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## Developmental regulators moonlighting as transposons defense factors

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### Abstract

**Background:** The germline perpetuates genetic information across generations. To maintain the integrity of the germline, transposable elements in the genome must be silenced, as these mobile elements would otherwise engender widespread mutations passed on to subsequent generations. There are several well-established mechanisms that are dedicated to providing defense against transposable elements, including DNA methylation, RNA interference, and the PIWI-interacting RNA pathway.

**Objectives:** Recently, several studies have provided evidence that transposon defense is not only provided by factors dedicated to this purpose but also factors with other roles, including in germline development. Many of these are transcription factors. Our objective is to summarize what is known about these “bi-functional” transcriptional regulators.

**Materials and methods:** Literature search.

**Results and conclusion:** We summarize the evidence that six transcriptional regulators—GLIS3, MYBL1, RB1, RHOX10, SETDB1, and ZBTB16—are both developmental regulators and transposable element-defense factors. These factors act at different stages of germ cell development, including in pro-spermatogonia, spermatogonial stem cells, and spermatocytes. Collectively, the data suggest a model in which specific key transcriptional regulators have acquired multiple functions over evolutionary time to influence developmental decisions and safeguard transgenerational genetic information. It remains to be determined whether their developmental roles were primordial and their transposon defense roles were co-opted, or vice versa.

### Keywords

LINE1; germ cell; primordial germ cell; pro-spermatogonia; spermatogonial stem cell; transcription factor; transposon

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#### AUTHOR CONTRIBUTIONS

Both authors researched data for the article, substantially contributed to the discussion of content, and wrote, reviewed, and edited the manuscript before submission.

#### CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

## 1 | INTRODUCTION

The processes of development and transposon defense are distinct functions that have traditionally been studied separately. In this review, we discuss transcriptional regulators that have the surprising ability to function in both processes. We first discuss the known or putative developmental functions of these transcriptional regulators in male germ cells. We then discuss the evidence that these factors silence transposons, what classes of transposons they act on, and how their defense functions might be linked with their developmental functions.

### 1.1 | Germ cell development and spermatogenesis

Germ cell development initiates during early embryogenesis when primordial germ cells (PGCs)—the common precursors of spermatozoa and oocytes—are first formed.<sup>1,2</sup> These PGCs migrate to the gonadal ridge, whereupon they receive signals that initiate one of two distinct orchestrated programs - oogenesis or spermatogenesis. At ~E12.5 in mice, male PGCs convert into pro-spermatogonia (ProSG; also called gonocytes), which undergo three successive stages – multiplying (M)-ProSG, primary transitional (T1)-ProSG, and secondary transitional (T2)-ProSG. These three ProSG stages are distinguished by whether or not they undergo cell proliferation, as well as their epigenetic reprogramming status.<sup>3</sup> At the perinatal stage in mice, T2-ProSG gives rise to spermatogonial stem cells (SSCs), the cell type responsible for supporting spermatogenesis throughout adult life. Like all stem cells, SSCs have the ability to undergo both self-renewal and differentiation.<sup>3,4</sup> Their self-renewal is greatly influenced by the niche-factor glial cell line-derived neurotrophic factor (GDNF),<sup>5</sup> as well as several non-GDNF-stimulated transcription factors (TFs), including DMRT1, FOXO1, MYC, ZBTB16, and TAF4B.<sup>6–10</sup> The differentiation of SSCs leads to the formation of spermatogonial progenitors, which further differentiate to form spermatocytes. The latter undergo meiosis to generate haploid cells called round spermatids, which later differentiate to form elongated spermatids, the immediate precursors of spermatozoa.<sup>3</sup> This process of spermatogenesis is a highly orchestrated process that is governed by many TFs, including DMRT1, SOHLH1, SOHLH2, and SOX3.<sup>11–13</sup>

### 1.2 | Transposons

Most eukaryotic genomes are dominated by repetitive DNA, most of which is interspersed between coding regions.<sup>14</sup> This repetitive DNA is predominantly derived from the activity of transposable elements (TEs) – many of which are parasitic genetic units that replicate and transpose the new copy to another site by a ‘copy and paste’ mechanism. This process leads—over evolutionary time—to the accumulation of hundreds of thousands of TE copies dispersed through the genome.<sup>15</sup> TEs are regarded as a double-edged sword. On the positive side, TEs sometimes acquire functions over evolutionary time that are useful to the host organism. For example, some TEs have been shown to evolve into regulatory sequences and protein-coding domains that have been co-opted to serve the purposes of the host.<sup>16</sup> But on the negative side, TEs can be highly deleterious. This follows from the fact that TEs transpose in a relatively random manner in the genome. Thus, they can disrupt gene function or alter gene expression, contributing to genetic disease and cancer.<sup>16,17</sup>

TEs are broadly characterized as either retrotransposons or DNA transposons. Retrotransposons are transcribed into an RNA intermediate, which can be reverse-transcribed and integrated at a new site in the genome.<sup>18</sup> Retrotransposons can be further divided into those that either contain or lack a long terminal repeat (LTR). An example of an LTR-containing retrotransposon is intracisternal A-particles (IAPs), which originally were derived from infectious retroviruses that integrated into the germline.<sup>19</sup> Non-LTR retrotransposons include long interspersed nuclear elements (LINEs) and short interspersed nuclear elements (SINEs).<sup>18</sup> In contrast to retrotransposons, DNA transposons are mobile DNA elements that generate a single-or double-stranded DNA intermediate to move to another genomic location.<sup>20</sup> DNA transposons use a 'cut and paste' mechanism and thus, unlike retrotransposons, do not accumulate in copy number.

### 1.3 | Transposon defense

To protect against the negative effects of TEs, multiple epigenetic and RNA-mediated mechanisms have been postulated to have evolved.<sup>21</sup> Such silencing mechanisms are particularly critical for the germline, as they reduce the transmission of TE-induced mutations to subsequent generations.

DNA methylation is a major mechanism that curbs TE transposition.<sup>22</sup> The majority of DNA methylation in the mammalian genome occurs in repetitive sequences, which are largely TEs, and encompass the bulk of heterochromatin. The re-methylation of repeat elements following genome-wide demethylation in PGCs occurs progressively in fetal ProSG and is completed by the newborn stage.<sup>23</sup> DNA methylation is catalyzed by DNA methyl transferases, including DNMT1, DNMT3A, and DNMT3B.<sup>24</sup> DNMT1 is considered to be mainly involved in maintaining methylation marks after DNA replication, and thus it assures faithful propagation of existing DNA methylation patterns. It preferentially methylates hemi-methylated DNA from the parental strand onto the newly synthesized daughter strand during DNA replication. In contrast, DNMT3A and DNMT3B are de novo DNA methyl transferases that generate new DNA methylation marks. DNMT3A and DNMT3B are largely functionally redundant in silencing TEs in germ cells.<sup>24</sup>

Another DNMT family member—DNMT3L—lacks DNA methyltransferase activity and instead serves to activate other DNMT family members. DNMT3L is particularly important for driving DNA methylation in the germline. Global loss of DNMT3L leads to moderate to severe hypomethylation of repeat elements in ProSG.<sup>23</sup> This DNA hypomethylation is associated with reactivation of TEs in male germ cells, meiotic arrest, and male sterility.<sup>23,25,26</sup> Thus, DNMT3L is critical for TE defense in germ cells.

An opportunity for TEs to transpose at a high rate is when DNA methylation marks are globally erased in PGCs as a means to epigenetically reprogram the genome.<sup>27</sup> This hypomethylated state is maintained when PGCs first become ProSG.<sup>28</sup> While genome-wide DNA demethylation provides the benefit of reprogramming the genome for the next generation, it opens the door for TE activation and transposition. Thus, mechanisms have evolved to defend against TEs becoming active when PGCs and ProSG are in this hypomethylated state.

One means by which TEs are held in check is through histone modifications. Evidence suggests that PGCs and ProSG make use of several histone modifications to suppress TE expression and protect genomic integrity. For example, H2A (H4R3me2s) is a repressive histone mark catalyzed by the arginine methyltransferase, PRMT5, that silences *LINE1* and IAP TEs in PGCs.<sup>29</sup> Another histone modification that silences TEs, including *LINE1* elements, is the dimethylation of lysine (K) 9 in histone 3 (H3) (H3K9me2).<sup>30</sup> This histone modification is widely present in spermatogonia (SG) and early spermatocytes (preleptotene to zygotene) but largely disappears at the pachytene stage.<sup>31</sup>

Another major mechanism that protects against TEs in PGCs and ProSG is the Piwi-interacting (pi) RNA pathway. This pathway is mediated by short (24–31 nt) non-coding RNAs called “piRNAs” that associate with members of the PIWI RNA-binding Argonaute protein family to suppress transposons and thereby maintain germline genome integrity.<sup>32</sup> In mice, there are three PIWI family members—PIWIL1, PIWIL2, and PIWIL4 (also known as MIWI, MILI, and MIWI2, respectively)—the latter two of which are expressed in PGCs and/or ProSG. The *Piwil2* gene is first detectably expressed in gonadal tissue at E12.5 when PGCs transit to form M-ProSG,<sup>33</sup> whereas *Piwil4* expression is not detectable until E15.5 in T1-ProSG.<sup>34</sup> Both PIWIL2 and PIWIL4 are critical for the suppression of *LINE1* elements and other TEs in ProSG, based on analysis of *Piwil2*- and *Piwil4*- knockout (KO) mice.<sup>34,35</sup> Both KO strains suffer from male sterility.<sup>34,35</sup>

Mouse germ cells express two distinct populations of piRNAs: “pre-pachytene” and “pachytene.” The pre-pachytene piRNAs are present prenatally in ProSG and postnatally in SG, originate largely from TEs, and primarily target TEs.<sup>36</sup> In contrast, pachytene piRNAs are present in more advanced postnatal germ cells (pachytene spermatocytes and round spermatids); while some of this class are derived from repetitive TEs, they mainly originate from unique genomic clusters and are thought to mainly target coding mRNAs.<sup>37</sup>

PiRNAs suppress gene expression either transcriptionally or post-transcriptionally.<sup>38</sup> piRNAs mediate the former by inducing heterochromatin formation. piRNAs mediate the latter by cleaving RNAs complementary with their sequence. One means by which piRNAs repress transcription is by triggering DNA methylation. In ProSG, the PIWIL4-associated protein, SPOCD1, drives piRNA-guided TEs methylation and transcriptional silencing.<sup>39</sup>

## 2 | BI-FUNCTIONAL TRANSCRIPTIONAL REGULATORS

Below, we summarize the evidence that some transcriptional regulators are bi-functional factors: they function in germ cells, including in germ cell development, and were subsequently found to function in TE defense (Figure 1). These transcriptional regulators act at different stages of germ cell development (Figure 1). GLIS3, SETDB1, ZBTB16, RB1, and RHOX10 act in ProSG and/or undifferentiated SG, including SSCs. MYBL1 acts in postnatal germ cells at the pachytene stage.

### 2.1 | RHOX10 and the *RHOX* cluster

The hallmark of homeobox proteins is they contain a 60 amino-acid domain that binds to specific sequences in DNA or RNA.<sup>40</sup> The best-known homeobox protein subfamily is the

HOX subfamily. The genes encoding these TFs were originally discovered in flies, where they control key embryonic developmental events that correspond to their position in *HOX* gene clusters<sup>41</sup>. Subsequent studies showed that most metazoans contain *HOX* gene clusters and that these gene clusters encode TFs that drive key embryonic developmental events just as they do in flies.<sup>41</sup>

More recently, the reproductive homeobox (*RHOX*) subfamily was discovered.<sup>42</sup> The homeobox proteins in this subfamily are all encoded by a single gene cluster on the X chromosome.<sup>42–46</sup> Unlike the *HOX* gene cluster, the *RHOX* gene cluster is largely confined to mammals, suggesting it is a “mammalian invention” providing functions specifically important for mammals.

All known members of the mouse and human *RHOX* gene clusters are primarily expressed in cell types in the reproductive tract and placenta,<sup>47</sup> raising the possibility that the *RHOX* proteins are TFs devoted to reproductive functions. In support of the former, *RHOX* proteins have been shown to function as TFs that bind to specific sequences.<sup>48,49</sup> In support of the latter, several studies have shown that *RHOX* proteins regulate specific genes in Sertoli cells, germ cells, and epididymal cells.<sup>48–52</sup>

The biological functions of *RHOX* proteins have primarily been studied in mice. This has been challenging, as there are 33 genes in the mouse *Rhox* cluster.<sup>47</sup> One approach that has been taken is to mutationally inactivate individual *Rhox* genes. This approach, which has only been applied to a small number of *Rhox* genes, has revealed that some *Rhox* genes function in spermatogenesis,<sup>50,53,54</sup> while mutational inactivation of *Rhox9* has no discernable effects in mice in vivo.<sup>55</sup> Another approach to examining the function of *Rhox* genes has been to knock down their expression in vivo. This approach has successfully demonstrated that the Sertoli cell-expressed *Rhox8* gene functions in spermatogenesis,<sup>56</sup> but failed to define a function for the germ-cell expressed *Rhox3* paralogs because of experimentally validated off-target effects.<sup>57</sup>

As another approach to defining the biological functions of *Rhox* genes, Song et al. deleted the entire *Rhox* cluster in mice.<sup>53</sup> They found that global deletion of the *Rhox* cluster caused strain-specific embryonic lethality. In a mixed genetic background that permitted embryo survival, global *Rhox*-cluster KO mice were runted and had low viability, but those that survived were found to have progressive spermatogenic defects. Testes weight failed to increase with age; some spermatozoa were produced but the number of spermatozoa failed to increase as these adult *Rhox*-cluster KO mice aged. Most strikingly, these KO mice exhibited a progressive increase in seminiferous tubules devoid of (or mostly lacking) germ cells, a phenotype consistent with an SSC defect. As further evidence for an SSC defect, tubules that *did* contain germ cells tended to lack *early* germ cells. These defects were reproduced in mice conditionally lacking the *Rhox* cluster specifically in germ cells, demonstrating that these SSC-associated defects are cell autonomous.

While, in principal, any of the 33 homeobox genes in the *Rhox* cluster could be responsible for the SSC-associated defects in *Rhox*-cluster KO mice, Song et al. considered the best candidate to be *Rhox10*, as it was the only *Rhox* gene known to be highly expressed in

undifferentiated SG<sup>58</sup>. To test its role, Song et al. mutationally inactivated *Rhox10* and found that this caused the same progressive spermatogenic decline phenotype as *Rhox*-cluster KO mice.<sup>53</sup>

To determine the exact nature of the defect, a battery of assays was used. Analysis of SSC markers, including *GFRα1*, Id4-eGFP, and nuclear FOXO1, demonstrated that *Rhox10*-null mice had reduced SSCs in newborn mice, indicative of an SSC establishment defect. This was confirmed by germ-cell transplantation analysis, which showed that postnatal day (P) 7–8 *Rhox10*-null mice had a striking decrease in transplantable colonies. Such an SSC defect could be caused by reduced germ-cell proliferation, reduced germ-cell survival, or impaired germ-cell differentiation. Analysis with proliferation and apoptosis markers indicated that loss of RHOX10 did not have a measurable impact on either the proliferation or apoptosis of newborn germ cells, suggesting instead that RHOX10 is critical for germ cell progression. To directly test this, single-cell RNA-sequencing (scRNAseq) analysis was employed. scRNAseq analysis of FACS-purified Id4-eGFP+ cells at different time points revealed an accumulation of a cell cluster with the molecular characteristics of T1-ProSG in *Rhox10*-null mice at a time point (P3) when T1-ProSG have already progressed to form later stages of germ cells in wild-type (wt) mice.<sup>53</sup> This abnormal accumulation of T1-ProSG in *Rhox10*-null mice suggests that RHOX10 serves to drive T1-ProSG to form T2-ProSG. Given that T2-ProSG are regarded as the direct precursors of SSCs,<sup>3</sup> the T1-to-T2 ProSG progression defect in *Rhox10*-null mice explains the low frequency of SSCs in newborn *Rhox10*-null mice.

The notion that RHOX10 drives ProSG differentiation was further supported by Tan et al.<sup>49</sup> Using newly identified ProSG markers—DNMT3L and ETV4<sup>59</sup>—they confirmed that *Rhox10*-null mice accumulate ProSG at P2 and P3 in vivo, a phenotype recapitulated in vitro. Further, using a battery of genome-wide approaches, Tan et al. also identified high-confidence RHOX10 target genes that are candidates to drive ProSG differentiation. Through “rescue” experiments, the authors demonstrated that at least two of these genes act downstream of RHOX10 to promote ProSG differentiation.<sup>49</sup>

A new wrinkle to this story was provided by Tan et al., who found that RHOX10 suppresses the transposition of *LINE1* elements, suggesting this homeobox gene functions to silence parasitic DNA in the germline.<sup>60</sup> Using an *SN1* single-copy *LINE1* transgene reporter mouse line,<sup>61</sup> Tan et al. found that *SN1 LINE1* copy number was significantly higher in the testes in *Rhox10*-null mice as compared to littermate wt mice,<sup>60</sup> indicative of increased *LINE1* transposition as a result of *Rhox10* loss. This effect was specific to the testes, not the other adult tissues tested. Mice lacking the entire *Rhox*-cluster specifically in germ cells also displayed a testes-specific increase in *LINE1* copy number. Time-course analysis showed that the increased *SN1 LINE1* copy number occurs between E13.5 and E16.5, and was sustained at P0 and adult stages. This indicated that RHOX10 suppresses *LINE1* transposition in ProSG. Bisulfite analysis of the *LINE1* transgene promoter in testes from E16.5 *Rhox10*-null mice revealed that this promoter exhibited significantly decreased methylation upon *Rhox10* loss. This suggested that one mechanism by which RHOX10 suppresses *LINE1* transposition in ProSG is by promoting *LINE1* promoter methylation at this stage of germ-cell development.

To identify genes involved in RHOX10-mediated *LINE1* suppression, Tan et al. performed RNA sequencing (RNAseq) and ChIP-qPCR analysis of *Rhox10*-null ProSG in vivo and in vitro, respectively, and found that RHOX10 directly drives the expression of *Piwil2*, which encodes a key component in the piRNA pathway that protects against TEs.<sup>60</sup> Through rescue experiments, they found that RHOX10 inhibits *LINE1* transposition through the activation of *Piwil2* expression. Thus, the authors identified a *Rhox10-Piwil2-LINE1* circuit that serves to provide TE defense in the fetal germline.

In addition to RHOX10, Tan et al. identified several other mouse RHOX family members that also have the ability to suppress *LINE1* expression, albeit in vitro.<sup>60</sup> Interestingly, many of these other *LINE1*-suppressing RHOX family members are expressed in different germ-cell developmental stages, for example, the *Rhox3* paralogs and *Rhox11* are expressed in round spermatids. This raises the possibility that the *RHOX* gene cluster expanded over evolutionary time as a means to defend the genome against *LINE1* elements at different stages of germ cell development. Other *Rhox* family members are expressed in somatic cells in the reproductive tract, including granulosa cells and Sertoli cells.<sup>56,62</sup> Given that *LINE1* elements actively transpose in many somatic cells,<sup>63,64</sup> this raises the possibility that RHOX TFs expanded over evolutionary time to be expressed in somatic cells in the reproductive tract to defend against TEs.

Tan et al. also found that *LINE1* transposition is suppressed by human *RHOX* family members, indicating that *LINE1* suppression is a conserved function of the RHOX protein family. Intriguingly, the ability of human RHOXF2 to suppress *LINE1* transposition is lost in mutant forms of RHOXF2 present in infertile human patients. This raises the intriguing possibility that loss of RHOXF2 causes human infertility, in part, because it allows uncontrolled *LINE1* transposition in the male germline.

In summary, RHOX10 is a multi-functional homeobox TF that has at least two functions. First, RHOX10 drives the differentiation of ProSG and consequent SSC formation through a molecular mechanism involving at least two downstream genes. Second, RHOX10 defends ProSG against parasitic *LINE1* elements in ProSG, and thereby acts as a guardian of the genome. The ability to suppress TE transposition extends to several other RHOX family members, raising the intriguing possibility that the primary impetus for the initial formation and subsequent evolution of this homeobox TF family was to suppress the deleterious effects of TEs.

## 2.2 | ZBTB16

ZBTB16 (also known as PLZF) is a member of the POZ and Kruppel zinc finger (POK) protein family. All members of this TF family contain both a Kruppel-like zinc finger and a BTB/POZ domain. The former binds to specific DNA sequences as well as specific proteins, and the latter is an evolutionarily conserved protein-protein-interaction domain that promotes the dimerization of POK family members.

Two studies demonstrated that ZBTB16 has an essential role in SSCs.<sup>65,66</sup> Costoya et al. showed that *Zbtb16*-null mice undergo a progressive loss of SG with age, increased germ cell apoptosis, and the subsequent appearance of “Sertoli cell-only syndrome”

seminiferous tubules.<sup>65</sup> The appearance of seminiferous tubules lacking all germ cells raised the possibility that *Zbtb16*-null mice have an SSC defect. This was confirmed by germ-cell transplantation analysis, which showed that germ cells from these mutant mice are unable to repopulate germ cell-depleted recipient testis.<sup>65</sup> Buaas et al. came to the same conclusion, based on their analysis of a naturally occurring mouse mutant—*luxoid* (*lu*)—that lacks the *Zbtb16* gene. These authors obtained several lines of evidence that adult homozygous *lu* mutant mice have a SSC defect: (i) they largely lack primitive SG in their seminiferous tubules, (ii) some tubules completely lack germ cells, and (iii) germ-cell transplantation analysis showed that *lu* homozygous mutant germ cells were not able to successfully colonize recipient testes.<sup>66</sup> Together, these two studies provide strong evidence that ZBTB16 is essential for SSC maintenance in vivo.

Subsequently, Puszyk et al. discovered that ZBTB16 has a second role in male germ cells – it promotes the silencing of *LINE1* retrotransposons.<sup>67</sup> These investigators generated a loss-of-function *Zbtb16* mutant—which they called “PLZF<sup>OFF</sup>”—that encodes a form of ZBTB16 that lacks DNA-binding activity. They found that these PLZF<sup>OFF</sup> mice recapitulate both the testicular phenotype of *Zbtb16*-null mice<sup>65</sup> and the biallelic loss phenotype (PLZF<sup>-/-</sup>) in humans.<sup>68</sup> Analysis of testes and bone marrow from these PLZF<sup>OFF</sup> mice revealed that many *LINE1* elements were upregulated. This was accompanied by hypomethylation of *LINE1* promoters, suggesting that ZBTB16 suppresses *LINE1* elements by promoting their methylation. MeDIPseq analysis demonstrated that the hypomethylation of *TE* elements triggered as a result of loss of ZBTB16 was pervasive in both testes and bone marrow. Not only did this methylation de-regulation impact *LINE1* elements, but also other interspersed repetitive elements, including *SINEs*. However, it was not determined whether interspersed repetitive elements besides *LINE1* elements were elevated in expression in *Zbtb16*-null mice. Interestingly, coupled hypomethylation and transcriptional upregulation were not restricted to repetitive elements, as well-established developmental and cell-cycle regulators (such as *c-Kit*, *Crabp1*, and *Myc*) also underwent this kind of regulation in PLZF<sup>OFF</sup> mice. This dysregulation may have a causal role in the SSC defects caused by ZBTB16 loss.

Given that ZBTB16 is a TF, one might expect that it would repress *LINE1* transcription by binding to *LINE1* promoters. However, ChIPseq analysis by Puszyk et al. demonstrated that rather than occupying *LINE1* promoters, ZBTB16 occupies a subregion of the *LINE1* open reading frame (ORF) 2.<sup>67</sup> This occupancy was associated with increased DNA methylation and decreased histone H3 acetylation level at the 5' UTR regulatory region of *LINE1* elements. This suggests (but does not prove) that ZBTB16 occupancy of the *LINE1* coding region serves to suppress *LINE1* expression by a mechanism that somehow methylates *LINE1* promoter elements. As evidence of functionality, the PLZF-binding sites in the *LINE1* ORF2 region were found to be conserved in mice and humans.<sup>67</sup> In the future, it will be intriguing to dissect how the binding of ZBTB16 in *LINE1* coding regions drives downstream events in *LINE1* promoters to ultimately suppress *LINE1* transcription.

Cellular stress has previously been implicated in unleashing TEs to undergo transposition.<sup>69</sup> This led Puszyk et al. to wonder if ZBTB16 could have a role in stress-induced *LINE1* activation. In support of this hypothesis, a previous study showed that cellular stress (in the form of the cytokine IL-3) elicits the re-localization of ZBTB16 from the nucleus to



the cytoplasm of human CD34<sup>+</sup> CD71<sup>-</sup> myeloid progenitors.<sup>70</sup> Puszyk et al. replicated this finding in the human hematopoietic cell line, KG1a, exposed to heat stress. Given that ZBTB16 can only directly regulate transcription in the nucleus, such a nuclear-to-cytoplasm localization shift would be predicted to unleash *LINE1* elements and allow their transposition, which Puszyk et al. found to be the case. This was further validated using a *LINE1* reporter in HEK293T cells.<sup>67</sup> Together, these data support a model in which nuclear ZBTB16 is recruited to *LINE1* elements to promote their methylation, transcriptional repression, and impaired transposition.

In summary, ZBTB16 is an intriguing multi-functional TF that has critical roles in SSC maintenance and TE defense. In the future, it will be critical to dissect its exact role(s) in SSC maintenance (e.g., SSC self-renewal vs. inhibition of SSC differentiation). It will also be important to determine whether it drives related processes, such as the initial formation of SSCs perinatally. In support, ZBTB16 was recently shown to act downstream of RHOX10 to drive ProSG differentiation and SSC establishment in vitro.<sup>49</sup> With regard to ZBTB16's *LINE1* defense role, it will be interesting to determine what stages of germ cell development are impacted and whether its *LINE1* defense role is conserved in humans. Finally, it will be important to determine the molecular mechanisms employed by ZBTB16 to suppress *LINE1* elements. In the Perspective, below, we discuss the possibility that ZBTB16 acts downstream of RHOX10 to drive *LINE1* defense.

### 2.3 | RB1

RB1 (also known as simply “RB”) is a transcriptional co-receptor that serves to inhibit the cell proliferation of a wide array of cell types.<sup>71</sup> It performs this inhibitory role through its interaction with members of the E2F family of transcription factors.<sup>72</sup>

To determine whether RB1 has a role in the proliferative block that ProSG undergoes when they transition between the M- and T1-ProSG stage (see Introduction), Spiller et al. made use of global *Rb1*-null mice.<sup>73</sup> Given that these mice suffer from embryonic lethality at ~E14.5, these authors addressed the role of RB1 using *ex vivo* organ cultures of testicular cells from *Rb1*-null mouse fetuses.<sup>73</sup> They elected to generate these organ cultures from E14.5 embryos, as this is the time point when proliferative quiescence normally initiates in ProSG; that is, the M-ProG/T1-ProSG transition. Using the proliferation markers MKI67 and pHH3, as well as bromodeoxyuridine incorporation, they observed that wt E14.5 ProSG exhibited little proliferation in these organ cultures, as expected. In contrast, E14.5 *Rb1*-null ProSG were actively proliferating, indicative of loss of the G1/G0 arrest that normally occurs in T1-ProSG. In an attempt to simulate ProSG reaching the fully quiescent state (which normally occurs at ~E16.5 in vivo), the authors also cultured these organoids for 2 days. They found that this two-day culture caused the proliferative quiescence phenotype of the mutant ProSG to be lost. One interpretation of this finding is that RB1 only functions to promote the initial period of mitotic arrest in T1-ProSG. Another possibility is that this in vitro study failed to fully capture RB1's functions in vivo.

To determine the role of RB1 in germ cells in vivo, a subsequent study—Du et al.—conditionally knocked out *Rb1* at the stage proceeding to the ProSG stage – in PGCs (using *Blimp1*-Cre mice). They found that these *Rb1*-cKO<sup>*Blimp1*</sup> mice have normal germ

cell number and germ cell proliferation at the M-ProSG stage (at E13.5).<sup>74</sup> However, at the beginning of the T1-ProSG stage (E14.5), when proliferation normally begins to taper, only 14% of *Rb1*-cKO<sup>*Blimp1*</sup> ProSG were non-proliferative (as compared to 41% of wt control germ cells, based on the proliferation marker MKI67). The proliferative quiescence defect was even more striking in T1-ProSG at E16.5: only 35% of *Rb1*-cKO<sup>*Blimp1*</sup> ProSG were MKI67<sup>-</sup> as compared with 98% MKI67<sup>-</sup> ProSG in control mice. Du et al. also found that a 6-fold higher proportion of *Rb1*-cKO<sup>*Blimp1*</sup> ProSG undergoes apoptosis than control ProSG (at E16.5, but not at E14.5). Thus, RB1 not only promotes mitotic arrest of ProSG but also promotes their survival.

ProSG gives rise to two types of SG in rodents. One type is SSCs, the testicular stem cells that permit long-term spermatogenesis. The other type is differentiating SG which gives rise to the first wave of spermatogenesis. To investigate the role of RB1 in this bifurcation of the spermatogenic pathway, Yang et al. conditionally knocked out *Rb1* in mouse ProSG (by mating *Ddx4*-Cre mice with *Rb1*-floxed mice).<sup>75</sup> Yang et al. found these cKO mice are able to undergo the first wave of spermatogenesis, but not subsequent SSC-dependent spermatogenesis, as indicated by a progressive germ cell-loss phenotype.

It remains to be determined why RB1 is required for SSC-dependent spermatogenesis. One possibility is ProSG must undergo a proliferative quiescence “priming stage” before they can form SSCs. Another possibility is that RB1 drives SSC-dependent spermatogenesis by a mechanism independent of its mitotic arrest-promoting role. For example, it might regulate the transcription of specific genes (through its known role as a transcriptional co-receptor) in ProSG to permit SSC generation, or alternatively, in SSCs, to promote SSC maintenance. Thus, a fundamental question is: does RB1 *only* function in ProSG or does it *also* have functions after the ProSG stage?

To directly investigate whether RB1 functions *after* the ProSG stage, Yang et al.<sup>75</sup> conditionally knocked out *Rb1* at the spermatogonial progenitor stage (by mating with *Neurog3*- and *Stra8*-Cre mice). They found that *Rb1*-cKO<sup>*Neurog3*</sup> and *Rb1*-cKO<sup>*Stra8*</sup> male mice are fertile but have modest but significantly reduced testes weight (by 9% and 21% compared to controls, respectively). The seminiferous tubules in these mutant mice had heterogeneous morphology; some with disrupted spermatogenesis, others with normal spermatogenesis. Evidence suggests that RB1 functions in SSCs. For example, Yang et al. found that RB1 knockdown in undifferentiated SG cell cultures reduced SSC frequency, as assessed by the germ-cell transplantation analysis. In further support of a role in SSCs, Hu et al.<sup>76</sup> conditionally knocked out *Rb1* in ProSG (using *Ddx4*-Cre mice) and found that these *Rb1*-cKO male mice become sterile by ~2 months of age. Histological analysis showed that these *Rb1*-cKO mice displayed a striking depletion of early spermatogenic cell types by P28, consistent with an SSC defect. This interpretation was verified by analysis of *Rb1*-cKO mice between P5 and P28. GFR $\alpha$ 1<sup>+</sup> A<sub>single</sub> SG was present at a normal density at P5 but thereafter declined dramatically in *Rb1*-cKO testes. These authors found the loss of GFR $\alpha$ 1<sup>+</sup> A<sub>single</sub> SG in *Rb1*-cKO testes is not due to their inhibited proliferation or increased apoptosis. Instead, these cells lacked the capacity for self-renewal, based on BrdU-tracing analysis of the mitotic progeny of *Rb1*-mutant A<sub>single</sub> SG.

In addition to its developmental roles in germ cells, Montoya et al. obtained evidence that RB1 functions in TE defense. First, Montoya et al. found that molecular complexes composed of RB1 and E2F transcription factors interact with *LINE1* elements.<sup>77</sup> Second, Montoya et al. found that triple-KO fibroblasts harboring mutations in *Rb1* and its orthologs, *p107* and *p130*, have reduced heterochromatic marks (e.g., H3K9me3) at *LINE1* elements, accompanied by increased *LINE1* expression.<sup>77</sup> Third, RB1 is known to cooperatively regulate several genes in conjunction with ZBTB16,<sup>78</sup> which, as described in the “ZBTB16” section above, is known to transcriptionally silence *LINE1* elements and inhibit their transposition in germ cells.<sup>67</sup> This raises the intriguing possibility that RB1 and ZBTB16 cooperate to defend against *LINE1* elements, but this remains untested (see Perspective). Also, untested is whether RB1 provides transposon defense in germ cells. At present, the only evidence that RB1 functions in transposon defense come from experiments in somatic cells.<sup>77</sup>

In summary, RB1 is a transcriptional co-receptor that drives one of the hallmark features of ProSG—their mitotic silencing—and is also critical for long-term spermatogenesis. RB1 is also essential at a later stage of germ-cell development—in undifferentiated SG—where it functions to allow for normal spermatogenesis, but its precise role in undifferentiated SG is not yet clear. There is tantalizing evidence that RB1 is a bi-functional factor that not only has developmental roles but also functions in *LINE1* defense, but whether it performs this role in germ cells remains to be tested.

## 2.4 | GLIS3

GLIS Family Zinc Finger 3 (GLIS3) is a nuclear protein harboring 5 C2H2-type zinc finger domains that serve as both a transcriptional activator and repressor.<sup>79,80</sup> In the male reproductive tract, GLIS3 is transiently expressed in PGCs and then re-expressed in ProSG. GLIS3 expression persists until the undifferentiated SG stage and then is downregulated before the differentiating SG stage postnatally.<sup>81,82</sup> Two studies have examined the function of GLIS3 in germ cells.

Kang et al. conducted their studies on *Glis3*-hypomorphic male mice.<sup>81</sup> They observed that these mutant mice have reduced numbers of germ cells (TRA98<sup>+</sup> cells) relative to control wt mice at P1; a defect that persists at P4 and then becomes more severe by P7. While an obvious possible mechanism for this reduction in germ cell number would be either increased germ cell apoptosis or impaired germ cell proliferation, Kang et al. found no evidence for either of these mechanisms, implying instead that germ cells in *Glis3*-hypomorphic mice have a germ-cell progression defect. In support, P7 *Glis3*-hypomorphic mice were found to have an excess of germ cells expressing cytoplasmic FOXO1, a ProSG marker,<sup>83</sup> and fewer germ cells expressing nuclear FOXO1, an SSC marker.<sup>83</sup> This raised the possibility that *Glis3*-hypomorphic mice have an SSC establishment defect, which was supported by the finding that *Glis3*-hypomorphic mice exhibit low expression of many undifferentiated SG-enriched genes at P7. This defect persists even at P28, indicative of a developmental block, not a developmental delay. Together, these data suggest that GLIS3 promotes the ProSG-to-SSC conversion step, but more work needs to be done to confirm this.

A more recent study—Ungewitter et al.—examined a strain of *Glis3*-mutant mice with a null mutation in *Glis3*<sup>82</sup>. Their analysis revealed that these *Glis3*-null mice have a ~40% reduction in ProSG at E15.5, implying that GLIS3 not only functions in late-stage (T2) ProSG (above) but also in early-stage (T1) ProSG. These authors did not observe increased ProSG apoptosis in *Glis3*-null mice, suggesting that the ProSG defect in these KO mice is instead due to decreased ProSG proliferation or impaired ProSG progression. In support of the latter, they found that critical differentiation genes, including *Nanos2*, *Nanos3*, *Stra8*, and *Rec8*, are dis-regulated in E14.5 *Glis3*-null mice testes. Furthermore, GLIS3 has been implicated in the differentiation of other cell lineages.<sup>84,85</sup>

Intriguingly, Ungewitter et al. also found that *Glis3*-null mice exhibit upregulation of a large battery of TE defense genes. RNAseq analysis revealed that all three major retrotransposon classes—*LINE*, *SINE*, and LTR (*ERV*)—exhibited upregulated expression in *Glis3*-null E14.5 testes. This suggests that GLIS3 is involved in broad TE silencing in ProSG. With regard to mechanism, these authors found that several genes involved in piRNA biosynthesis, catabolism, and metabolism are downregulated in *Glis3*-null mice, raising the possibility that GLIS3 suppresses TEs by positively regulating the piRNA pathway. Downregulated piRNA pathway genes include *Ddx4*, *Piwi1*, *Piwi2*, *Piwi4*, *Mael*, *Tdrd1*, *Tdrd5*, and *Tdrd9*. Other downregulated genes include *Dnmt3l* and *Morc1*, the former of which promote TE methylation and subsequent transcriptional repression; and the latter of which encodes a retrotransposon silencer.<sup>86</sup> As additional evidence that GLIS3 serves as a TE defense factor, the authors found that increasing GLIS3 expression at E12.5 (using a Rosa26-driven *Glis3* mouse line crossed with Dppa3-creER) increases the expression of several TE defense genes, including *Dnm3tl*, *Piwi1*, and *Piwi4*. Together, these data support the possibility that GLIS3 is a key TF that suppresses TEs by broadly transcriptionally activates several TE defense pathways in ProSG and perhaps later stages.

In summary, GLIS3 appears to be a multi-functional TF involved in both germ cell development and TE defense. GLIS3 is essential for normal T1-ProSG accumulation. Some evidence suggests it promotes the T2-ProSG-to-SSC conversion step. Its loss causes widespread activation of TEs in ProSG, accompanied by repressed piRNA pathway functions, strongly suggesting that GLIS3 serves to defend the male germline against TEs.

## 2.5 | SETDB1

SETDB1 (also known as KMT1E or ESET) is a histone lysine N-methyltransferase responsible for the di- and tri-methylation of H3K9. Consistent with the well-established role of methylated H3K9 in transcriptional repression, SETDB1 has been found to promote gene silencing through heterochromatin formation in a wide variety of cell lineages.<sup>87</sup>

Given that SETDB1 catalyzes the formation of a key repressive histone mark, Mochizuki et al. hypothesized that this methyltransferase has functions in the early embryo.<sup>88</sup> To test this hypothesis, these investigators conditionally knocked out *Setdb1* in the epiblast (by breeding *Setdb1*-floxed mice with *Sox2-Cre* mice). They found that these conditional (c) KO mice exhibited a drastic reduction in nascent PGCs, indicating that SETDB1 is critical for PGC formation.

To test whether SETDB1 has roles in germ cells *after* the PGC formation stage, Liu et al. conditionally knocked out *Setdb1* in migrating PGCs (by breeding *Setdb1*-floxed mice with *Tnap*-Cre mice, which express CRE as early as E9.5).<sup>89</sup> They found that these cKO mice had reduced M-ProSG number (at E13.5), reduced postnatal gonad size (at P10), and reduced testis size (examined at 5 weeks and in adults) compared to wt littermate mice. Histological analysis of P10 and P35 testes showed that many seminiferous tubules in *Setdb1*-cKO mice are devoid of germ cells. While it is uncertain why not all seminiferous tubules in these cKO mice have this phenotype, it is likely to be due to incomplete CRE-mediated recombination, as the authors did not observe transmission of the deleted allele in offspring. They found that neither proliferation nor apoptosis genes were broadly dysregulated, suggesting that SETDB1 does not act in ProSG by driving their proliferation or survival. This implied that, instead, SETDB1 acts by promoting ProSG progression, but this requires further experimentation verification.

By what molecular mechanism does SETDB1 act in ProSG? Past studies have shown that SETDB1 catalyzes the formation of the repressive histone marks, H3K9me2 and H3K9me3, in other cell lineages.<sup>90</sup> Thus, a likely mechanism is SETDB1 does the same in the germline lineage. In support, Mochizuki et al. and Liu et al. found that cKO mice conditionally lacking functional *Setdb1* in developing PGCs and ProSG, respectively, have decreased H3K9me3 occupancy and an overall increase in gene expression.<sup>89,88</sup> SETDB1 was found to also increase the level of another repressive histone mark—H3K37me3—in the germline,<sup>89</sup> probably through an indirect mechanism, as it is known that SETDB1 modulates PRC2, a regulator of H3K27me3 level.<sup>91</sup>

Mochizuki et al. pinpointed three SETDB1-regulated genes as key regulators of PGC formation.<sup>88</sup> These three genes—*Dppa2*, *Otx2*, and *Utf1*—were found to be involved in the BMP signaling events required to drive PGC formation. Through gain- and loss-of-function experiments in cell aggregates containing PGC-like cells, Mochizuki et al. found that SETDB1 negatively regulates and occupies the flanking regions of these three genes. They then showed that these three genes serve to negatively regulate BMP signaling. Together, the data support a model in which SETDB1 promotes PGC formation by repressing the transcription of three BMP signaling regulators, which leads to positive BMP signaling, a key event in PGC generation.

Not only does SETDB1 serve as a developmental regulator of PGCs, but it may regulate self-renewal vs. differentiation decisions in undifferentiated SG. An et al. found that the knockdown of SETDB1 in undifferentiated SG cultures reduces the frequency of SSCs in these cultures, as determined by germ-cell transplantation analysis.<sup>92</sup> This strongly suggests that SETDB1 promotes SSC maintenance. While the molecular mechanism for this function is not known, a tantalizing possibility is that SETDB1 silences SG differentiation genes to allow for SSC self-renewal.

Together, the data from Mochizuki et al.,<sup>88</sup> Liu et al.,<sup>89</sup> and An et al.<sup>92</sup> support the possibility that SETDB1 has many developmental roles in germ cells, including promoting the initial formation of PGCs, their progression in the embryo, as well as SSC maintenance after birth.

Intriguingly, Liu et al. found that SETDB1 not only has developmental roles, but it silences retrotransposons<sup>89</sup>. These authors found that ablation of *Setdb1* decreases H3K9me3 occupancy at widespread *ERV* loci (including the *ERVK* and *ERV1* subfamilies), accompanied by upregulation of their expression in ProSG. The repressive histone mark, H3K27me3, was also present at increased levels at *ERV* loci, presumably by an indirect mechanism (see above). *LINE1* elements also had decreased H3K9me3 and H3K27me3 occupancy, but most *LINE1* elements exhibited little or no increased expression. A possible explanation for the lack of *LINE1* activation is epigenetic compensation. In support, most *LINE1* elements in *Setdb1*-cKO mice were found to exhibit *increased* DNA methylation. This hypermethylation response was widespread, as *Setdb1*-cKO ProSG exhibited a ~2.5-fold increase in DNA methylation globally. In contrast, *ERV* family members typically avoided this putative counter-regulatory mechanism. Indeed, Liu et al. found that the *ERV* family members exhibiting the highest level of H3K9me3 in wt mice and strongest transcriptional induction in *Setdb1*-cKO mice (e.g., *IAPLTR1* and *IAPLTR1a*), exhibited *decreased* DNA methylation.

In summary, SETDB1 is a chromatin regulator involved in both germ cell development and TE defense. Evidence suggests it has roles in PGC formation, ProSG progression, and SSC maintenance. In ProSG, SETDB1 serves to defend the genome specifically against *ERV* TEs.

## 2.6 | MYBL1

MYBL1 (also called A-MYB) is a TF that binds to DNA through a helix-loop-helix domain. This TF is part of a small protein family that includes two other family members - MYB (also called C-MYB) and MYBL2 (also called B-MYB).

MYBL1 is unique among these three family members in being highly expressed in male germ cells, raising the possibility it has roles in spermatogenesis. Indeed, Toscani et al. showed that *Mybl1*-null mice have meiotic arrest at the early pachytene stage, and generate no spermatozoa.<sup>93</sup> The morphology of the seminiferous tubules in *Mybl1*-null mice is abnormal, including vacuolization of the Sertoli cell cytoplasm. While SG and pre-leptotene spermatocytes are normal, most pachytene primary spermatocytes exhibit degeneration, thereby pinpointing the defect between the pre-leptotene to the pachytene stage. This was confirmed by the lack of detectable expression of the pachytene markers PGK-2 and HSP70-2, both of which begin expression at the leptotene stage. Post-meiotic germ-cell stages (i.e., spermatids and spermatozoa) were completely absent, indicative of a complete block.

As another approach to discern the function of MYBL1, Bolcun-Filas et al. examined mice harboring a missense mutation in *Mybl1* (derived from a large-scale mutagenesis genetic screen to identify infertile mice). These *Mybl1*<sup>repro9</sup> mutant mice were found to have subtle defects in autosomal synapsis at the pachytene stage, a high incidence of un-synapsed sex chromosomes, incomplete double-strand break repair on synapsed pachytene chromosomes, and little DNA crossing over.<sup>94</sup> As further evidence of a pachytene defect, microarray analysis of whole testes from P14 and P17 *Mybl1*<sup>repro9</sup> mutant and wt mice revealed that meiotic genes were preferentially misregulated in KO testes. Using CHIP-chip analysis,

putative direct targets of MYBL1 in pachytene spermatocytes were identified, including genes encoding proteins known to be involved in the processes found to be a defect in these *Mybl1<sup>repro9</sup>* mutant mice, including double-stranded DNA repair, synapsis, crossing over, and pachytene cell-cycle progression. These genes encode proteins that are candidates to act downstream of MYBL1 in meiosis.

Together, the results from Bolcun-Filas et al.<sup>94</sup> and Toscani et al.<sup>93</sup> suggested that MYBL1 is a master transcriptional regulator that controls several different events necessary for the progression of germ cells through the pachytene stage of meiosis.

A subsequent study—Li et al.—found that MYBL1 is critical for another function during meiosis - pachytene piRNA production.<sup>95</sup> This study found that MYBL1 drives the production of two classes of RNAs critical for piRNA biosynthesis: (i) piRNA precursors and (ii) mRNAs encoding core piRNA biogenesis factors, including PIWIL1. MYBL1 was found to also promote the expression of several other RNA-silencing-pathway genes, including *Tdrd1*, *Tdrd5*, *Tdrd6*, *Tdrd12*, *Pld6*, and *Mael*. MYBL1 is likely to directly regulate all these genes, as it was found to occupy their promoter regions.

The finding that MYBL1 promotes the transcription of *both* piRNA genes and genes encoding piRNA biogenesis proteins led Li et al. to propose that MYBL1 operates in a coherent feedforward loop that ensures robust accumulation of pachytene piRNAs.<sup>95</sup> Such feedforward loops are known to amplify initiating signals to increase target gene expression. They also serve to act as switches that respond to sustained signals but ignore transient signals. The notion that MYBL1 is regulated by a feedforward loop is also supported by the finding that MYBL1 positively autoregulates its own expression.<sup>95</sup>

Recently, another study—Maezawa et al.<sup>96</sup>—obtained evidence that MYBL1 drives piRNA biogenesis through the action of super-enhancers (SEs) – enhancer clusters that drive unusually high levels of transcription<sup>97</sup>. Maezawa et al. defined a SE as any extensive chromatin region enriched for acetylated [ac] H3K27, which is generally regarded as an enhancer mark. Among the SEs this study identified were “meiotic SEs,” based on their being present in pachytene spermatocytes, not earlier or later stages. The TF most enriched within H3K27ac peaks at meiotic SEs was MYBL1. As evidence that MYBL1 is functionally important at these meiotic SEs, *Mybl1*-null pachytene spermatocyte chromosome spreads had dramatically reduced H3K27ac signal as compared to wt pachytene spermatocyte chromosome spreads. Intriguingly, many of the 518 MYBL1-bound meiotic SEs overlapped with pachytene piRNA gene clusters and piRNA biogenesis genes, suggesting that the expression of these piRNA-related genes is driven by SEs through the action of MYBL1. Together, the data from Maezawa et al.<sup>96</sup> suggests that MYBL1 drives the formation of meiotic SEs that, in turn, drive the high transcription of pachytene piRNA pre-mRNA genes and piRNA biogenesis factor genes. It remains to be determined what proportion of piRNAs depend on this MYBL1/SE-dependent mechanism for their expression during meiosis.

In summary, MYBL1 appears to be a master TF critical for several events during the pachytene stage of meiosis, including (i) sex-chromosome synapsis, (ii) double-strand

DNA break repair on synapsed pachytene chromosomes, (iii) DNA crossing over, (iv) transcriptional induction of piRNA precursor genes during meiosis, and (v) transcriptional induction of genes encoding key components of the pachytene piRNA pathway as well as other RNA-silencing pathways. Given that a proportion of pachytene piRNAs are derived from TEs,<sup>98,99</sup> the latter two functions raise the possibility that MYBL1 provides defense against TEs during the process of meiosis. However, there is, as of yet, no direct evidence for this.

### 3 | PERSPECTIVE

In this review, we make the case that some transcriptional regulators have the unique ability to both regulate developmental events in germ cells and protect these cells from deleterious TEs. Below are some questions about these “bi-functional” factors to consider for the future.

#### **First, might some of the putative bi-functional factors described in this review work together?**

One likely example of this is the TFs RHOX10 and ZBTB16, as RHOX10 was recently shown-through rescue experiments-to act upstream of ZBTB16 to drive ProSG differentiation and SSC establishment in vitro.<sup>49</sup> While it remains to be determined whether this molecular circuit operates in vivo, it is known that ZBTB16 is expressed in ProSG in vivo<sup>65</sup> and RHOX10 drives ProSG differentiation and the initial formation of SSCs in vivo.<sup>49,53</sup> It will also be intriguing to determine whether ZBTB16 also acts downstream of RHOX10 to provide *LINE1* defense. A third factor that may also be involved is the mitotic silencing factor RB1. Evidence suggests that RB1 silences *LINE1* elements,<sup>77</sup> and RB1 is known to cooperatively regulate several genes in conjunction with ZBTB16.<sup>78</sup> Thus, it is conceivable that RB1, ZBTB16, and RHOX10 all function together in a molecular circuit that confers defense against TEs.

#### **Second, did the bi-functional factors described in this review originally have only one activity and then acquired a second activity over time?**

For example, were they originally developmental regulators and then acquired a TE defense role later? Or were these factors originally TE defense factors that later acquired other roles, such as the ability to influence germ cell development and differentiation? Functional analysis of these factors in different species has the potential to provide an answer to this question.

#### **Third, do the developmental defects in organisms lacking these factors result from TE-induced defects?**

We think this is unlikely to be true for RHOX10 for at least two reasons. First, the molecular mechanisms by which RHOX10 acts in development and TE defense have been partially defined, and they are different. RHOX10 drives ProSG diff through the TFs DMRT1 and ZBTB16,<sup>49</sup> while it suppresses *LINE1* transposition through the RNA-silencing factor PIWIL2.<sup>60</sup> Second, RHOX10 acts on these two events at different developmental time points: its *LINE1* defense role is exerted at fetal stages, while its ProSG differentiation role is exerted perinatally.<sup>49,60</sup> In the case of the other factors we have discussed, it is possible,



in some cases, that loss of their TE defense role leads to the defects observed. For example, the reduction in ProSG number in *Glis3*-null mice and *Setdb1*-null mice could be due to the activation of *LINE1* elements or other TEs in these mutant mice. In support, it is known that elevated expression of *LINE1* ORFs can cause deleterious endonuclease and reverse transcriptase activities.<sup>100–102</sup> In particular, overly active *LINE1*s are known to cause DNA damage accumulation, checkpoint activation, and cell death.<sup>101,102</sup>

**Fourth, might some of these factors regulate developmental events by acting on repetitive elements that have been integrated into developmental genes over evolutionary time?**

This possibility is supported by the finding that one-quarter of expressed genes contain a *LINE1* retrotransposon in their 3'UTR or in their introns.<sup>103</sup> Thus, perhaps some of the factors we have described in this review regulate developmental genes by binding to former TEs that have been incorporated into the regulatory circuitry of these developmental genes. If this was the case, such factors would regulate development and promote TE defense by a common mechanism – by binding to repetitive elements.

**Fifth, what is the breadth of TE defense provided by the bi-functional factors described in this review?**

Might the factors we described here function in cell stages or types beyond those demonstrated in the studies to date? For example, since evidence suggests that ZBTB16, GLIS3, and RB1 have developmental roles in SSCs,<sup>65,66,75,81</sup> these factors might also have TE defense roles in SSCs. A related question is – what is the breadth of TEs impacted by the factors discussed here? For example, ZBTB16 was found to suppress *LINE1* element expression,<sup>67</sup> but other TEs and repetitive elements were not examined. Given that *Zbtb16*-mutant mice not only exhibit hypomethylation of *LINE1* elements but also *SINEs*, this raises the possibility that *SINE* activity is also suppressed by ZBTB16. In some cases, the co-regulation could be causal. As a case in point, *LINE1* elements are known to facilitate the transposition of at least two other subtypes of non-LTR retrotransposons: *SINES* and *SVAs*.<sup>104–106</sup>

The field has just begun to uncover how developmental decisions and “selfish DNA” defense might be linked. It is only natural that evolutionary forces might select proteins that function in both processes.

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## DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

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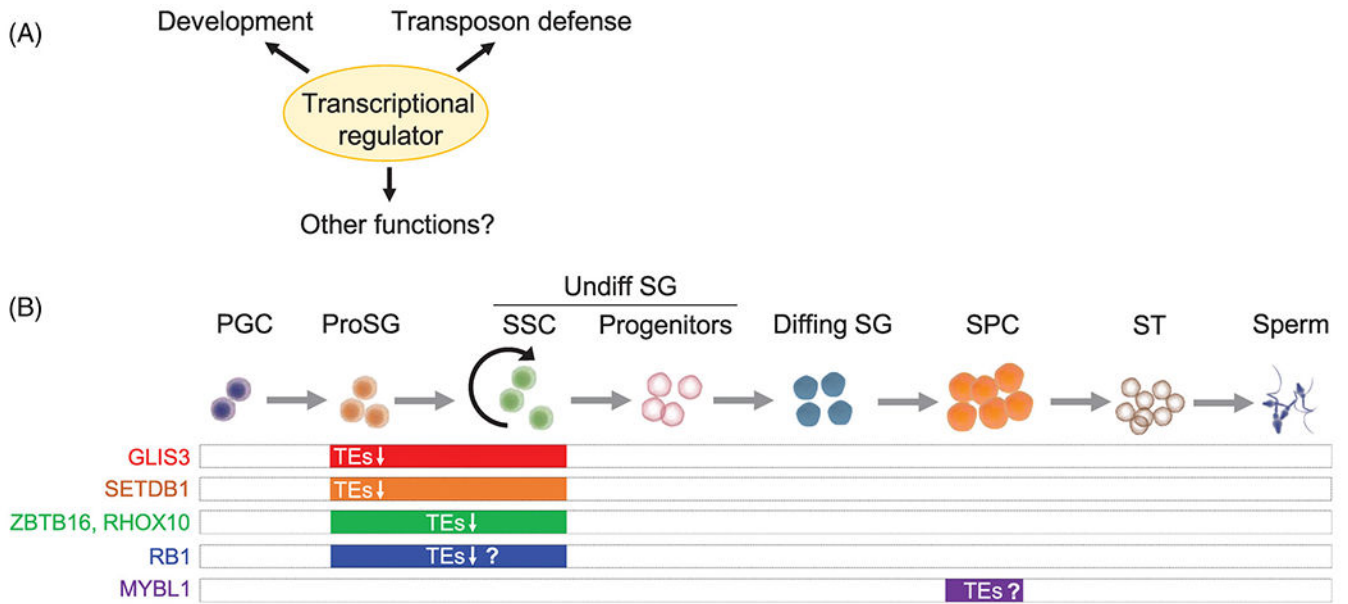
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**FIGURE 1.** Multi-functional factors acting in the germline. (A) We propose that some individual transcriptional regulators are capable of providing the functions depicted. (B) The 6 germ-cell transcriptional regulators that we discuss in this review. The germ cell stage(s) that they appear to act—including in transposable element (TE) suppression—are indicated. RB1 has functions in ProSG and SSCs, and thus is hypothesized to also suppress TEs in these stages, but its TE suppression function is only based on experiments in somatic cells. In the case of MYBL1, it is known to drive the production of a large subset of piRNAs, raising the possibility that MYBL1 indirectly functions in TE defense, but this has not been directly demonstrated.