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Research Highlights
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Research Highlights

UHRF1: a jack of all trades, and a master epigenetic regulator during spermatogenesis

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Since its initial discovery in mouse and human cells as nuclear protein 95 (Np95; [1]) and inverted CCAAT box-binding protein 90 (ICBP90; [2]), respectively, ubiquitin-like PHD and RING finger domain-containing protein 1 (UHRF1) has quickly risen to current prominence as a key epigenetic regulator in diverse cellular and developmental processes (reviewed in [3]). A recent in vivo study further revealed a critical role of UHRF1 during mouse spermatogenesis [4].

Much like the famed Swiss Army knife, the multidomain UHRF1 protein functions through its five arms, including from N-terminus to C-terminus, a ubiquitin-like (UBL) domain, a tandem Tudor domain (TTD), a plant homeodomain (PHD), a SET- and RING-associated (SRA) domain, and a really interesting new gene (RING) domain (Figure 1A). Initial studies focused on the characterization of individual domains and their interacting partners in vitro and ex vivo. Later studies uncovered the incredible amount of coordination and crosstalk among these domains, as UHRF1 carries out its multifaceted functions through precise interactions with DNA, histones, and other effector proteins.

UHRF1 is best known for its function in DNA methylation maintenance. It is a critical partner for the maintenance DNA methyltransferase DNMT1, which methylates the newly synthesized daughter strand following semi-conservative DNA replication. UHRF1 preferentially binds to hemi-methylated DNA through its SRA domain [5–7]. The SRA domain also interacts directly with DNMT1's replication focus targeting sequence (RFTS) [7, 8], thus tethering DNMT1 to newly replicated DNA. The physical interaction between UHRF1 and DNMT1 increases the activity and specificity of DNMT1 for methylating hemi-methylated CG sites [9, 10]. In addition, UHRF1 may regulate do novo DNA methylation through interactions with DNMT3A and DNMT3B under specific cellular contexts [11].

Hemi-methylated DNA is not the only cue that UHRF1 takes from the genome. To guide DNMT1 to the right spot in the nucleus, UHRF1 also understands the highly complex language of chromatin and employs its other domains to decipher the combinatorial state of post-translational histone modifications (i.e., the histone code). Its TTD domain preferentially binds to a single histone H3 N-terminal tail with di- or tri-methylated lysine 9 (H3K9me2/3) and unmethylated lysine 4 (H3K4me0) [12–15]. The importance of this interaction has been shown in human HeLa cells [15] and mouse embryonic stem cells (ESCs) [16]. There is also evidence that UHRF1 interacts with G9a, one of the histone methyltransferases responsible for H3K9me2 [17]. There are additional layers of crosstalk among the domains. The PHD domain, which has an affinity to unmodified arginine 2 on the H3 tail (H3R2me0) [14, 18–20], facilitates the interaction between TTD and H3K9me3 [21]. Importantly, structural studies illustrate that the full-length UHRF1 protein adopts a closed conformation due to intramolecular interactions in the absence of ligands. Binding to H3K9me3 is blocked by an intramolecular interaction of TTD with a polybasic region (PBR) between the SRA and RING domains [22, 23]. Meanwhile, binding to H3R2me0 is inhibited by an intramolecular interaction of the PHD domain with the SRA domain [22]. Binding to hemi-methylated DNA shifts UHRF1 to an open state, which promotes H3K9me3 recognition by UHRF1 [22, 23]. Thus, UHRF1 integrates the two major epigenetic silencing pathways by its dynamic interactions with hemi-methylated DNA and H3K9me2/3.

UHRF1's role in maintenance DNA methylation is also dependent on two other domains: the RING finger domain and the UBL domain. Its RING finger domain has E3 ubiquitin ligase activity toward histone H3 [24], DNMT1 [25, 26], and UHRF1 itself [27]. UHRF1-dependent H3 ubiquitination is a prerequisite for DNMT1 binding to DNA replication sites [28]. Hemi-methylated DNA stimulates UHRF1 ubiquitin ligase activity on H3 by directing TTD-PHD-bound H3 substrate to the active site of E2 ubiquitin ligase [29]. The tandem monoubiquitin marks on H3 are recognized by DNMT1 via a ubiquitin interaction motif (part of the RFTS binding module) [30, 31], which enhances DNMT1 recruitment and high-fidelity maintenance of DNA methylation. Indeed, it has been demonstrated that UHRF1's E3 ubiquitin ligase activity is required for maintenance DNA methylation at retrotransposons and satellite repeats

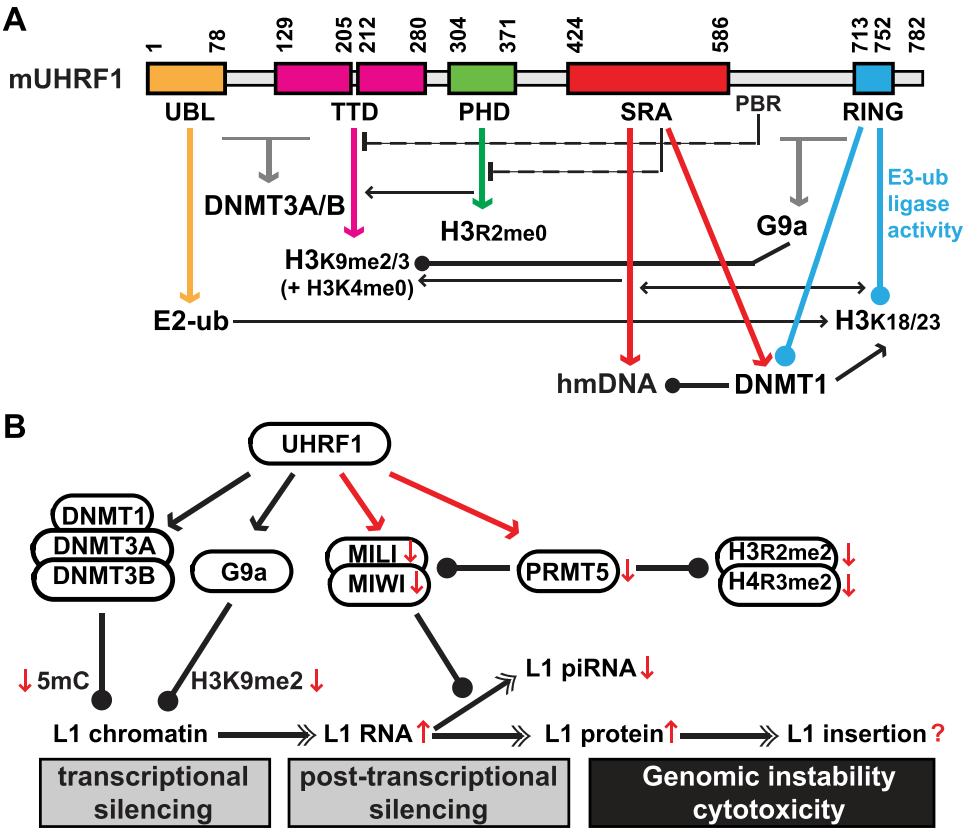

Figure 1. UHRF1 regulates multiple epigenetic pathways during spermatogenesis. (A) Mouse UHRF1 domains and interacting partners. Domain boundaries are labeled above the domain structure as amino acid coordinates per UniProt (https://www.uniprot.org/uniprot/Q8VDF2). Drawn to scale. Six domains/regions are labeled below the domain structure: UBL, ubiquitin like; TTD, tandem Tudor domain; PHD, plant homeodomain; SRA, SET- and RING-associated; PBR, polybasic region; and RING, really interesting new gene. Domains and corresponding interactions (down arrows) are color coded. Interactions with DNMT3A/3B and G9a have not been mapped to defined domains, thus in gray arrows. Ball-headed lines indicate enzymatic modifications. Crosstalk is annotated as either stimulatory (arrow) or inhibitory (T-end dashed lines for intramolecular interactions between TTD and PBR, and between PHD and SRA). hmDNA, hemi-methylated DNA. E2-ub, E2 ubiquitin conjugating enzyme. (B) Model for UHRF1-mediated epigenetic silencing of L1 during spermatogenesis. In postnatal testes, L1 retrotransposons are transcriptionally silenced by DNA methylation and H3K9me2/3, and post-transcriptionally by the pachytene piRNA pathway. In the light of the recent work by Dong and colleagues [4], UHRF1 has emerged as the master regulator of these epigenetic pathways. Newly reported interactions of UHRF1 with PIWI proteins and PRMT5 are highlighted (in red). The conditional loss of UHRF1 function causes meiotic catastrophe and germ cell death. Many changes are observed at molecular levels (↑, upregulation; ↓, downregulation). In particular, L1s are de-repressed at both RNA and protein levels. However, whether there is an increase in L1 insertion and to which degree L1 retrotransposition contributes to genomic instability in *Uhrf1*-deficient germ cells remain unknown.

[28, 31]. In contrast, the ubiquitination of DNMT1 by UHRF1 regulates DNMT1 stability and promotes its degradation [25, 26]. The role of the UBL domain was only recently revealed. It is required for H3 ubiquitination by UHRF1 RING E3 ligase in stabilizing the E2-E3-chromatin complex, and also for maintenance DNA methylation on retrotransposons [32, 33].

So far, only a few studies have explored UHRF1's function in vivo, in sharp contrast to aforementioned numerous in vitro and ex vivo studies that helped to illuminate UHRF1's biochemical and cellular functions. UHRF1 is essential for early development. Global loss of function causes early developmental arrest shortly after gastrulation in mice [6, 34]. Thus, conditional knockout (cKO) strategies have been adopted to investigate the role of UHRF1 in other stages of development, including during the proliferation and maturation of colonic regulatory T cells [35], chondrocyte differentiation and limb growth [36], neuronal differentiation and survival [37], oocyte growth [38], and, most recently, spermatogenesis [4].

DNA methylation in the germline of mammalian genomes is not static. Globally, the germline genome undergoes two rounds of DNA methylation reprogramming during development. The first round occurs in preimplantation embryos and the second in migrating/post-migratory primordial germ cells (PGCs). Recent data indicate the erasure of global methylation in PGCs is largely due to replication-coupled passive DNA demethylation (reviewed in [39]). In rapidly proliferating PGCs between embryonic day (E) 9.5 and E11.5, both UHRF1 and de novo methyltransferases DNMT3A/3B are downregulated and become undetectable [40, 41]. Later, in the female germline, an oocyte-specific methylation pattern is established in growing oocytes (GOs) and completed in fully grown oocytes (FGOs) via de novo DNA methyltransferases DNMT3a/DNMT3L [42, 43]. To study the role of UHRF1 in oocyte and preimplantation embryonic development, the *Zp3*-Cre line was used, which is expressed exclusively in GOs [38]. In *Uhrf1* cKO females, oogenesis is unaffected. At the molecular level, there is 20% reduction in global CG methylation and 15% reduction in global non-CG methylation in FGOs. As oocyte growth does not involve maintenance DNA methylation (no DNA replication in meiotic prophase I), these data suggest that UHRF1 participates in de novo CG and non-CG methylation in oogenesis. Importantly, in vitro fertilization of *Uhrf1* cKO oocytes with wild-type sperm leads to the developmental arrest in 80% of the embryos [38]. Thus, the maternally derived UHRF1 protein is also required for preimplantation

embryonic development. The loss of maternal UHRF1 leads to a global reduction in CG methylation, encompassing genic, intergenic, tandem repeats, retrotransposons (L1, B1, and IAP), and imprinted sequences, suggesting maternal UHRF1 is required for maintenance CG methylation in preimplantation embryos. However, its impact on retrotransposon expression was not assessed by the study [38].

DNA methylation is an important mechanism of controlling the proliferation and differentiation of stem cells, not only ESCs but also adult stem cells. Indeed, in the mouse brain, UHRF1 is expressed specifically in fetal and adult neural stem cells (NSCs) [37, 44]. To investigate whether early epigenetic mechanisms impact the long-term behavior of NSCs and derivatives, a recent study used *Emx1-Cre* to specifically abolish UHRF1 function in the dorsal telencephalon [37]. The *Emx1* promoter is expressed exclusively in the dorsal telencephalon from embryo to adulthood. The conditional deletion of UHRF1 starts as early as E10 in the NSCs of the developing cerebral cortex. However, no gross morphological defect is observed during the embryonic development. The first morphological phenotype becomes detectable at postnatal day (P) 7 as the thickness of the cerebral cortex is reduced. The delayed neurodegeneration appears to be the result of increased cell death at the onset of embryonic neurogenesis and the subsequent escalated cell death during the neuronal maturation at postnatal stages. At the molecular level, reflecting an essential role of UHRF1 in maintaining DNA methylation, global DNA methylation, is significantly reduced, including 35% loss on L1, B1, and IAP retrotransposons. Surprisingly, there is little change in transcription of L1, B1, and annotated genes. The only exception is for IAP retrotransposons, more specifically from the IAPEz family, which shows ∼130-fold increase in the cKO cortices. The extraordinary magnitude of change contrasts sharply with more modest alterations in *Uhrf1* KO ESCs (2-fold) and embryos (4∼8-fold) [6, 45]. These results indicate that UHRF1 is critical for IAP repression during neurogenesis [37].

Until recently, the physiological role of UHRF1 in spermatogenesis has not been explored. The testis boasts the highest level of UHRF1 RNA expression among a panel of mouse tissues when examined by northern blot [1]. Indeed, UHRF1 protein is present not only in proliferating spermatogonia but also in meiotic spermatocytes and differentiating spermatids [46], implicating a potential function during spermatogenesis. In a landmark paper, Dong and colleagues conditionally abrogated UHRF1 in the differentiating spermatogonia and spermatocytes of postnatal testes using a *Stra8*-Cre mouse line [4]. UHRF1 protein abundance and subcellular localization during male germ cell development were thoroughly analyzed. Interestingly, wild-type UHRF1 protein is detected in nearly all developmental stages, encompassing fetal, neonatal, and adult stages, from prospermatogonia to early round spermatids. Notably, there is a dynamic change in subcellular localization, beginning as mainly cytoplasmic in fetal prospermatogonia, nuclear in mitotic spermatogonia, back to cytoplasmic at the onset of meiosis (preleptotene, leptotene, zygotene, and early pachytene spermatocytes), and then nuclear again (late pachytene and round spermatids), although the regulation and significance of the cytoplasmic/nuclear shuttling remain unclear (see later discussions in the context of UHRF1's interactions with PIWI proteins and PRMT5).

Phenotypically, the conditional deletion of UHRF1 in differentiating spermatogonia leads to a spermatogenic arrest at the pachytene spermatocyte stage and ultimately male infertility [4]. At the histological level, abnormality in seminiferous tubules becomes apparent as early as P14, when the first cohort of pachytene spermatocytes is formed. Cell marker analyses indicate that both spermatogonial differentiation and meiotic initiation are unaffected in cKO testes. However, staining of phosphorylated histone H2A.X (γ-H2A.X), a chromatin marker for adjacent DNA double-strand breaks (DSBs), shows an unusual nucleus-wide distribution in cKO pachytene spermatocytes, instead of being confined to the XY body. Such a pattern is indicative of a systemic failure in DNA damage repair and the persistence of genome-wide unrepaired DSBs. Many meiotic mouse mutants are accompanied with extensive asynapsis and failure in meiotic sex chromosome inactivation (MSCI), which is thought to trigger germ cell apoptosis at the mid-pachytene stage [47]. However, despite widespread γ-H2A.X signals, the autosomal synapsis appears to be complete in the residual *Uhrf1* cKO pachytene spermatocytes. Additionally, the number of RPA2 foci is reduced in the *Uhrf1* cKO spermatocytes, indicating a defect in meiotic recombination in meiotic prophase I. Regardless of the checkpoint pathways, the developmental defect in *Uhrf1* cKO spermatocytes can be attributed to elevated apoptosis (approximately 3-fold higher than the wild-type control at P14), which persisted until at least P35 [4].

Given the role of UHRF1 in DNA methylation, Dong and colleagues first examined methylation levels by immunostaining of methylated cytosines (5mCs) [4]. DNA methylation in the *Uhrf1* cKO appears normal at P10 but reduced globally in P14 testes. No apparent change is found in differentiating spermatogonia but 5mC signals become nearly undetectable in leptotene, zygotene, and pachytene spermatocytes. Bisulfite sequencing shows a substantial reduction in DNA methylation in the L1 5'UTR promoter region from 86 to 45% at P18, which is comparable to that seen in *Uhrf1* KO embryos (85 to 40%) [6]. DNA methylation is also reduced from 90 to 70% at IAP elements, although the magnitude is much less than that in *Uhrf1* KO embryos (95 to 20%) [6]. Mild reduction in methylation is observed in the typically paternally methylated intergenic differentially methylated region (DMR) between the imprinted genes *Dlk1* and *Gtl2* (93 to 68%). These data suggest that UHRF1 is required for DNA methylation maintenance during the postnatal male germ cell development. The current study did not examine methylation for other genes or heterochromatic regions, such as major or minor microsatellites. The magnitude of methylation loss in germ cells might have been substantially underestimated due to the presence of testicular somatic cells. To fully understand the role of UHRF1-mediated methylation in postnatal germ cells, it is imperative to pinpoint the precise timing of the methylation loss as well as to identify the genomic regions that are affected, preferably using a methylomics approach on stage-specific germ cell populations.

Following DNA methylation reprogramming in PGCs, in the postnatal testis, the male germline genome undergoes at least two additional episodes of dynamic DNA methylation. The first episode occurs as spermatogonia transition from undifferentiated (Kit^-) to differentiating (Kit^+) spermatogonia [48, 49]. Among the reported DMRs from whole-genome bisulfite sequencing analyses, more genomic regions lose methylation than gain methylation at this transition. Only 40 genomic regions gain >30% CG methylation, while 1352 regions lose >30% CG methylation [49]. The second wave is at the onset of meiosis, which features a transient reduction in DNA methylation (TRDM), equivalent to a genome-wide 12% of CG methylation loss, primarily in preleptotene spermatocytes [50]. The TRDM is thought to occur via DNA replication-dependent DNA demethylation due to a delay in maintenance DNA methylation. The global loss in CG methylation is fully regained in pachytene spermatocytes [50].

UHRF1 has been shown to play a role in spermatogonial differentiation in a *Uhrf1* inducible KO (iKO) model [51]. In this iKO model, no Kit$^+$ cells are identified, suggesting the loss of UHRF1 completely blocks spermatogonial differentiation and/or required for the survival of differentiating spermatogonia. The phenotype in the iKO model is much more severe than that observed in the cKO model by Dong and colleagues. However, the interpretation of the iKO results may be confounded by an unexpected adverse effect of tamoxifen itself on spermatogenesis [52]. In the paper of Dong and colleagues, *Stra8*-Cre is used to ablate *Uhrf1* function genetically. Endogenous STRA8 protein is expressed only in differentiating spermatogonia through leptotene spermatocytes [53, 54]. However, the *Stra8*-Cre clearly functions in a subset of undifferentiated spermatogonia in the transgenic mice [55], potentially due to the lack of some endogenous regulatory elements in the promoter sequence used in the transgene. Thus, it is reasonable to suspect that *Stra8*-Cre-mediated deletion of UHRF1 may occur prior to the type A to A1 transition, which marks the beginning of spermatogonial differentiation [56]. Therefore, the impact from a loss of UHRF1, in the current model, points to a role of maintenance DNA methylation in differentiating spermatogonia as well as in meiotic prophase. Again, further analysis of DNA methylation changes is warranted to dissect the differential contribution of UHRF1 during these two developmental stages.

What is the impact of reduced methylation on gene expression? To this end, the authors examined the effects on gene expression by RNA-seq [4]. Few genes show deregulation at P9, but 200 genes are upregulated by >2-fold at P12. Additionally, many retrotransposon families are upregulated. The upregulation of L1 and IAP is also confirmed by qPCR, ranging from 3–7-fold in P18 and P56 testes. As retrotransposon expression is regulated in germ cells by the PIWI-interacting RNA (piRNA) pathway (reviewed by [57, 58]), the authors checked PIWI protein abundance and piRNA production [4]. Unexpectedly, both MILI and MIWI, two PIWI proteins that are expressed in spermatocytes, are downregulated in *Uhrf1* cKO spermatocytes. In addition, the piRNA biogenesis is compromised. How does UHRF1 crosstalk with the piRNA pathway? In this regard, the authors offered novel evidence for the physical interactions of UHRF1 with MILI and MIWI [4]. Therefore, it is likely that such interactions in normal germ cells help to stabilize MILI and MIWI and facilitate the generation of pachytene piRNAs. What process might mediate this stabilization? Based on the involvement of PRMT5 (see below), the data support a model in which the stabilization of MILI and MIWI by UHRF1 is mediated via its recruitment of PRMT5 and subsequent symmetric dimethylation of these PIWI proteins (Figure 1B).

Indeed, the authors presented compelling evidence for an interaction between UHRF1's TTD domain and PRMT5's SAM domain [4]. In wild-type leptotene and zygotene spermatocytes, both PRMT5 and UHRF1 are enriched in the cytoplasm. In pachytene spermatocytes, PRMT5 and UHRF1 colocalize in the nucleus. In P18 *Uhrf1* cKO testes, both *Prmt5* mRNA and protein are downregulated, so are the PRMT5-mediated symmetrically dimethylated H3R2me2 and H4R3me2 marks. The impact of altered histone modifications in the *Uhrf1* cKO testis is unclear. Interestingly, *Prmt5* cKO, mediated by *Stra8*-Cre, phenocopies the *Uhrf1* cKO, showing a similar meiotic arrest [59]. At P10, loss of PRMT5 has no impact on H3R2me2, although H4R2me2 is lost. L1 and IAP expression are also unaffected at P10. The authors of the *Prmt5* cKO study did not examine PRMT5-mediated histone modifications and retrotransposon expression at later stages [59]. Therefore, it is still unclear whether the downregulation of PRMT5 in developing germ cells directly contributes to derepression of retrotransposons. It is also noteworthy that UHRF1 has a preference for unmethylated H3R2 [14, 18–20]. Both symmetrically and asymmetrically dimethylated H3R2s impede the interaction between UHRF1 and H3 tail in vitro [19]. In addition, H3R2me2 is only present in the cytoplasm of wild-type spermatocytes [4], suggesting H3R2 is not a substrate for PRMT5 in these cells. Taken together, it is more likely that the reduction of PRMT5 impacts substrates other than H3, including PIWI proteins and spliceosomal Sm proteins [60, 61]. The proposed model further predicts that the symmetrical dimethylation of MILI and MIWI is compromised in the *Uhrf1* cKO spermatocytes, which should be experimentally validated in future studies (Figure 1B).

In summary, the recent study by Dong and colleagues [4] adds to our knowledge about the essential roles that UHRF1 plays in vivo. Already recognized as a jack of all trades, this study establishes UHRF1 as a master regulator of multiple epigenetic pathways during male germ cell development. Importantly, the observed infertility phenotype is highly relevant to human male reproduction as genetic polymorphisms in the human UHRF1 gene have been associated with oligospermia [62]. As discussed above, there remain many unanswered questions. In particular, the role of retrotransposon activation in the *Uhrf1* cKO model awaits further elaboration. Deregulation of L1 retrotransposons can potentially impact germ cell well-being at three discrete stages of the L1 life cycle: transcription, translation, and retrotransposition [63] (Figure 1B). Previous studies have identified illegitimate meiotic recombination in *Dnmt3l* KO spermatocytes [64] and an increase in retrotransposition in *Mov10l1* KO spermatocytes [63]. Thus, it will be informative to examine levels of retrotransposition by either NextGen sequencing approaches or the new L1 reporter mouse [63, 65]. In parallel, many domain-specific *Uhrf1* mutants have been characterized in vitro and ex vivo. At least two knock-in (KI) mouse models have been established, including the SRA KI [45] and TTD KI [66]. Interestingly, the *Uhrf1* TTD KI (Y191A/P192A) mouse model displays no overt phenotype, despite a failure in binding to H3K9me2/3 by the mutant UHRF1 protein, suggesting the binding of UHRF1 to H3K9me2/3 is not essential for animal development and reproduction [66]. Nevertheless, there is a modest global reduction in DNA methylation in several somatic tissues examined, including retrotransposons [66]. These and additional new KI models should allow the molecular dissection of the function of UHRF1's individual domain module(s) during mammalian reproduction.

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