Stem Cell Reports
Article

Rbf Regulates *Drosophila* Spermatogenesis via Control of Somatic Stem and Progenitor Cell Fate in the Larval Testis

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http://dx.doi.org/10.1016/j.stemcr.2016.11.007

SUMMARY

The *Drosophila* testis has been fundamental to understanding how stem cells interact with their endogenous microenvironment, or niche, to control organ growth in vivo. Here, we report the identification of two independent alleles for the highly conserved tumor suppressor gene, *Retinoblastoma-family protein* (*Rbf*), in a screen for testis phenotypes in X chromosome third-instar lethal alleles. *Rbf* mutant alleles exhibit overproliferation of spermatogonial cells, which is phenocopied by the molecularly characterized *Rbf*[11] null allele. We demonstrate that Rbf promotes cell-cycle exit and differentiation of the somatic and germline stem cells of the testes. Intriguingly, depletion of Rbf specifically in the germline does not disrupt stem cell differentiation, rather Rbf loss of function in the somatic lineage drives overproliferation and differentiation defects in both lineages. Together our observations suggest that Rbf in the somatic lineage controls germline stem cell renewal and differentiation non-autonomously via essential roles in the microenvironment of the germline lineage.

INTRODUCTION

Adult stem cells are maintained via self-renewal in all metazoans but must also differentiate to perform specialized roles. The stem cell microenvironment, or niche, which was first elucidated in the *Drosophila melanogaster* gonad (Kiger et al., 2000; Tran et al., 2000; Xie and Spradling, 2000) is essential for stem cell homeostasis. Specifically, the niche provides the cellular architecture and secretes molecular signals to regulate stem cell behavior (Li and Xie, 2005; Matunis et al., 2012; Zoller and Schulz, 2012). Not surprisingly, defective niche function has been associated with abnormal development and disease, particularly tumor initiation and progression (Boyle et al., 2007; Voog et al., 2014; White and Lowry, 2015).

Forward-genetic screens in *Drosophila* have previously revealed factors required for adult testis development (Castrillon et al., 1993; Hackstein, 1991; Matunis et al., 1997; Wakimoto et al., 2004), however such screens of male-sterile alleles often fail to detect genes required for earlier stages of development. We identified factors required for testis stem cell development by analyzing third-instar larval (L3) testes of homozygous recessive late-larval or pupal-lethal ethyl methanesulfonate (EMS)-generated mutants in a screen (manuscript for the complete screen in preparation). Here, we discuss one complementation group represented by isolation of two mutant alleles mapping to the *Retinoblastoma-family protein* (*Rbf*) gene. The founding human RB tumor suppressor protein (RB1) was first identified as mutated in children with the rare eye cancer, retinoblastoma, and subsequently detected as a loss-of-function mutation in a wide range of cancers (reviewed in Weinberg, 1995). In addition to *RB1*, the vertebrate genome contains two paralogous genes, *retinoblastoma-like 1* (*RBL1/p107*), and *retinoblastoma-like 2* (*RBL2/p130*).

The *Drosophila* RB family is comprised of two genes, *Rbf* and *Rbf2* (Du and Dyson, 1999), which both exhibit structural conservation with the vertebrate proteins and function similarly to control cell-cycle gene expression. Rbf2 has evolved in *Drosophila* from the ancestral Rbf and has some differences in its C terminus in addition to regulating expression of unique targets (Du and Pogoriler, 2006; Wei et al., 2015). Loss of Rbf function in insects results in overproliferation and developmental defects across a broad range of tissues (Buttitta et al., 2007; Du and Dyson, 1999; Duman-Scheel et al., 2004; Firth and Baker, 2005; Martin-Castellanos and Edgar, 2002). Knowledge from *Drosophila* has shed light on Rbf-dependent mechanisms for coordinating proliferation during development and, given the strong homology with mammals, studies in flies have implications for understanding RB family dysregulation in human cancer. In particular, studies in flies have
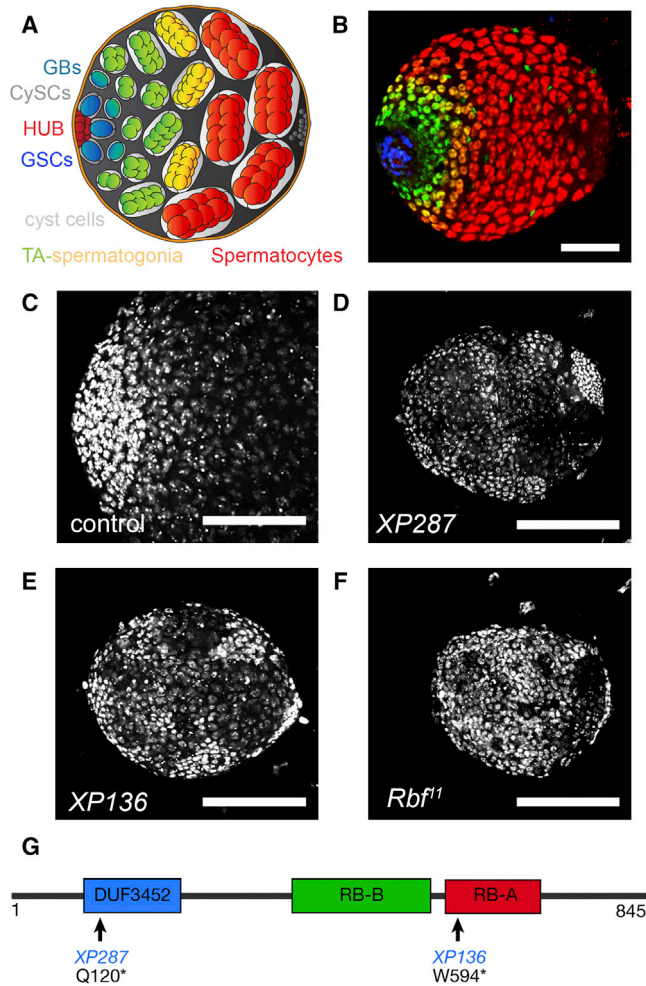
Figure 1. *Rbf* Is Required for Testis Development
(A) *Drosophila* L3 testis schematic. The niche is composed of hub cells (maroon). Germline stem cells (GSCs, blue) attached to the hub differentiate to give gonialblasts (GBs, teal), which divide to produce transit-amplifying (TA) spermatogonia (green/yellow). The somatic stem cells (CySCs, dark gray) surround the GSCs and differentiate to produce cyst cells (light gray) that encapsulate spermatogonia/spermatocytes. The spermatogonia differentiate into spermatocytes (red).
(B) Confocal image of L3 testis with GSCs and gonialblasts in blue (*esg-lacZ*), spermatogonia in green/yellow (Hnt/Topi), and spermatocytes in red (Topi).
(C) DAPI-stained L3 wild-type testis.
(D–F) Testis hemizygous for *Rbf* loss-of-function alleles *XP287* (D), *XP136* (E) identified in the screen, and (F) *Rbf* null allele, *Rbf^{11}*.
(G) Schematic of Rbf protein with conserved Retinoblastoma-associated protein A (RB-A) and B (RB-B) domains. Molecular lesions for the two *Rbf* alleles identified.
Scale bars, 100 μm.

enabled elucidation of connections between key growth signaling pathways and RB protein function during development of complex tissues and organs (Duman-Scheel et al., 2004; Firth and Baker, 2005).

The capacity to delay cell-cycle progression at the G1/S transition is central to tumor suppression by RB proteins, predominantly via interaction with, and inhibition of, the E2F family of S-phase transcriptional activators. In *Drosophila*, the role of Rbf proteins in cell-cycle regulation is considerably less complex than for mammals, with just two E2F subunits (compared with at least eight in mammals) and one DP cofactor (compared with two in mammals) (Dynlacht et al., 1994; van den Heuvel and Dyson, 2008). Rbf and RB1 share capacity to bind to E2F transcriptional activators, similarly RBL1/p107, RBL2/p130, and Rbf2 bind E2F repressor complexes (Du and Pogoriler, 2006). *Drosophila* E2F1 activates transcription by forming heterodimers with the DP transcriptional cofactor. In the absence of developmental growth signals, hypophosphorylated Rbf represses E2F-mediated transcription by binding and blocking the transcriptional activation domain of E2F/DP (Giacinti and Giordano, 2006). In response to mitogenic signals, G1-S Cyclin/cyclin-dependent kinase (CDKs) (e.g., CycD and CycE) can hyperphosphorylate Rbf, releasing the E2F1-DP complex to promote S-phase gene transcription (reviewed in Giacinti and Giordano, 2006). Flies have just one CDK inhibitor, Dacapo (Dap), which selectively inhibits CycE/Cdk2, but not CycD/Cdk4 (de Nooij et al., 1996).

The *Drosophila* testis provides a system for analysis of gene function in two distinct cell populations derived from adjacent stem cell types (the germline and somatic lineage) within their endogenous niche. The testis produces sperm throughout the lifetime of the adult male fly. From the L1 stage, the stem cell niche is composed of a cluster of somatic cells (the hub) that supports two stem cell populations: the germline stem cells (GSCs) and the somatic stem cells, also known as cyst stem cells (CySCs) (Gönczy and DiNardo, 1996; Hardy et al., 1979). Each GSC is enclosed by two CySCs, and both populations undergo asymmetric divisions to (1) maintain the stem cell pool and (2) differentiate into gonialblast daughter or somatic cyst cells, respectively (Fuller and Spradling, 2007; Hardy et al., 1979; Yamashita et al., 2003) (Figures 1A and 1B). The gonialblast exits the niche enclosed by a pair of cyst cells and, after four rounds of transit-amplifying (TA) mitotic divisions with incomplete cytokinesis, generates a 16-cell spermatogonial cyst (Hardy et al., 1979). Upon further growth and differentiation, spermatogonial cysts develop into spermatocytes, which undergo meiosis to produce sperm (Fuller and Spradling, 2007) (Figures 1A and 1B). Here, we demonstrate that although *Rbf* mutants display cell-cycle exit and differentiation defects in both

the germline and somatic lineages, Rbf function was only required in the somatic lineage for testes development. Thus, Rbf function in the somatic cell lineage is required non-autonomously for regulating cell-cycle exit and differentiation in the germline.

RESULTS

Rbf Is a Regulator of Spermatogenesis

Our forward-genetic screen of X chromosome mutants isolated two independent alleles of *Rbf*: *XP136* and *XP287* (Figures 1D and 1E). The condensed chromatin (detected by DAPI staining) characteristic of GSCs, mitotic spermatogonia, CySCs, and immature cyst cells in L3 testes suggested expansion of premeiotic germ cells (and/or immature somatic cells) in both *Rbf* mutant alleles (Figures 1D and 1E compared with control in 1C). Hemizygous *XP136* and *XP287* mutants are lethal at the pupal stage and contain point mutations encoding premature stop codons at amino acids 594 and 120, respectively (Figure 1G). Moreover, L3 testes from the molecularly characterized *Rbf[11]* null (Du and Dyson, 1999) phenocopied *XP247* and *XP136*, displaying expansion of premeiotic cells (Figure 1F compared with 1D and 1E). Together these data provide the first evidence that *Rbf* is essential for germline stem cell fate and spermatogenesis in *Drosophila*.

### Rbf Is Expressed in Germline and Somatic Lineages and Required for Germline Differentiation

Rbf protein localization has not been previously reported for testes, although peptides for Rbf were detected in mass spectroscopic analysis of the sperm proteome (Wasbrough et al., 2010). We detected Rbf protein in both the somatic and germline lineages of the L3 testis (Figures 2A–2C″) using an anti-Rbf monoclonal antibody (gift from Nick Dyson). Rbf protein was particularly abundant in somatic cells (Figures 2B and 2C). Consistent with the *Rbf[11]* allele being a complete deletion of the *Rbf* coding sequence (Du and Dyson, 1999), Rbf protein was below the level of detection in hemizygous *Rbf[11]* L3 testes (Figures 2D–2D″). To characterize potential changes in GSC and/or progenitor cell fate in the *Rbf* mutants, we used *3.6-1*, an *esg-lacZ* reporter, to mark GSCs and their immediate progeny (Bunt and Hime, 2004). Strikingly, the majority of *Rbf[11]* L3 gonads were composed of *esg-lacZ*-positive cells (Figures 2F–2F″ compared with control in 2E–2E″), suggesting a germline differentiation defect and associated accumulation of GSCs and/or their immediate progeny. Furthermore, TA spermatogonial cells (marked with Bam; McKearin and Ohlstein, 1995) and terminally differentiated spermatocytes (marked with Topi; Jiang et al., 2007) were decreased in

prevalence in *Rbf[11]* mutants (Figures 2F–2F″ compared with control in 2E–2E″).

Expansion of *neuralized-lacZ* (*neur[a101]*), which is upregulated in GSCs and their immediate daughters (Terry et al., 2006), was also observed in *Rbf[11]* L3 testes (Figures S1A and S1B). Further confirmation that *Rbf[11]* L3 testes had ectopic GSC and/or spermatogonial cells was provided by the presence of Spectrin-rich spectrosomes characteristic of GSCs and gonialblasts, or small fusomes with few branches characteristic of spermatogonia (Hime et al., 1996), far from the niche in the *Rbf* mutant testes (Figures S1C and S1D). The somatic cell boundaries were also marked using Dlg (Papagiannouli and Mechler, 2010, 2009) (Figures S1E and S1F) and E-cadherin/Traffic jam (Figures S1G and S1H) to provide a measure of spermatogonial cyst size. Normal-sized cysts of synchronously proliferating spermatogonia and spermatocytes were not observed in *Rbf[11]* mutants (Figures S1F, S1I, and S1J), providing further evidence that *Rbf* loss of function results in defective stem cell differentiation and the inability to generate germline cysts.

Germline Cells Proliferate Distant from the Stem Cell Niche in *Rbf* Mutant Testes

Our analysis of differentiation markers suggests that Rbf is necessary for germline differentiation in L3 testes. The differentiation block was also associated with a proliferation defect in the *Rbf[11]* mutants, with ectopic DNA synthesis (detected via bromodeoxyuridine [BrdU] incorporation) (Figures 3B and 3B′ compared with 3A and 3A′) and mitosis (measured by PH3 staining; Figures 3D and 3D′ compared with the control in 3C and 3C′, quantified in 6D). Wild-type testes do not exhibit any overlap of PH3 and Traffic jam (Tj) (somatic cells) away from the stem cell niche (Figures 3E and 3E′ white arrow), however we observed mitotically active (PH3) somatic cells (Tj) distant from the niche in *Rbf[11]* mutant testes (Figures 3F and 3F′, white arrowhead). As Rbf has been most strongly implicated as a negative regulator of cell-cycle progression via its capacity to inhibit E2F1, we examined a reporter for E2F1 transcriptional activity: the DNA replication factor Proliferating Cell Nuclear Antigen (PCNA-GFP) (Thacker et al., 2003). In the wild-type background, PCNA-GFP expression was strongly detected in close proximity to the niche, i.e., the stem and progenitor cells (Figures 3G and 3G′), while in *Rbf[11]* mutants, there was an expansion of PCNA-GFP expression (Figures 3H and 3H′). In *Drosophila* larval imaginal wing discs, E2F1 drives progression of both G1 to S phase (via CycE) and G2 to mitosis (via the String/cdc25 phosphatase) (Reis and Edgar, 2004). In accordance with increased E2F1 driving G1 to S-phase progression by positively regulating the S-phase cyclins, we observed elevated CycE in *Rbf[11]* gonads (Figure S2B). String (Stg), which is rate
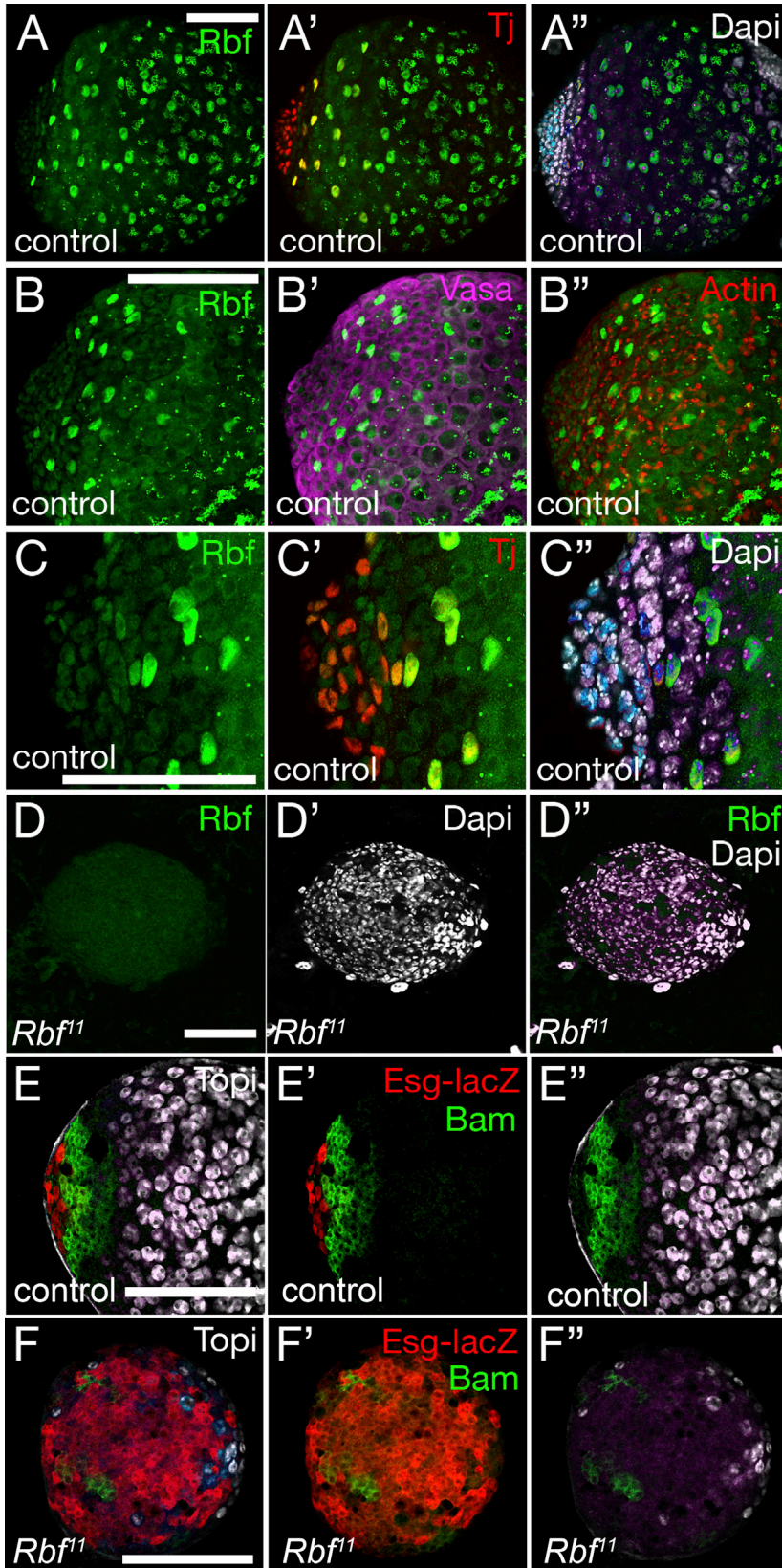
Figure 2. *Rbf* Regulates Germline Stem Cell Differentiation

(A–A″) Wild-type L3 testis stained for Rbf (green), Traffic jam (Tj, red), and DAPI (white).

(B–B″) Wild-type stained for Rbf (green), Vasa (purple), and Actin (red).

(C–C″) Close up of wild-type niche stained with Rbf (green) and Tj (red).

(D) *Rbf¹¹* L3 testis stained for Rbf (green) and DAPI (white).

(E and F) Wild-type (E) and *Rbf¹¹* L3 (F) testis marked with *esg-lacZ* (red) for GSCs, Bam (green) for spermatogonia, and Topi (white) for differentiated spermatocytes.

Scale bars, 100 μm. See also Figure S1.
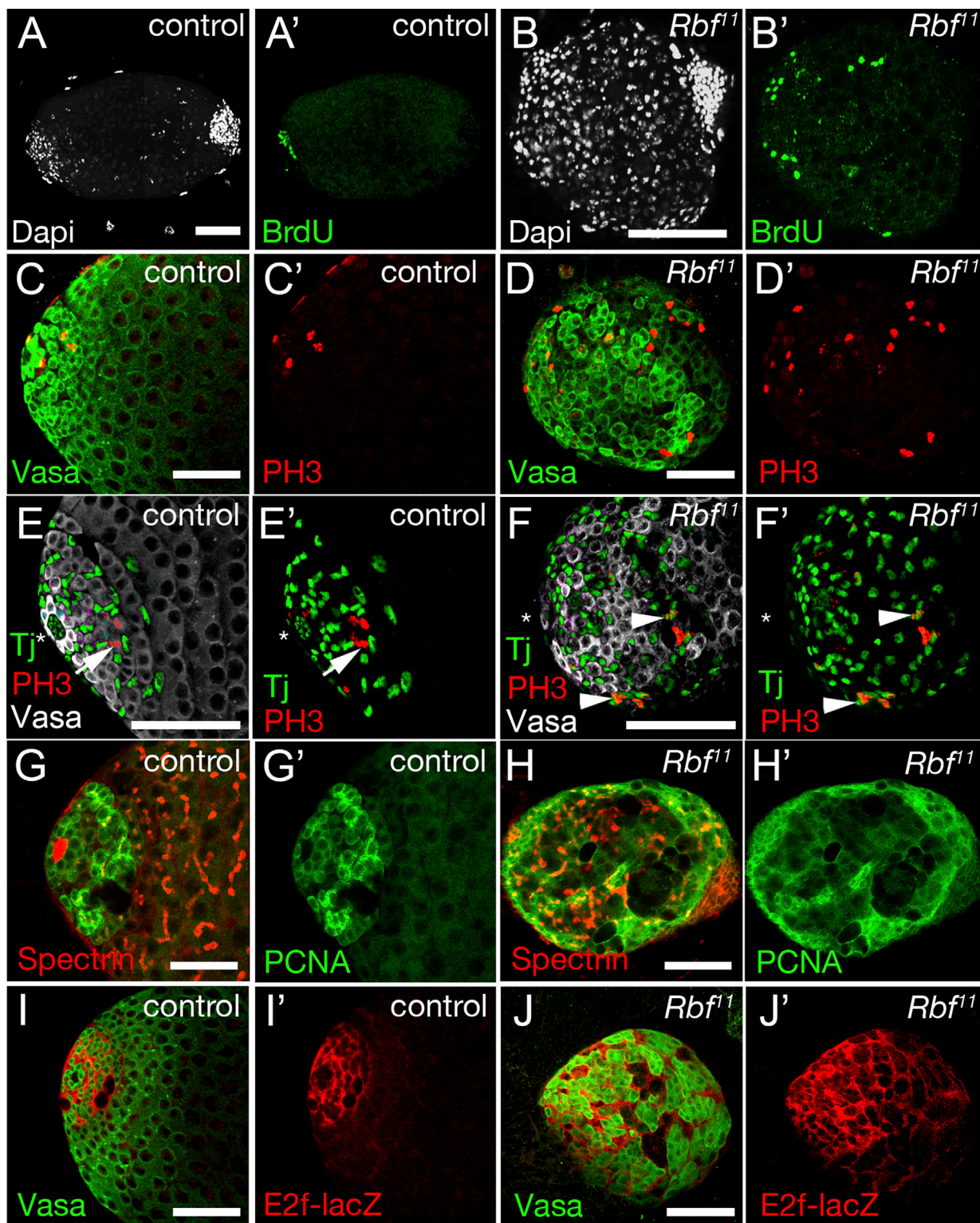
Figure 3. *Rbf* Mutant L3 Testes Overproliferate

(A and B) Wild-type (A and A') and *Rbf^11* (B and B') with S phase marked by BrdU (green) and co-stained with DAPI (white).

(C and D) Wild-type (C and C') and *Rbf^11* (D and D') L3 testes stained with anti-phosphorylated histone H3 (PH3, red) to mark mitotic cells and Vasa (green) for germ cells.

(legend continued on next page)

limiting for G2-M progression and cell division (Reis and Edgar, 2004), was normally limited to stem and progenitor cells in close proximity to the niche, but was observed throughout Rbf^{11} mutant testes (Figure S2A). Thus, Rbf also behaves as a tumor suppressor in the gonad, potentially via its well-characterized role as an inhibitor of the transcription factor and cell-cycle regulator, E2F1.

Transcription of mammalian E2F1 is subject to autoregulatory control, being repressed in early G1 when most E2F1 protein is in complex with RB, and activated in response to G1 CDK activity to ensure the G1 to S-phase transition (Bertoli et al., 2013; Johnson et al., 1994). Intriguingly, given the germline phenotype in Rbf mutants, $E2f1$ promoter activity (detected using an $E2f1-lacZ$ enhancer trap) was predominantly detected in the CySCs and early cyst cells that surround the stem cell niche (Figures 3I and 3I′) rather than the germline. Moreover, the pattern of $E2f1$ promoter activity was disrupted in Rbf^{11} mutant testes, with activity being detected away from the niche and again most strongly in somatic cells (Figures 3J and 3J′). To provide additional evidence that the ectopic $E2f1-lacZ$ activity was present in somatic cells distant to the niche, we co-labelled testes with rhodamine-phalloidin to detect filamentous actin outlining the cyst cells (Figures S2C and S2D). These data indicate that the phenotype observed in the Rbf^{11} mutant may primarily be due to a disruption of CySC differentiation.

Rbf Is Required in the Somatic Lineage, Not the Germline, for GSC Differentiation

We next sought to dissect the relative contribution of Rbf loss of function in the somatic or germline lineages to the phenotype observed. Surprisingly, we did not observe a germline differentiation defect following depletion of Rbf specifically in the germline (using $nanos$-Gal4 to drive a UAS-Rbf hairpin RNAi; Figures 4B–4B″ compared with 4A–4A″). Moreover, expression of Rbf specifically in the somatic lineage (with $C587$-$Gal4$) was sufficient to rescue the differentiation defect in the Rbf^{11} mutant (Figures 4E–4E″ compared with 4D–4D″). Zfh-1 marks the CySCs and their immediate progeny (in addition to large pigment cell nuclei), while Tj marks CySCs, their progeny, and older cyst cells. In an Rbf^{11} testis, we observed expansion of the domain of Zfh-1 expression to encompass all of the Tj-positive cells, however Zfh-1 appeared relatively less abundant in cells distant from the niche compared with CySCs.

Importantly, $C587$-driven overexpression of Rbf rescued the ectopic Zfh-1 normally observed in the Rbf^{11} background (Figures 4G–4G″).

Therefore, we investigated Rbf loss of function specifically in the somatic lineage (using $C587$-$Gal4$ to drive the UAS-Rbf hairpin RNAi; Figure 5). Indeed, depletion of Rbf in somatic cells resulted in a germline differentiation defect, even though Rbf remained abundant in the germline lineage (compare Figures 5A–5A″ with 5C–5C″ and 5B and 5B′ with 5D and 5D′). Rbf protein was normally most abundant in mature cyst cells and spermatocytes (Figures 5A–5A″ and 5B–5B′). Although few spermatocytes were found in Rbf null mutants (Figure 2F), they were observed in the $C587$>Rbf-$RNAi$ knockdown (Figures 5C and 5D). The germline differentiation defect in $C587$>Rbf-$RNAi$ testes was not as severe as in the Rbf^{11} null mutants, as we observed large patches of germ cells marked by Bam (compare Figure 5G with 2F–2F″). The morphology of the testes were, however, severely disrupted (compare Figures 5G and 5H with 5E and 5F) and large clusters of Bam-positive germ cells were observed (Figure 5G), dissociated from the stem cell niche, unlike in control testes (Figure 5F), indicating significantly impaired germ cell differentiation.

As observed for the Rbf null mutant, somatic Rbf knockdown resulted in ectopic expression of Tj (CySC and immature cyst cell marker) throughout the disorganized testis, which coincided with a delay in differentiation of germ cells (compare Figures 5H–5H″ with 5F–5F″). Mitotic activity is normally only observed in somatic and germ cells near the niche and spermatogonial within the apical third of the larval testis (Figures 5I–5J′). $C587$>Rbf-$RNAi$ testes exhibited varying levels of ectopic mitosis, depending upon the plane of optical section, but overproliferating germ cells (Vasa expressing) were found throughout the testis (Figures 5K–5M and 6D for quantification of mitosis). Together these data demonstrate that Rbf in the somatic lineage is required non-cell autonomously for controlling germline proliferation and differentiation.

To further investigate whether loss of Rbf resulting in E2F1 dysregulation in the somatic cells might be a major factor in the Rbf mutant phenotype, we tested whether depletion of E2F1 in the somatic cell lineage altered the germline defect in the Rbf^{11} hemizygote. Strikingly, depletion of E2F1 specifically in the somatic cells could rescue the proliferation and differentiation defect in the Rbf^{11}

(E and F) Wild-type (E, E′) and Rbf^{11} (F, F′) stained for Tj (green), PH3 (red), and Vasa (white). Asterisk marks the hub. Arrows mark mitotic germ cells and arrowheads mitotic somatic cells.
(G and H) Wild-type (G and G′) and Rbf^{11} (H and H′) in the $PCNA$-GFP (green) background to mark E2f1 activity, co-stained with α-Spectrin (red).
(I and J) Wild-type (I and I′) and Rbf^{11} (J and J′) with $E2f1-lacZ$ reporter (red) and Vasa (green).
Scale bars, 100 μm. See also PH3 quantification in Figures 6D and S2.
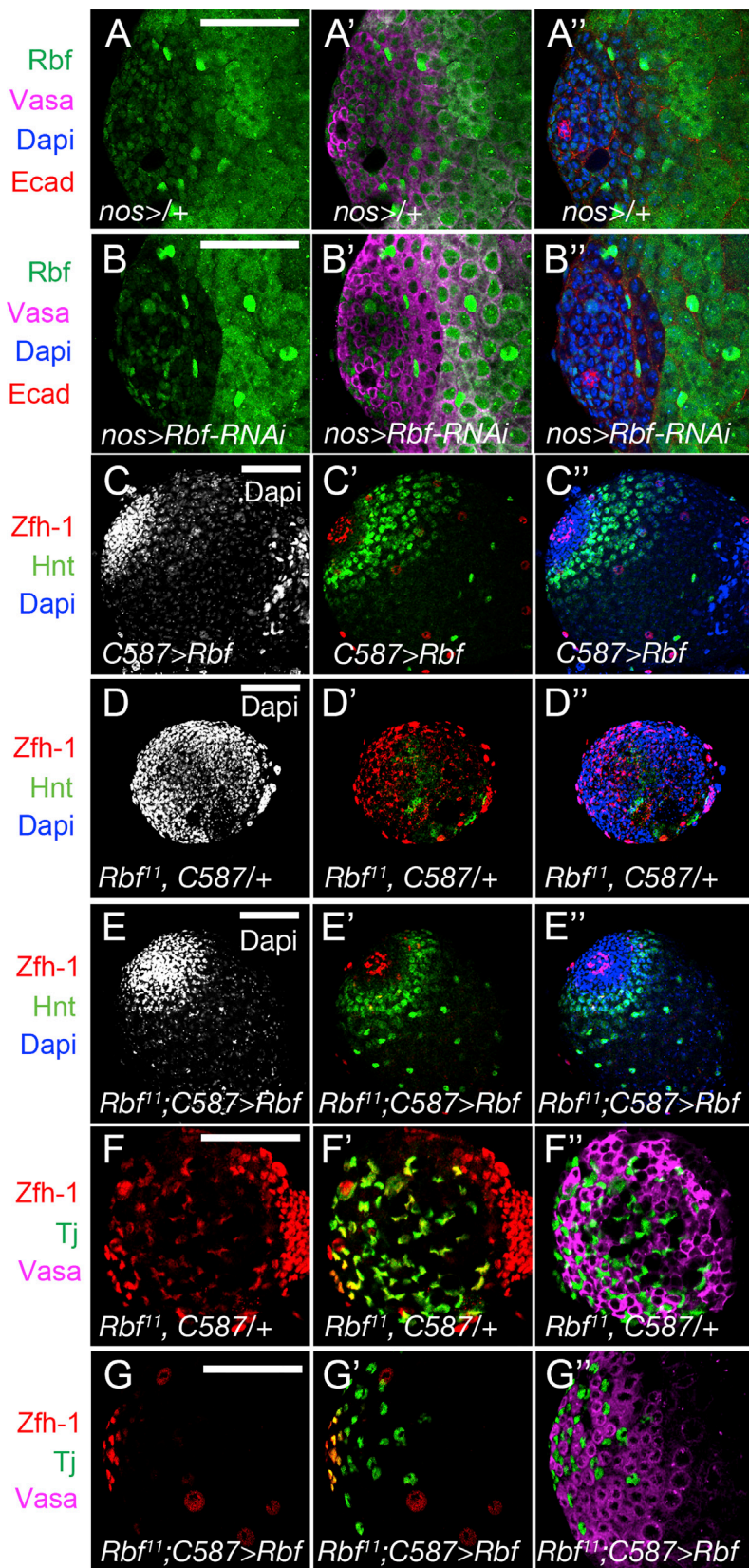
Figure 4. *Rbf* Expression in the Somatic Lineage of the L3 Testes Rescues the GSC Differentiation Defect

(A and B) *nos-Gal4/+* (A–A'') compared with *Rbf* knockdown in the germline via RNAi with the *nos-Gal4* driver (B–B''), with Rbf (green) antibody staining, Vasa (purple), E-cadherin (red), and DAPI (blue).

(C–E) *C587-Gal4* driven *UAS-Rb* wild-type transgene in the somatic lineage alone (C–C''), *Rbf^{11}* mutant alone (D–D''), and *Rbf^{11}* with *C587-Gal4* driven overexpression of Rbf (E–E''), stained with Zfh-1 (red), Hindsight (Hnt, green), and DAPI (white).

(F and G) *Rbf^{11}* testes with the *C587* driver alone (F–F'') or with *C587*-driven overexpression of Rbf (G–G''), stained with Zfh-1 (red), Tj (green), and Vasa (purple).

Scale bars, 100 μm.
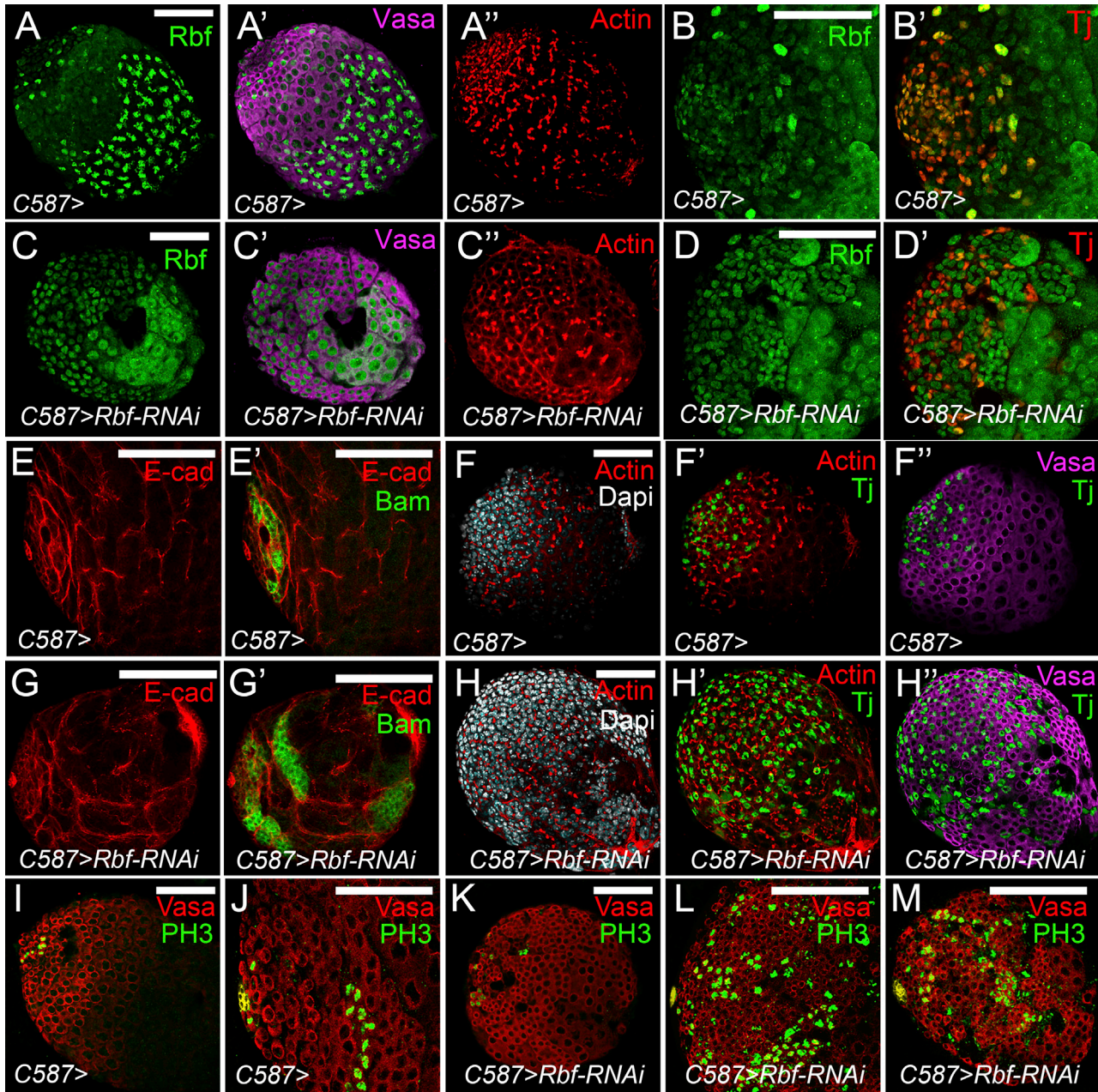
**Figure 5. *Rbf* Depletion in Somatic Cells Disrupts GSC Proliferation and Differentiation**

(A–D) Rbf (green) antibody staining for *C587>* control (A–A″, B–B′) or following *Rbf* depletion in the somatic lineage (C–C″, D–D′), stained with Actin (red) and Vasa (purple), or Tj (red).

(E–M) Control and (G and H) *C587*-driven *Rbf* knockdown stained with Bam (green) and E-cadherin (red) (E–E′ and G–G′) or Tj (green), Actin (red), Vasa (purple), and DAPI (white) (F–F″ and H–H″). (I and J) control and (K–M) *Rbf* knockdown with PH3 (green) and Vasa (red). Scale bars, 100 μm. See also PH3 quantification in Figure 6D.

mutant. Bam-positive cells were detected adjacent to the niche (although some structural defects were still evident) and terminally differentiated spermatocytes were again observed throughout the testis (compare Figures 6A–6A‴ and 6C–6C‴), and mitotic cells were reduced to control levels (Figure 6D). Note that knockdown of E2F1 alone did not result in an observable phenotype (Figures 6B–6B‴). Both the structure of the stem cell niche (Figures 6C″–6C‴) and the production of spermatocytes (Figure 6C′) in *Rbf^{11}* testes (Figures 6A–6A‴) were rescued by reduction
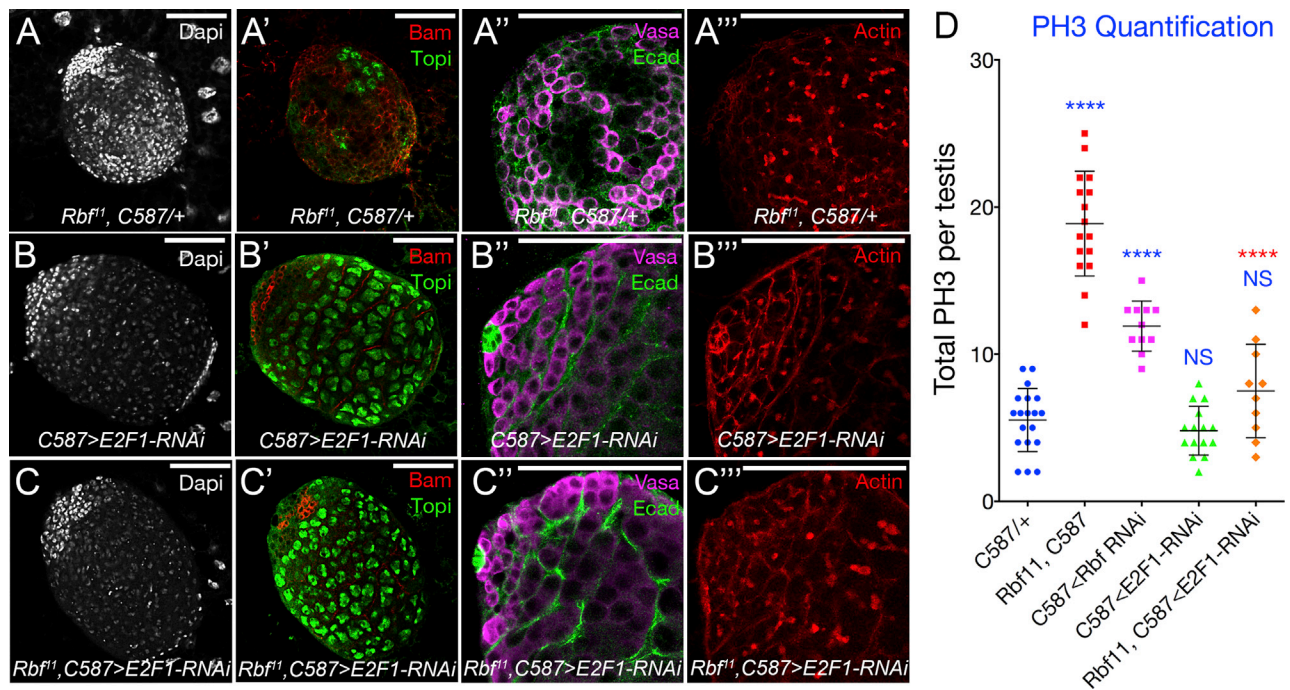
Figure 6. Depletion of *E2f1* in the Somatic Lineage Is Sufficient to Rescue the Germline Proliferation and Differentiation Defects in *Rbf* Mutants

(A–C) *Rbf[11]* mutant alone (A–A‴), knockdown of *E2f1* with *C587-Gal4* alone (B–B‴), or knockdown of *E2f1* in the somatic lineage of *Rbf[11]* mutants (C–C‴), marked with Topi (green), Bam (red), and DAPI (white), or with E-cadherin (green), Vasa (purple), and Actin (red). (D) Quantification of mitosis in the genotypes indicated. Statistical tests were performed with GraphPad Prism 6 using unpaired two-tailed t test with 95% confidence interval. Error bars represent SEM and according to the GraphPad classification of significance points. Blue asterisks (****$p < 0.0001$) indicate significance compared with *C587/+*. Red asterisks (****$p < 0.0001$) indicate significance compared with *Rbf[11], C587>*. NS indicates no significant difference compared with *C587/+*. Scale bars, 100 μm.

of E2F1 in somatic cells, suggesting restored differentiation of both early and late cyst cells. In summary, these data demonstrate that although Rbf is expressed in both the somatic cells and germline cells of the L3 testis, expression in the somatic cells is critical for maintenance of spermatogenesis, while potential roles for Rbf in the early germline lineage are relatively minor.

DISCUSSION

Analysis of EMS-induced male-sterile mutant collections suggest most alleles elicit meiosis or spermiogenesis phenotypes, but relatively few hits disrupt the stem cell niche (Wakimoto et al., 2004). This is not surprising, as many stem cell determinants and signaling pathways essential to niche function also have critical functions in earlier development, hence loss-of-function alleles are associated with embryonic or larval lethality and will be absent from fertility screens. In contrast, the unique features of meiotic cells (only spermatocytes and oocytes undergo

meiosis) or post-meiotic spermatids often derive from factors specific to these processes. Thus, although several genetic screens for male-sterile alleles have identified genes that function in the stem cell niche to regulate maintenance, proliferation, and differentiation of GSCs and/or somatic stem cells, these are often hypomorphic alleles (Castrillon et al., 1993; Kiger et al., 2001).

Our screening strategy permitted identification of two alleles of Rbf, a core cell-cycle regulator required for metamorphosis and survival, which would be precluded from sterility-based screens. The overproliferating germ cells present in the *Rbf* null allele did not divide synchronously and appear to have characteristics of GSCs. Few mutants have been previously reported with such phenotypes, with the exception of those affecting JAK-STAT signaling in CySCs (Kiger et al., 2001; Tulina and Matunis, 2001). STAT activity in CySCs is mediated through induction of its target genes *zfh-1* and *chinmo* (Flaherty et al., 2010; Leatherman and DiNardo, 2008). Ectopic expression of *upd1, zfh-1*, or *chinmo* results in formation of both CySC- and GSC-like tumors (Kiger et al., 2001; Leatherman and DiNardo, 2008;

Tulina and Matunis, 2001). Mutations that prevent the ability of the somatic cells to encyst the germline also result in spermatogonial proliferation defects (Sarkar et al., 2007). Thus, cyst cells provide a signal to the germline to facilitate germ cell differentiation, which can be disrupted by cell autonomous defects within the soma (Gonzalez et al., 2015). GSCs can also be maintained adjacent to the hub in the testis after ablation of CySCs and cyst cells, and germ cells away from the hub overproliferate with dot-like or dumbbell-shaped fusomes, indicating that they have GSC or gonialblast identity (Lim and Fuller, 2012). These phenotypes have similarities to *Rbf* deficient testes, which show variable encystment of germ cells, suggesting *Rbf* is required in the soma to provide signals for germ cell differentiation.

Intriguingly, although Rbf was detected in both somatic and germ cells, albeit at higher levels in the somatic population, the *Rbf* mutant phenotype was largely rescued by restoration of Rbf in somatic cells. Moreover, while depletion of Rbf in the germline did not generate observable phenotypes, depletion of Rbf in somatic cells was sufficient to drive asynchronous expansion of GSC-like cells distant to the GSC niche. However, the phenotype observed following RNAi depletion was less severe than for the null allele, allowing some differentiation to disorganized cysts of Bam-positive spermatogonia, which suggests that depletion of Rbf in the somatic lineage using the Rbf RNAi is incomplete and/or occurs at a later time point.

Rbf regulates cell-cycle progression by inhibiting E2F1 transcription factor activity. Phosphorylation of Rbf by G1 Cyclin/CDKs results in release of E2f1 and activation of S-phase gene transcription, including auto-regulation of E2f1 expression to generate a positive feedforward loop on DNA replication and S-phase entry (Bertoli et al., 2013). *E2f1* promoter activity was normally abundant in somatic cells surrounding the stem cell niche, while activity was expanded in the *Rbf*[11] testes. Together these data suggest that mediation of E2f1 activity in the somatic cell lineage by Rbf is essential for cell-cycle exit and differentiation of somatic stem cells. This is supported by the expansion of the CySC marker Zfh-1 in *Rbf*[11] testes.

Indeed, E2f1 activity was fundamental to the *Rbf*[11] phenotype as depletion of E2F1 in the somatic cells of *Rbf*[11] testes was sufficient to restore both somatic and germline differentiation. The Rbf mutant testes phenotype thus arises as a consequence of ectopic E2F1 activity blocking cell-cycle exit in CySCs. Continued proliferation of the somatic lineage prevents differentiation and establishment of the signaling networks necessary for cell-cycle exit and differentiation of the neighboring germline cells. Thus, perdurance of a stem cell-like state in *Rbf* mutant somatic cells non-cell autonomously induces germ cell tumors.

RB family proteins have differential roles in vertebrate stem cell populations. Conditional loss of *Rb* in the mouse male germline reduces the capacity of spermatogonial stem cells to undergo self-renewal and is associated with transient increases in progenitor spermatogonial proliferation, without a differentiation block (Hu et al., 2013). Complete loss of RB activity in mouse embryonic stem cells (that lack all three RB family members: RB, p107, and p130) results in failed withdrawal from the cell cycle and impaired differentiation (Dannenberg et al., 2000). The presence of closely associated stem cell populations in the *Drosophila* testis (GSCs and CySCs) provided the opportunity here to investigate potential roles for *Rbf* in these two cell types.

Retinoblastoma proteins complex with E2F and DP family transcriptional repressor proteins (Bertoli et al., 2013) to both activate and repress gene expression associated with regulation of cell-cycle repression and differentiation. Rbf-E2F1-DP complexes regulate transient gene expression necessary for cell-cycle progression, whereas E2F2 complexes are proposed to generate a more stable program of transcriptional repression of non-cell-cycle targets (Georlette et al., 2007). Our observations that CySCs in *Rbf* mutants fail to enter quiescence indicate that the main role of Rbf in these cells is to regulate cell-cycle progression, which may subsequently prevent signals being sent to the germline to initiate germ cell differentiation. Together our data demonstrate Retinoblastoma-family protein activity in the niche is essential for cell-cycle exit and differentiation of neighboring stem cells, and suggest that RB function may also be important in cancer microenvironment(s) for tumor progression.

EXPERIMENTAL PROCEDURES

Fly Stocks and Husbandry

Unless otherwise stated the lines were obtained from the Bloomington Stock Center. Other stocks used include 3.6-1 (*esg-lacZ*) (gift from S. Hayashi), *C587-Gal4* (Song and Xie, 2003), *UAS-Rbf*[RNAi] (VDRC 10696), *Rbf*[11] (de Nooij et al., 1996), *stg-lacZ* line 6.4 (Bruce Edgar), *E2f1-lacZ* (Bob Duronio).

Forward-Genetic Screening Strategy

Mutant fly stocks were generated by EMS mutagenesis. *FRT19A* male flies were mutagenized with 16 mM EMS in 1% sucrose. The males were crossed to *y, gt, rst/FM7C, Kr-GFP* virgins. Approximately 3,000 stocks were established by crossing individual *FM7C, Kr-GFP* carrying virgin females from the F1 progeny, back to *FM7C, Kr-GFP/Y* males. The stocks were scored for lethality and ~350 late-larval or pupal-lethal stocks were screened. Male L3 gonads were screened for spermatogenesis defects via dissection and staining with DAPI to enable visualization of the intensely stained early germ cells and somatic cells, in contrast to weakly stained spermatocytes.

Immunohistochemistry and Imaging

Testes were dissected and fixed in 4% formaldehyde in PBST (PBS with 0.2% Triton X-100) for 15 min, blocked for 1 hr in 4% normal horse serum in PBST prior to incubation with the appropriate primary antibodies overnight and fluorescently conjugated secondary antibodies as per Bunt and Hime (2004). Antibodies used were as follows: rat anti-Topi 1:500 (gift from H. White-Cooper), rabbit anti-Zfh1 1:5,000 (gift from R. Lehmann), mouse anti-Rbf (DX11) 1:20 (gift from N. Dyson), rat anti-CycE 1:500 (gift from H. Richardson), guinea-pig anti-Traffic jam 1:10,000 (gift from D. Godt), rabbit anti-β-gal 1:5,000 (Cappel), chicken anti-β-gal 1:5,000 (Abcam), rabbit anti-phosphohistone H3 (1:2000, Upstate), goat anti-Vasa (dC-13) 1:100 (Santa Cruz Biotechnology). The hybridoma supernatants for FasIII (7G10) 1:25, Dlg (DLG1), Bam 1:25, E-cadherin (Dacd2) 1:100, Hnt 1:100, Spectrin (3A9) 1:100, were obtained from the Developmental Studies Hybridoma Bank. Actin was detected with rhodamine-conjugated phalloidin. Secondary antibodies conjugated to Alexa 488, Alexa 564, Alexa 633 (Molecular Probes) were used at 1:500. Testes were mounted in Prolong Gold with DAPI (Molecular Probes) to reveal DNA. Imaging was performed on a Zeiss 510 Meta confocal microscope, and image processing was performed with Adobe Photoshop CS3 and CS4.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2016.11.007.

AUTHOR CONTRIBUTIONS

J.E.L.M., W.G.S., L.M.Q., and G.R.H. wrote the manuscript and contributed to experimental design, and J.E.L.M., W.G.S., H.W., F.Y., Y.C., N.A.S., J.H., M.T., and N.D. contributed to mutant isolation, characterizations, and experimental design. W.G.S. designed the original genetic screen (manuscript for the complete screen in preparation). N.D., J.E.L.M., L.M.Q., and G.R.H. contributed equally.

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