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Emerging evidence for clinical significance of histone methyltransferase PRDM9 in reproductive system and cancer development*

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PRDM9 is a histone methyltransferase able to trimethylate histone 3 at lysine-4 and lysine-36 residues. Since its discovery in 2005, there has been significant progress in the characterization of the structure and functions of PRDM9. The primary studies all pointed out at its role in meiosis and recombination; however, there are indications that it may be involved in other cellular processes as well. Such an additional role of PRDM9 in other types of cells and tissues apart the germ cells is still an open question. Small cohort of studies compared to the entire literature for PRDM9 hints for

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possible expression of this gene in other locations and having clinical significance in reproductive and cancer biology fields. In this review, we summarize the current knowledge about the meiotic and non-meiotic functions of PRDM9, effect of stress on the achievements and we list the remaining questions waiting to be answered.

KEY WORDS: cancer / infertility / meiotic recombination / PRDM9

In 2005, the team of Matsui *et al*. [Hayashi and Matsui 2006] discovered transcripts of a new germ-cell specific histone methyltransferase. These transcripts were detected in both the female fetal gonads and in the postnatal testis mouse germ cells entering meiotic prophase. The protein product was named Meisetz [meiosis-induced factor containing PR/SET domain and zinc-finger motif] and was shown to trimethylate lysine-4 on histone 3. More importantly, its absence in mice caused sterility in both sexes due to impaired double-strand break repair pathway, homologous chromosome non-pairing and abolished sex body formation. Five years later, three groups independently discovered that Meisetz, from then on known under its nomenclature name *Prdm9* (PR/SET domain 9), is the major factor determining the positioning of meiotic recombination hotspots along the chromosome in humans and mice [Baudat *et al.* 2010, Myers *et al*. 2010, Parvanov *et al.* 2010]. *Prdm9* belongs to the PRDM genes family, originated in metazoans and expanded in vertebrates with some duplications in primates [Fumasoni *et al*. 2007]. The genes of this family are coding for proteins, mostly transcriptional regulators, some of which are involved in tumorigenesis [reviewed in [Fog *et al.* 2012, Casamassimi *et al*. 2020]. PRDM9 was found to play a role in controlling recombination hotspots in number of species like non-human primates [Auton *et al.* 2012, Groeneveld *et al.* 2012, Schwartz *et al.* 2014, Heerschop *et al.* 2016, Stevison *et al*. 2016], rodents [Buard *et al.* 2014, Capilla *et al*. 2014, Kono *et al.* 2014], ruminants [Sandor *et al*. 2012, Ma *et al*. 2015, Ahlawat *et al*. 2016a, Ahlawat *et al.* 2016b, Ahlawat *et al.* 2017, Zhou *et al.* 2018], equids [Steiner and Ryder 2013, Beeson *et al*. 2019] and even whales [Damm *et al*. 2022]. PRDM9 protein uniquely combines three main domains – a KRAB domain responsible for protein-protein interactions [Imai *et al*. 2017, Parvanov *et al*. 2017], a PR/SET domain with histone methyltransferase activity [Hayashi and Matsui 2006, Wu *et al*. 2013, Eram *et al.* 2014, Powers *et al.* 2016] and a DNA-binding domain representing an array of zinc fingers [Berg *et al.* 2010, Parvanov *et al.* 2010, Berg *et al*. 2011, Fledel-Alon *et al.* 2011, Hinch *et al*. 2011] – Figure1. Between the KRAB and the PR/SET domains, there is NLS/SSXRD motif with potential nuclear-localization signal and behind the PR/SET domain there is a single zinc finger with unknown role. Sequencings of the *Prdm9* gene revealed that its N-terminal part representing the KRAB and PR/SET domains is more or less conserved. In contrast, the C-terminal part containing the zinc finger array is hypervariable and the individual alleles differ in both the number and the DNA binding specificities of the individual fingers. The sequences of mouse *Prdm9* revealed five major haplotypes based on laboratory mouse inbred strains [Parvanov *et al*. 2010]. Further sequencings of "wild" *Prdm9* mouse

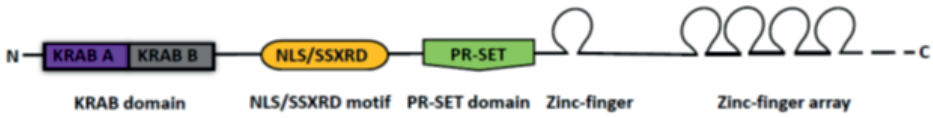

Fig. 1. PRDM9 consists of several domains and motifs. At the N-terminus is the The Krüppel associated box (KRAB) domain containing subdomains A and B. It is a transcriptional repression domain, which in PRDM9 is responsible for protein-protein interactions. The KRAB domain is followed by NLS/SSXRD motif, which probably serves as nuclear localization signal. The PR-SET domain is the catalytic subunit of the protein, which transfers methyl group from S-adenosyl methionine to lysine 4 and lysine 36 of histone 3 and thus bringing them to trimethylated state. After the PR-SET is positioned conserved zinc-finger of unknown function. At the C-terminus is the zinc-finger array, which consists of variable number of highly polymorphic zinc fingers and it defines the DNA-binding specificity of PRDM9.

alleles revealed one hundred nine variants based on 268 sequenced zinc finger arrays [Buard *et al*. 2014, Kono *et al.* 2014, Mukaj *et al.* 2020]. Studies in humans revealed two major *PRDM9* alleles and a multitude of alleles with very low frequencies [Alleva *et al.* 2021]. The great part of the nucleotide differences is localized at positions coding for the amino acids that ensure direct contact with the DNA and thus contribute for the different specificity of DNA binding of the different protein variants of PRDM9. The variety of *Prdm9* alleles provides source for new hotspots, thus avoiding depletion for existing ones (every repair via the homolog leads to disappearance of the hotspot sequence and eventually infertility [Boulton *et al.* 1997]).

PRDM9 in meiotic recombination

The discovery that PRDM9 is the major determinant for meiotic hotspots placement spurred numerous studies exploring the mechanism of action of PRDM9, that shed light on how it affects the recombination and meiosis in gametes. PRDM9 is expressed early in meiotic prophase I – at leptonema – when it binds its recognition DNA sequence, trimethylates the adjacent nucleosomes on lysine-4 and lysine-36 of histone 3 [H3K4me3 and H3K36me3]. This methylation mark is a signal for to the DNA recombination machinery to create DSB by SPO11 and the subsequent repair of the break to result in recombination either as equal exchange between the two homologs [crossover], or unidirectional transfer of information (noncrossover) [Hayashi and Matsui 2006, Keeney 2008, Baker *et al*. 2014, Lange *et al*. 2016, Powers *et al.* 2016]. Still, the full mechanism of the initiation of recombination by PRDM9 is lacking. Current data indicate that in early leptonema PRDM9 bind its recognition sequence as a multimer [Baker *et al.* 2015, Schwarz *et al*. 2019], and then trimethylates H3K4 and H3K36 on both sides of its binding site. The methylation of two to four nucleosomes on each side [Baker *et al*. 2014, Powers *et al*. 2016] is restricted perhaps via the interaction of the PRDM9 KRAB domain with CDYL and EHMT2 [Parvanov *et al.* 2017]. Interestingly, the cumulative length of the crossover tracts coincides with the average length defined by the PRDM9 methylated nucleosomes [Baker *et al.* 2014].

By the end of leptonema, CDYL and EHTM2 are probably removed from the complex and the nucleus and an interaction between PRDM9 and another protein, EWSR1, helps associate PRDM9-bound hotspots with the chromosome axis [Tian *et al*. 2021]. This association occurs through an interaction with the meiotic cohesin protein REC8, which helps integrate the hotspots to the axis in the presence of SYCP3 [Bhattacharyya *et al*. 2019]. After DSBs are formed by SPO11, PRDM9 disappears from the nucleus by late zygonema, while EWSR1 probably remains bound further, as it is shown that the lack of EWSR1 at pachynema affects the formation and resolution of Holiday junctions [Tian *et al*. 2021]. The described mechanism of hotspot activation and processing is dependent on open chromatin. Hotspot activity in actively transcribed genes is more likely to result in DSB formation and recombination compared to that in moderately or low expression genes in meiosis [Walker *et al.* 2015].

PRDM9 and infertility

The proper DSB formation and repair assures exchange of genetic information between the homologous chromosomes and their accurate segregation later on. Defects in any of the components of the recombination machinery lead to blockage of the meiotic course, impaired or missing gametes and sterility in the phenotype. *Prdm9* depleted mice of both sexes are sterile with abrogated double-strand break (DSB) repair. Cytologically, the main observed features are disrupted sex-characteristic body features formation and retained DMC1 foci [Hayashi and Matsui 2006]. Two clinical studies in early 2000s found patients with azoospermia associated with individual SNPs in *PRDM9,* which was enriched in patients compared to the control group. However, the causative role of these SNPs could not be determined unequivocally [Miyamoto *et al.* 2008, Irie *et al.* 2009]. Therefore, the studies in mice provided a better tool for study the effect of *Prdm9* on fertility.

Several studies pointed *Prdm9* as involved in ovarian insufficiency and depletion. A study [Wei *et al*. 2021] shows that 17 months old female mice have two-fold higher expression of *Prdm9* than 3 months older ones, while older female mice have twice higher trimetylation on lysine4 on histone 3. This observation still requires validation since PRDM9 is present only in fetal ovaries when meiosis I is performed. Intriguing question is the *Prdm9* RNA may have some regulatory role. Another study [Wang *et al.* 2021] on single cell sequencing of fetal ovaries found that germ cells expressing strongly *Prdm9, Spo11* and *Dmc1* undergo apoptosis. In humans, the end screening of over thousand patients with primary ovarian insufficiency by whole exome sequencing found two distinct monoallelic mutations in *ANKRD31* in three unrelated women and three heterozygous mutations in *PRDM9* in four unrelated patients [Wang *et al.* 2021]. Studies of the effects of these mutations *in vitro* showed reduced histone methytransferase activity.

Sterility caused by *Prdm9* was observed by the group of Forejt et al. when exploring the phenomenon of "hybrid sterility". It is a result of intersubspecific cross between two

inbred mouse strains, where in the male hybrid offspring the combination is between PWD allele of *Prdm9* by inbred strain originating from *Mus musculus* subspecies and B6 allele by inbred stain originating from *Mus domesticus* branch [Mihola *et al.* 2009]. This phenotype concerns only the male sex and represented by small testes, meiotic arrest at mid- to late pachytene, complete absence of sperm [Forejt 1996, Mihola *et al.* 2009], chromosome asynapsis of up to 90% and impairment of transcriptional inactivation of the sex chromosomes [Bhattacharyya *et al*. 2013, Gregorova *et al*. 2018]. The females are fertile although still having synaptic problems during meiosis [Bhattacharyya *et al*. 2013]. There are two other major components of the hybrid sterility – heterozygosity of the genome and presence of X-linked factor [Storchova *et al*. 2004, Dzur-Gejdosova *et al*. 2012, Bhattacharyya *et al*. 2014, Gregorova *et al.* 2018, Lustyk *et al.* 2019]. The leading role of *Prdm9* for hybrid sterility was confirmed in intersubspecific hybrids between *M.m.musculus* and *M.m.domesticus* wild-derived strains [Mukaj *et al.* 2020]. While the reason for sterility is clear in the absence of *Prdm9*, less is known about the nature of incompatibility between these two *Prdm9* alleles. Several theories attempt to give explanation [Davies *et al.* 2016, Smagulova *et al.* 2016, Yamada *et al*. 2017] and though they address different molecular aspects of the mechanism of hybrid sterility, we still lack the complete picture of this phenomenon. Open question is if this phenomenon acts indeed in nature when the two wild subspecies of mice are in contact – the high heterozygosity of the wild genomes make the chance of low having "pure" *musculus* or *domesticus* types in crosses and also the effect of hotspot erosion in the nature would be ignorable low [Forejt *et al*. 2021].

Although the function of *Prdm9* in meiosis and recombination in humans, mice and other mammals has been well documented, it is still unknown how general it is. Sequenced exomes of over 3000 British adults of Pakistani origin with high parental relatedness found over 1000 rare-variant homozygous genotypes with 781 gene knock-outs (loss of function) in many genes considered important for life or fertility. A healthy mother with loss-of-function mutation in the PR/SET domain of *PRDM9* had no apparent fertility problems and had three children [Narasimhan *et al.* 2016]. Another study in mice found that male animals lacking functional *Prdm9* were fertile and sired pups [Mihola *et al.* 2019]. The genetic background was from PWD inbred mouse strain and differed from the used so far B6 genome. Though the animals had increased DSB formation at promoter areas, shown to be problematic in sterile mouse hybrids [Smagulova *et al.* 2016], they were able to bypass meiotic arrest and produce functional sperm. Apart from genetic background, sex-specific modifiers can reduce the requirement for *Prdm9* leading to *Prdm9*-independent recombination in mouse female meiosis and fertility despite the absence of PRDM9 [Powers *et al.* 2020]. This raises the question to what extent *Prdm9* is important for meiosis and how the genetic background affects it. *Prdm9* is evolutionary product in vertebrate animals and among them in many cases it lost its function (dogs, amphibians, birds, crocodiles) – Baker *et al*. [2017]. Accordingly, we can divide the species into two categories – performing meiosis with *Prdm9*-dependent and *Prdm9*-independent homologous recombination.

It is still unclear how the genetic background affects the function of *Prdm9* in species that use this pathway and what are the conditions for the cell to perform meiotic recombination without it. Additional studies are required to clarify the effect of PRDM9 on the human health and fertility and reveal the mechanisms of its action.

PRDM9 and its potential role in cancer

PRDM9/Prdm9 belongs to the family of PR/SET histone methyltransferases. The majority of these histone modifier proteins are associated with tumor-suppressing features, while others are shown to have oncogenic potential (reviewed in Di Tullio *et al.* [2022]). *PRDM9/Prdm9* has been known to be expressed only in germ cells; however, data meta-analysis showed presence of its mRNA in cancerous tissues. Analysis of the regulation of cancer/testis genes and in particular meiotic cohort of them finds *PRDM9* as aberrantly expressed in lymphoma and leukemia cell lines hinting possibility for oncogenic effect [Feichtinger *et al.* 2012]. Global study analysed 1879 cancer samples in 39 different cancer types and found *PRDM9* expressed in 20% of them [Houle *et al*. 2018]. The *PRDM9* expression in cancer tissues was significantly higher than in the surrounding healthy tissues and healthy non-germ tissues. The study also found many mutations in cancer samples within the 5Mb region of *PRDM9* on chromosome 5, as well as in other genes involved in the meiotic pathway. Importantly, they observed association between the aberrant *PRDM9* expression and presence of PRDM9-specific DNA motif in structural variant (SV) breakpoints [Houle *et al*. 2018]. Analysis of exome- and RNA-seq public datasets at the Cancer Genome Atlas portal (TCGA) [https://portal.gdc.cancer.gov/] revealed *PRDM9* together with *PRDM2, PRDM3/MECOM, PRDM16* and *ZFMP2/FOG2* as the genes among the PR/SET family most often mutated in cancers [Sorrentino *et al.* 2018]. *PRDM9* had the highest mutation rate in uterine corpus endometrial carcinoma (10%), lung adenocarcinoma (14.2%), and in skin cutaneous melanoma (15.4%). *PRDM9* was also found to be highly mutated in stomach adenocarcinoma samples [Reyes *et al.* 2021] by another bioinformatics study on public data from CBioPortal [https://www.cbioportal.org/] and TCGA databases. The same TCGA database was used to analyze samples of head and neck squamous cell carcinoma and found that the patient survival was associated with mutations in several genes, including *PRDM9* [Zou *et al.* 2016]. *PRDM9* was and among the six genes found to be with possible role in bladder cancer progression based on integrative analysis of 426 cancer samples data from TCGA database [Ding *et al.* 2019].

Though the listed studies (Tab. 1) provide information about presence and level of *PRDM9* mRNA and mutational rate, functional evidence or characterization for the possible effect on *PRDM9* in cancer remains to be evaluated further. Most observations relating *PRDM9* genetic variants to cancer are associational. Exome sequencing data from families with children affected by B-cell precursor acute lymphoblastic leukemia (B-ALL) discovered significant excess of rare allelic forms

Table 1. Potential oncogenic effects of *PRDM9* with expression in somatic cells

PRDM9 alteration	Cancer type	Reference
Elevated expression	Lymphoma and Leukemia cell lines, Liver and Ovarian Tumors, Bladder Cancer	Feichtinger *et al*. [2012], Houle *et al*. [2018], Ding *et al.* [2019]
Mutations in *PRDM9*	Uterine Corpus Endometrial Carcinoma, Lung adenocarcinoma, Skin Cutaneous Melanoma, Stomach Adenocarcinoma, Bladder Cancer, Head and Neck Squamous Cell Carcinoma	Houle *et al*. [2018], Sorrentino *et al*. [2018], Reyes *et al*. [2021], Zou *et al*. [2016], Ding *et al.* [2019]
Copy number variation	Stomach Adenocarcinoma, Bladder Cancer	Reyes *et al*. [2021], Ding *et al.* [2019]
Overlap of meiotic crossover sites with SV breakpoints	Glioblastoma Multiforme, Head and Neck Squamous Cell Carcinoma, Liver Hepatocellular Carcinoma, Breast Cancer, Kidney Cancer, Colorectal Cancer, Retinoblastoma, Leukemia	Houle *et al*. [2018], Hagstrom, Dryja [1999], Howarth *et al*. [2009], Paulsson *et al.* [2011]
Rare allelic forms of *PRDM9*	B-cell Precursor Acute Lymphoblastic Leukemia, High Hyperdiploid Childhood Acute Lymphoblastic Leukemia	Hussin *et al*. [2013], Woodward *et al*. [2014]

of *PRDM9* [Hussin *et al.* 2013]. Sequencing of the parents and the children showed that the alleles are transmitted to the affected children in half of the cases. Moreover, high hyperdiploid childhood acute lymphoblastic leukemia (HeH ALL) patients were associated with rare allelic forms of *PRDM9*, though with lower statistical power [Woodward *et al.* 2014]. The observations of Hussin *et al*. [2013] and Woodward *et al.* [2014] establish the hypothesis that PRDM9-defined sites of recombination in parents probably increase the genome instability in the offspring and cancer in pediatric age. As such, these findings imply the possible role of *PRDM9* variation in risk of aneuploidies or genomic rearrangements leading to childhood leukemia. An example of possible effect of altered PRDM9-driven recombination is the non-disjunction of chromosome 21 leading to maternally derived trisomy 21 [Oliver *et al.* 2016]. The rare forms of *PRDM9* in that case may cause lower recombination along chromosome 21 and thus contributing to higher risk of chromosome 21 non-disjoining.

Other remarks

The meiotic function of PRDM9 is to recognize and bind specific DNA sequences, which subsequently anchor to the chromosomal axes where the DSB should occur. A study pointing at the chromatin loop anchor points as fragile sites in the genome, which are prone to DNA breakage in normal and cancer cell lines [Kaiser and Semple 2018] and also some of them overlap with the known DNA-binding motifs of PRDM9, which raises the question if *PRDM9* is expressed on protein level in certain tumors and if any other protein is associated to these motif during the tumor progression. In fact, this overlaps with previous studies that found correlation between acquired SV

breakpoints and sites of meiotic crossovers, across recurrent breaks in retinoblastoma [Hagstrom and Dryja 1999], colorectal cancer [Howarth *et al.* 2009] and within individual somatic and meiotic recombination maps [Paulsson *et al.* 2011].

The very important question is whether indeed *PRDM9* is expressed on protein level in cancers, where its mRNA is elevated? This is yet to be demonstrated. If so, a possible mechanism of action could involve an interaction with the meiotic cohesin REC8, known also to be expressed in some cancers, and thus contribute to the disruption of the mitotic chromosomal architecture. If no, does *PRDM9* has any additional regulatory role for the cell metabolism? Known case of mitotically inhibited meiotic protein is *Rec8* in fission yeast [Harigaya *et al.* 2006, Hiriart *et al*. 2012] where post-transcriptional mRNA degradation occurs. One study showed the presence of PRDM9 on protein level [Feichtinger *et al*. 2012] in cancerous cell line, but further studies are required to validate this observation and prove it as a general phenomenon in cancers, or exception met in cell lines.

Remaining question is also if the deregulated expression of *PRDM9* represents only transcriptional malfunction, or also involves impairment of the translation repression program, which otherwise assures the protein to be present only in spermatocytes. The existing studies were conducted in human cell lines and tumors. However, we should keep mind that *PRDM9* duplicated in primate evolution giving rise to a homolog - *PRDM7* [Fumasoni *et al*. 2007, Heerschop *et al.* 2021]. The duplication contains internal duplication leading to frameshift and new splicing site, which defines two isoforms – one without and one with four zinc fingers [Blazer *et al.* 2016]. This homolog has methytransferase activity on lysine 4 of histone 3 [Blazer *et al*. 2016] and distribution outside testes like melanocytes [Fumasoni et al. 2007]. Further studies are required to map the distribution of PRDM7 in different tissues and find its functional relation to PRDM9.

Conclusion

In conclusion, PRDM9 is enzyme known for almost two decades since its discovery. Though it got considerable attention and more and more groups studied its structure, function, disease associations, etc. still a lot of its potential clinical roles remain uncovered, especially outside the field of meiosis, where it was primarily studied and characterized. The continuing exploration of *PRDM9* as potential oncogene needs inhibitors specific to PRDM9 which would validate its role in tumorigenesis. The recent development of such inhibitor [Allali-Hassani *et al.* 2019] makes the validation of the oncogenic function of PRDM9 as high priority task.

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