaz *et al*., 2010). This phenotype may be due to developmental defects since *Prep1*$^{-/-}$ ES show an altered timing of expression of developmentally fundamental genes which can be partially rescued by reintroduction of Prep1 (Laurent *et al*., 2015). Hopwever, a mechanism similar to that described by Palmigiano *et al.,* (2018), i.e accumulation of DNA damage, may also be responsible for this phenotype. Other developmental phenotypes observed in various tissues of *Prep1* mutant mice are accompanied by defects in cell cycle status and proliferation; hematopoietic stem/progenitor cells (Modica *et al*., 2014; Yoshioka *et al*., 2015), thymocytes (Penkov *et al*., 2005; Penkov *et al*., 2008) and spermatogonial cells (Kawai *et al*., 2018). Both in mouse and zebrafish (Ladam *et al*., 2018) embryos the pre- and a post-gastrulation *Prep1* phenotypes may depend on different mechanisms. Should the DNA binding properties of TALE proteins in zebrafish be recapitulated in the mouse, the molecular mechanisms and biological properties of *Prep1* in a pre- v. post-gastrulation stages would be coordinated with its differential DNA-binding mode. This hypothesis, suggested by the differential DNA-binding distribution in zebrafish (Ladam *et al*., 2018) requires, and is worth of, further investigation.

Because paralogs of the TALE proteins might substitute for a missing one, their gene expression must also be considered in the interpretation of the KO phenotypes. *Prep1* is expressed ubiquitously in an e8.5 developing embryo as opposed to the *Prep2*, *Meis1* and *Meis2* genes which have a more restricted expression pattern (Fernandez-Diaz *et al*., 2010). The combined expression of all *Pbx* genes, instead, recapitulates that of *Prep1*.

Conclusions

A review of the phenotypic analysis of mice mutants suggests that Prep1 regulates basic cellular functions like DNA replication and DNA damage thereby regulating biological functions like embryonic development and tissue homeostasis in the adult. However, it is also clear that Prep1 has been generally under-investigated and that a myriad of other effects and mechanisms await to be uncovered. The precise scope and nature of the Prep1-Tp53 axis, the exact molecular mechanism of DNA damage accumulation in Prep1 down-regulated cells etc need to be investigated thoroughly. It must be noted that the number of labs working on Prep1 is very small. We hope that more labs will become interested to join this field of research in the near future.

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