Development 138, 2441-2450 (2011) doi:10.1242/dev.056572

Sequential changes at differentiation gene promoters as they become active in a stem cell lineage

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SUMMARY

Transcriptional silencing of terminal differentiation genes by the Polycomb group (PcG) machinery is emerging as a key feature of precursor cells in stem cell lineages. How, then, is this epigenetic silencing reversed for proper cellular differentiation? Here, we investigate how the developmental program reverses local PcG action to allow expression of terminal differentiation genes in the *Drosophila* male germline stem cell (GSC) lineage. We find that the silenced state, set up in precursor cells, is relieved through developmentally regulated sequential events at promoters once cells commit to spermatocyte differentiation. The programmed events include global downregulation of Polycomb repressive complex 2 (PRC2) components, recruitment of hypophosphorylated RNA polymerase II (Pol II) to promoters, as well as the expression and action of testis-specific homologs of TATA-binding protein-associated factors (tTAFs). In addition, action of the testis-specific meiotic arrest complex (tMAC), a tissue-specific version of the MIP/dREAM complex, is required both for recruitment of tTAFs to target differentiation genes and for proper cell type-specific localization of PRC1 components and tTAFs within the spermatocyte nucleolus. Together, the action of the tMAC and tTAF cell type-specific chromatin and transcription machinery leads to loss of Polycomb and release of stalled Pol II from the terminal differentiation gene promoters, allowing robust transcription.

KEY WORDS: tTAF, tMAC, Polycomb, Transcription, Spermatocyte, *Drosophila*, Spermatogenesis

INTRODUCTION

In stem cell lineages, proliferating precursors give rise to specific differentiating cell types. A major question is how the correct differentiation genes, kept silent in precursor cells, are selectively activated upon the switch to differentiation. Reversal of repression by the Polycomb group (PcG) epigenetic transcriptional silencing machinery has been implicated in the switch from proliferating precursor cells to differentiation in stem cell lineages. In embryonic stem cells (ESCs), PcG components are often associated with transcriptionally silent genes that will be turned on later during lineage specification (Boyer et al., 2006; Chamberlain et al., 2008; Lee et al., 2006). However, PcG action is unlikely to be the only mechanism that keeps the differentiation genes silent, as PcG proteins are not necessary for ESC self-renewal. Instead, PcG action appears to be required for the faithful execution of lineage-appropriate gene expression during ESC differentiation (Surface et al., 2010). In adult stem cell lineages, lack of the mammalian PcG protein Bmi1 results in loss of long-term self-renewing capacity in hematopoietic and neural stem cells, whereas overexpression of Bmi1 is associated with leukemia (Lessard and Sauvageau, 2003; Molofsky et al., 2003; Park et al., 2003). Consistent with the oncogenic features of Bmi1, several genes repressed by Bmi1 act to restrict the multipotency of progenitors in the hematopoietic lineage (Akala et al., 2008). Likewise, reversal of PcG-mediated epigenetic silencing by specific histone demethylases is required for keratinocyte differentiation in epidermis (Sen et al., 2008).

The PcG proteins act in at least two distinct but cooperating protein complexes: Polycomb repressive complex 1 (PRC1) and PRC2 (for a review, see Schwartz and Pirrotta, 2007). PRC2 initiates repression by methylating histone H3 at Lys27 (Cao et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2002; Muller et al., 2002). Recruitment of PRC1 may be initiated by the binding of Polycomb via its chromodomain to the trimethylated histone H3 Lys27 (H3K27me3) mark (Fischle et al., 2003; Min et al., 2003). PRC1 is found at silenced gene promoters even in the presence of the RNA polymerase II (Pol II) transcription machinery, suggesting that Polycomb might not block the formation of the preinitiation complex (Dellino et al., 2004). In the preinitiation complex, the C-terminal domain (CTD) of Pol II is in an unphosphorylated state. The CTD becomes phosphorylated at Ser5 as the polymerase initiates transcription of the first few nucleotides, and phosphorylation on Ser2 of the CTD is associated with Pol II fully engaged in transcript elongation (reviewed by Phatnani and Greenleaf, 2006). Thus, antibodies specific for the hypophosphorylated CTD provide a way to detect Pol II that is still at the promoter. In addition to the H3K27me3 mark placed by PRC2 activity, the PRC1 component dRing (Sce – FlyBase) acts as an E3 ubiquitin ligase to mono-ubiquitylate histone H2A at Lys119 (H2AK119ub) (Wang et al., 2004a), which blocks efficient transcriptional elongation (Stock et al., 2007). In addition to silencing gene expression by modifying specific histones, PcG complexes may repress transcription by compacting the chromatin and excluding transcriptional activators (Francis et al., 2004).

In stem cell lineages, if silencing by PcG action blocks the expression of differentiation genes, then the normal developmental program must reverse this epigenetic silencing in a gene-specific and lineage-appropriate fashion to allow differentiation of the proper cell type(s). Failure to do so might lead to abnormalities in development and underlie early pathological progression toward cancer.

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Accepted 29 March 2011

To understand the mechanisms that reverse silencing by PcG during differentiation in stem cell lineages, we investigated the sequence of events at the promoters of three terminal differentiation genes as cells progress from proliferating precursors to active differentiation in the *Drosophila* male germline. Male germ cells differentiate from adult stem cell precursors, first undergoing transit-amplifying mitotic divisions as spermatogonia, followed by switching to the spermatocyte program of cell growth and meiosis. In spermatocytes, a dramatic, cell type-specific transcription program is initiated in preparation for subsequent spermatid differentiation. This transcription program requires the cooperative activity of spermatocyte-specific homologs of components of the core transcription factor $TF_{II}D$ complex (the tTAFs) (Hiller et al., 2004; Hiller et al., 2001; Lin et al., 1996), as well as the testis-specific meiotic arrest (tMAC) complex, which resembles the mammalian MIP/dREAM (*Drosophila* RB, E2F and Myb) and the *C. elegans* SynMuv complexes (Ayyar et al., 2003; Beall et al., 2007; Jiang et al., 2007; Jiang and White-Cooper, 2003; Perezgasga et al., 2004; White-Cooper et al., 2000; White-Cooper et al., 1998). Expression of the tTAFs and testis-specific components of the tMAC complex, such as *always early* (*aly*), is turned on for the first time in spermatocytes, early in the G2 phase of meiosis I (White-Cooper et al., 2000; Hiller et al., 2001; Hiller et al., 2004; Chen et al., 2005). The tTAFs appear to activate the expression of terminal differentiation genes in part by counteracting repression by the PcG transcriptional silencing machinery. As a result of tTAF action, Polycomb is removed from differentiation gene promoters and sequestered to spermatocyte nucleoli, along with several other PRC1 components (Chen et al., 2005).

Here we show that a choreographed series of events under the control of a cell type- and stage-specific developmental program converts terminal differentiation genes from a transcriptionally silent state in precursor cells to a fully active state in spermatocytes. Our results in the germline stem cell (GSC) lineage provide a paradigm for how epigenetic silencing can be reversed in a gene-selective and stage-specific manner to allow expression of terminal differentiation genes and highlight the role of cell type-specific transcription machinery in this process.

MATERIALS AND METHODS

Fly strains and husbandry

Flies were raised on standard cornmeal molasses agar medium at 25°C unless stated otherwise. *Drosophila* strains are described previously (Ayyar et al., 2003; Gonczy et al., 1997; Hiller et al., 2004; Hiller et al., 2001; Jiang et al., 2007; Jiang and White-Cooper, 2003; Lin et al., 1996; McKearin and Ohlstein, 1995; McKearin and Spradling, 1990; Perezgasga et al., 2004; White-Cooper et al., 2000; White-Cooper et al., 1998). The allelic combinations for mutants were: $bam^{1}/bam^{\Delta 86}$, aly^{2}/aly^{5P}, can^{12}, *y,w*, $comm^{Z1340}$, $tomb^{GS12862}$, $topi^{143\text{-}68/157\text{-}89}$, $achi/vis^{Z3922}$, $mip40^{1a/4a}$. The $E(z)^{-/-}$ clones were induced by FLP recombinase that was exclusively expressed in germ cells using the germline-specific *nos-GAL4* driver with a *UAS-FLP* transgene in males of genotype *nos-GAL4*/Y; *UAS-FLP*; $E(z)^{731}$ *FRT2A*/*GFP3-13-7 FRT2A*.

Immunostaining

Immunofluorescence staining using anti-Sa, anti-Can or anti-Fib was performed using the methanol fixation procedure as described (Chen et al., 2005). Immunofluorescence staining using anti-E(z), anti-Su(z)12 or anti-H3K27me3 was performed using the formaldehyde fixation procedure as described (Hime et al., 1996).

Primary antibodies were anti-Can (1:1000), anti-Sa (1:100), anti-Fib (undiluted), anti-E(z) (1:100), anti-Su(z)12 (1:100) and anti-H3K27me3 (1:200; Millipore 07-449). Secondary antibodies were from the Alexa Fluor-conjugated series (1: 200; Molecular Probes).

Chromatin was visualized by DAPI (4′,6-diamidino-2-phenylindole) staining in VECTASHIELD medium (Vector Labs H-1200). Images were taken using the Leica TCS SP2 AOBS confocal system and processed using Adobe Photoshop.

Immunoblot

The nht^{1}/nht^{2} and nht^{1}/nht^{2}; $E(z)^{61}/E(z)^{731}$ flies were isolated at the day of eclosion at 25°C (permissive temperature) and shifted to 29°C (restrictive temperature) for 7 days. Twenty pairs of testes were dissected from each genotype and total protein was extracted, separated by 4-20% PAGE (Invitrogen, EC6025BOX) and transferred to a membrane (GE Healthcare RPM303F). The blot was probed with rabbit anti-H3K27me3 (1:400; Millipore 07-449) followed by HRP-conjugated anti-rabbit (1:5000, GE Healthcare NIF824). ECL reagents (GE Healthcare RPN2108) were used for detection. The blot was stripped for 30 minutes at 50°C with stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.7) and then re-probed with anti-H3 (1:5000; Abcam ab1791), followed by the same anti-rabbit secondary antibody (1:5000) and ECL detection.

Quantitative RT-PCR analyses

Twenty pairs of testes from *yw* (*wt*), nht^{1}/nht^{2} (*nht*) and nht^{1}/nht^{2}; $E(z)^{61}/E(z)^{731}$ [*nht; E(z)*] males (grown under the same conditions as described above) were dissected and used for total RNA extraction with TRIzol (Invitrogen 15596-018). cDNAs were synthesized with M-MLB reverse transcriptase (Promega M1701). Real-time PCR analyses were performed using TaqMan probes with Universal PCR Master Mix (Applied Biosystems 4304437) in an ABI 7300 machine. The TaqMan probes for *dj*, *fzo*, *Mst87F* and *Rpl32* are Dm02361628_s1, Dm02150476_s1, Dm02362808_s1 and Dm02151827_g1, respectively (Applied Biosystems). Each PCR reaction was performed in duplicate and the Ct numbers for each reaction were collected and averaged. The relative expression level of each target gene was calculated from $2^{\mathrm{Ct}(Rpl32)-\mathrm{Ct}(\text{target gene})}$, using *Rpl32* as an internal control.

Chromatin immunoprecipitation (ChIP) and data analyses

ChIP experiments using dissected testes were performed as described (Cao et al., 2002; Chen et al., 2005), except that Protein A Dynabeads (Invitrogen 100.01D) were used instead of Protein A Agarose beads. Each ChIP experiment used: 5 μl anti-Sa, 5 μl anti-Polycomb [from R. Kingston and R. Jones (Chen et al., 2005)], 5 μl anti-Pol II (ascites, 8WG16, Covance MMS-126R), 4 μl anti-Pol II (1 μg/μl, 4H8, Abcam ab5408), 5 μl anti-H3K4me3 (0.4 μg/μl, Abcam ab8580), 2 μl anti-H3K27me3 (1 μg/μl, Millipore 07-449), 1 μg anti-H3 (Abcam ab1791). All ChIP experiments were repeated at least three times in independent biological replicates.

Input DNA, mock precipitated DNA and ChIP DNA were analyzed using gene-specific primers (see below) with the Universal PCR Master Mix (Applied Biosystems 4304437) in an ABI 7300 machine. Quantitative PCR analyses of the ChIP experiments were performed as described (Chen et al., 2005). The ChIP and mock DNA were normalized to the input DNA and are plotted as a percentage of the input DNA (Input %) in Fig. S3 in the supplementary material. Further normalization was performed to convert the raw percentage input data to fold change relative to the constitutively expressed *CycA* gene from the same sample. For a given antibody, relative enrichment compared with *CycA* in the same sample is plotted in Fig. 3, with *CycA* set to 1 in order to compare across the different genotypes. Primers used were (5′ to 3′):

Mst87F: forward, GTCAAACCGATATACCTGTGCGTAA; reverse, ATGTGTTCAGGCCGAAAGGA; FAM, CCAGATTTTGTATCATTATTATTTG;

dj: forward, ACAAATAGTCTCCAGCTGTGGTTTT; reverse, CGACGTAAAATTAAAGCGGTTCTCT; FAM, CCAAAAGTTTTACAAAGAATTT;

fzo: forward, CCTCAAAAAGCGAGCAAAACAACAT; reverse, GTCAGATTCCGCCATTATGATTAGATATTACA; FAM, CTACAGTTGCCTATATTTCA; and

CycA: forward, CAACAGCAAGAAGGCAACGA; reverse, GAGTCCGATTATGCTCTGCTCTT; FAM, CCCTTCCTTCTCTCTTTCTC.

Microarray experiments and data analysis

Total RNA from ~200 pairs of fly testes (*bam*^{1}/*bam*$^{\Delta 86}$, *aly*^{2}/*aly*^{5P}, *sa*^{1}/*sa*^{2}, *y,w*) was extracted using TRIzol. The genomic DNA was degraded using 2 units DNase I (Fermentas EN0521, Glen Burnie, MD, USA) at 37°C for 20 minutes. RNA integrity was checked by gel electrophoresis (1% agarose). Approximately 4 μg total RNA from each biological replicate was used to generate labeling probes to hybridize with the Affymetrix GeneChip Drosophila Genome 2.0 Array according to the Affymetrix protocol. Three biological replicates were performed for each genotype. Microarray hybridization was processed at the Core Facility at the Stanford University School of Medicine and the raw data were exported from the Affymetrix Microarray Suite (MAS). The CEL files were used for signal normalization with RMA as part of the limma package (Bioconductor). The accession number for microarray data is GSE 28728 (Gene Expression Omnibus).

RESULTS

The PRC2 components E(z) and Su(z)12 are expressed in precursor cells but are downregulated as germ cells differentiate

Consistent with the model that terminal differentiation genes may be acted upon by the PcG transcriptional silencing machinery in precursor cells, immunofluorescence staining revealed abundant PRC2 proteins E(z) and Su(z)12 in GSCs, gonialblasts, spermatogonia and very early spermatocytes (Fig. 1A,B). Strikingly, E(z) and Su(z)12 protein levels abruptly dropped in spermatocytes in early G2 of meiotic prophase, immediately prior to the initiation of tTAF protein expression (arrowheads in Fig. 1A-A″,B-B″). *E(z)* and *Su(z)12* are likely to be downregulated at least in part at the level of mRNA expression or stability, as in situ hybridization revealed a decrease in mRNA levels in the spermatocyte region compared with the apical region of the testis, where stem and precursor cells are located (see Fig. S1 in the supplementary material). The downregulation of PRC2 components was not due to the action of either the tTAFs or the tMAC component Aly, as Su(z)12 protein levels still dropped abruptly in early spermatocytes in testes from tTAF [e.g. *ryan express* (*rye*, *TAF12L*)] or *aly* mutant males (see Fig. S2A-A″,B-B″ in the supplementary material). Conversely, loss of PRC2 did not allow precocious tTAF expression in precursor cells, as the tTAF Cannonball (Can, TAF5L) was not turned on in *E(z)* mutant spermatogonia (see Fig. S2C-C″ in the supplementary material). At least some of the *E(z)* mutant germ cells differentiated into round or elongating spermatids (see Fig. S2D-D″ in the supplementary material), indicating that *E(z)* function was not required for germ cell differentiation from spermatocyte to spermatid.

The abrupt decline in Su(z)12 and E(z) proteins in differentiating male germ cells was dependent on the switch to the spermatocyte program. The Su(z)12 and E(z) proteins remained high in the spermatogonial cysts that overproliferate in *bag of marbles* (*bam*) mutant testes (Fig. 1C,C′), in which the transition from spermatogonia to spermatocytes is entirely abolished (Gonczy et al., 1997; McKearin and Spradling, 1990).

Immunofluorescence staining revealed that the histone modification H3K27me3, which is made by the PRC2 component E(z), was abundant in early male germ cells and persisted into spermatocytes, even after the abrupt downregulation of E(z) and Su(z)12 proteins (Fig. 1D, arrowheads; Fig. 1E, cells on the left side). The fact that spermatocytes have already completed pre-meiotic S phase, which is the last DNA replication event of this lineage, prior to the downregulation of E(z) protein might contribute to the perdurance of the H3K27me3 modification in later-stage germ cells, including spermatocytes. Notably, marked clones of germ cells that were null mutant for *E(z)* had very much reduced H3K27me3, even as spermatocytes (Fig. 1D,E, dotted circles), indicating that *E(z)* encodes the sole or predominant histone methyltransferase in *Drosophila* male germ cells that generates the H3K27me3 epigenetic mark recognized by the antibody.

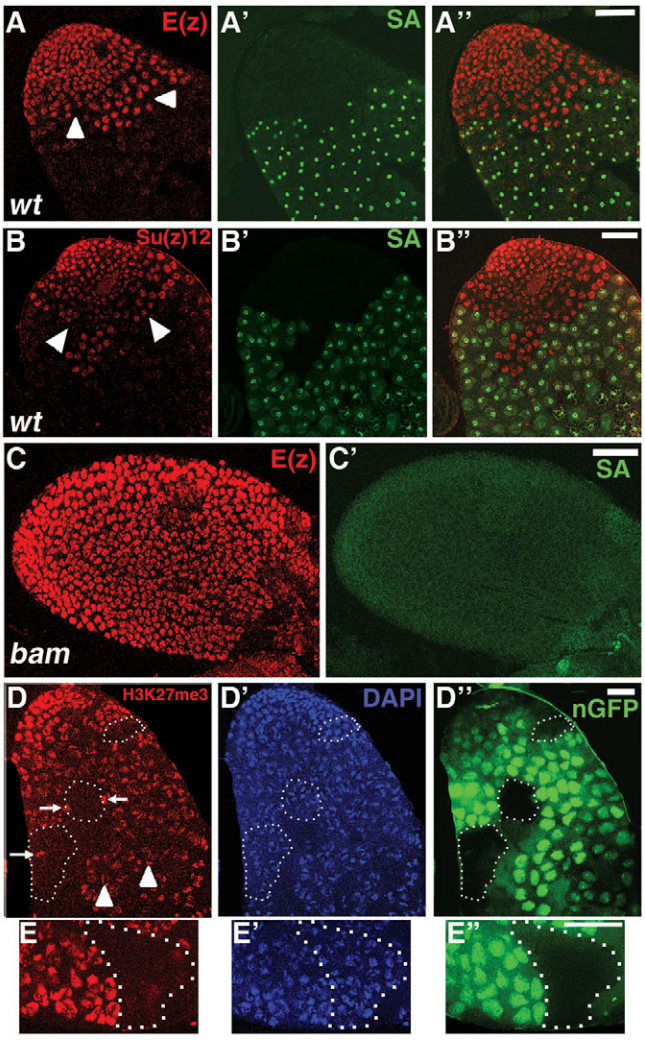

Fig. 1. Expression of the PRC2 components E(z) and Su(z)12 is downregulated as male germ cells differentiate. (**A-B″**) Apical region of wild-type (*wt*) *Drosophila* testis. (A) Anti-E(z); (A′) Sa-GFP; (A″) merge. (B) Anti-Su(z)12; (B′) Sa-GFP; (B″) merge. Arrowheads indicate downregulation of E(z) and Su(z)12 as tTAF (represented by Sa) expression is turned on. (**C**,**C′**) Apical region of *bam* testis. (C) Anti-E(z); (C′) Sa-GFP. Similar results were obtained for Su(z)12 in *bam* testis (data not shown). (**D-E″**) Testes from *E(z)*^{731}/+ males with marked clones of germ cells that are homozygous for the *E(z)*^{731} mutation. (D,E) Anti-H3K27me3; (D′,E′) DAPI; (D″,E″) nuclear GFP. Dotted lines outline cysts of germ cells that lack the nuclear GFP (nGFP) marker and are therefore *E(z)*$^{-/-}$. Arrows indicate *E(z)*/+ nuclei of somatic cyst cells associated with the germ cell clones. Arrowheads indicate spermatocyte chromosomes stained with anti-H3K27me3. (E-E″) High-magnification view of control (left) and *E(z)*$^{-/-}$ (dotted outline) spermatocytes, showing that the anti-H3K27me3 staining persists in *E(z)*$^{+/-}$ spermatocyte nuclei even after *E(z)* is downregulated and that the H3K27me3 staining in spermatocytes is largely dependent on *E(z)* function. Scale bars: 40 μm.

Although two PRC2 components, E(z) and Su(z)12, are downregulated after male germ cells switch from the spermatogonial mitotic proliferation to the spermatocyte cell

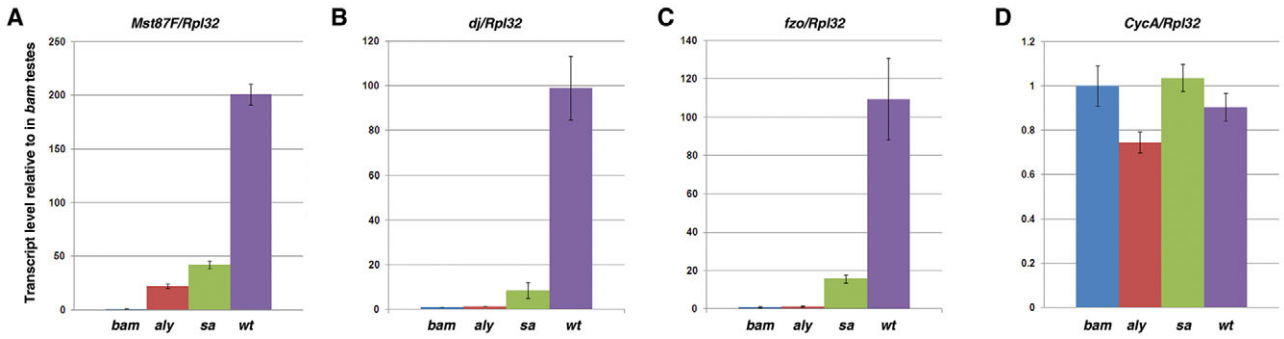

Fig. 2. Transcript levels for three tTAF target genes. Expression of (**A**) *Mst87F*, (**B**) *dj*, (**C**) *fzo* and (**D**) the non-target gene *CycA* in *bam*, *aly*, *sa* or wild-type (*wt*) *Drosophila* testes, as calculated from microarray data. The level of each transcript in each genotype was normalized to that of the house keeping gene *Rpl32* in the corresponding sample. Transcript levels are expressed as fold increase compared with the level of expression in *bam* mutant testes, which was set at 1 to facilitate comparison among the different genes. Error bars indicate s.d. from three independent biological replicates.

growth and differentiation program, the PRC1 components Polycomb, Polyhomeotic and dRing remain expressed in spermatocytes (Chen et al., 2005), indicating that the expression of PRC1 components and of certain PRC2 components are regulated differentially in the male GSC lineage.

H3K27me3 and Polycomb mark silent terminal differentiation genes in precursor cells

The state of the promoters of three terminal differentiation genes in precursor cells was assessed by chromatin immunoprecipitation (ChIP) using *bam* mutant testes, which are enriched with precursor cells but lack spermatocytes and spermatids. The terminal differentiation genes *Mst87F*, *don juan* (*dj*) and *fuzzy onions* (*fzo*) are not expressed in spermatogonia but are first transcribed in spermatocytes, in which they require wild-type function of both tMAC and tTAFs for normal expression (Hiller et al., 2004; Hiller et al., 2001; White-Cooper et al., 1998). Microarray analysis indicated that these differentiation genes are upregulated ~100- to 200-fold in wild-type compared with *bam* mutant testes (Fig. 2). *fzo* and *dj* were still essentially silent in testes from males mutant for the tMAC subunit *aly*. Although low levels of *Mst87F* transcript were detected by microarray in *aly* mutant testes (Fig. 2), expression was so low that *Mst87F* mRNA is not detected by northern in *aly* mutant testes although abundant transcript is detected in wild type (White-Cooper et al., 1998). The three differentiation transcripts were measurably expressed in tTAF [*spermatocyte arrest* (*sa*, *TAF8L*)] mutant testes, higher than in *aly* mutant testes but much lower than in wild-type testes, based on northern blot, in situ hybridization (White-Cooper et al., 1998; Hiller et al., 2001) and microarray analysis (Fig. 2).

To compare ChIP results among different genotypes, which by necessity had to be tested in separate reactions, enrichment of the promoter regions of the terminal differentiation genes was compared with enrichment of the *Cyclin A* (*CycA*) gene in the same sample (Fig. 3). Similar trends held when the ChIP data were plotted as a percentage of the input DNA (Input %) without normalizing to *CycA* (see Fig. S3 in the supplementary material); however, in this case it is harder to compare absolute levels among different genotypes. *CycA* was chosen for normalization for two reasons. First, our quantitative analysis of *CycA* mRNA levels by microarray (Fig. 2D) showed that levels of its transcript were similar in *bam*, *aly*, *sa* mutant and wild-type testes. In situ analysis of *CycA* mRNA levels also indicates its presence in both precursor cells and spermatocytes, as well as in tTAF and *aly* mutant spermatocytes (White-Cooper et al., 1998). Second, previous studies have shown that CycA protein is expressed in both spermatogonia and spermatocytes in wild-type, tTAF and *aly* mutant testes but not in spermatids (Lin et al., 1996), and that the level of CycA protein is equivalent between wild-type and either tTAF or *aly* mutant testes (White-Cooper et al., 1998). These features make *CycA* a good control on a per cell basis for the germ cells in which most transcription is taking place. Thus, normalizing for *CycA* across genotypes should correct for different numbers of spermatogonia and spermatocytes per testis in wild-type versus mutant genotypes, as well as for the presence of spermatids (which are mainly transcriptionally inactive but maintain stabilized mRNAs for many differentiation genes) in wild-type testes but not in mutant testes.

Reflecting the transcriptionally silent state of the terminal differentiation genes in proliferating spermatogonia, when antibodies against H3K4me3, a marker of transcriptionally active chromatin (Barski et al., 2007; Dou et al., 2006; Milne et al., 2002), were used in ChIP experiments with *bam* mutant testes, enrichment for the promoter regions of *Mst87F*, *dj* and *fzo* was much lower than at the promoter region of *CycA*, which is actively transcribed in spermatogonia (Fig. 3A and see Fig. S3A in the supplementary material). Likewise, ChIP from *bam* mutant testes with antibodies against the unphosphorylated form of Pol II (8WG16), which is the form in the preinitiation complex, revealed that Pol II occupancy at the promoter region of *Mst87F*, *dj* and *fzo* was near background and significantly lower than Pol II occupancy near the promoter of the *CycA* gene ($P<0.01$ in Fig. 3B; see Fig. S3B in the supplementary material). Similar results were obtained with antibodies (4H8) that recognize both unphosphorylated and Ser5 phosphorylated forms of Pol II (Guenther et al., 2007; Stock et al., 2007) ($P<0.05$ in Fig. 3C; see Fig. S3C in the supplementary material), suggesting that the differentiation genes do not have RNA polymerase at their promoters in precursor cells.

By contrast, ChIP with anti-H3K27me3 substantially enriched for *Mst87F*, *dj* and *fzo* compared with *CycA* in *bam* mutant testes (Fig. 3D and see Fig. S3D in the supplementary material), consistent with action of the PRC2 component E(z) on terminal differentiation genes in precursor cells. Likewise, ChIP with anti-Polycomb also enriched for *Mst87F*, *dj* and *fzo* compared with *CycA* in *bam* mutant testes (Fig. 3E and see Fig. S3E in the supplementary material), suggesting occupancy of these terminal differentiation genes by the PRC1 machinery. Together, the ChIP results suggest that the terminal differentiation genes tested lack the

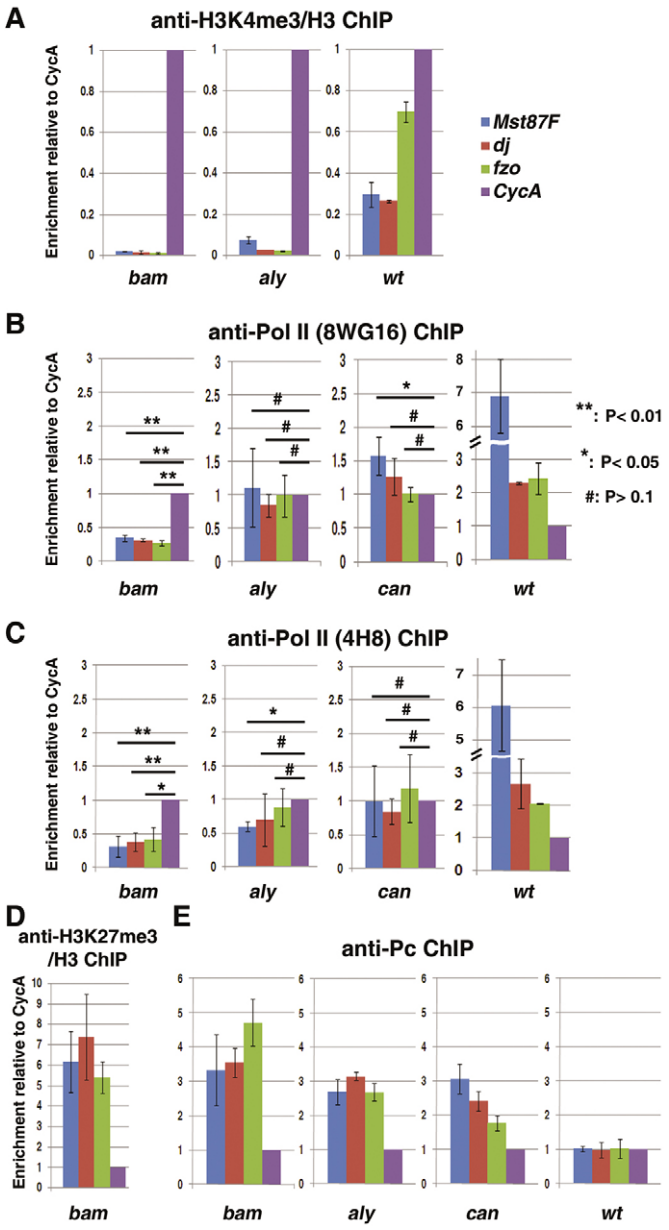

Fig. 3. Changes at the promoter regions of differentiation genes as spermatogonia become spermatocytes. Real-time PCR analysis of the enrichment of three tTAF target genes (*Mst87F*, *dj* and *fzo*) and one non-target gene (*CycA*) by ChIP using (**A**) anti-H3K4me3, (**B**) anti-RNA polymerase II (Pol II) (8WG16) specific for the form of Pol II in the preinitiation complex, which has an unphosphorylated C-terminal domain (CTD), (**C**) anti-Pol II (4H8) that recognizes both the unphosphorylated and Ser5 phosphorylated CTD of Pol II, (**D**) anti-H3K27me3, and (**E**) anti-Polycomb (Pc) from *bam*, *aly*, *can* or wild-type (*wt*) testes. To compare values across different genotypes, for each data point the level of ChIP DNA (ChIP DNA/input) at the target gene (*Mst87F*, *dj* or *fzo*) was first normalized to the level of ChIP DNA at the *CycA* gene from the same sample, and then the normalized values from at least three independent ChIP reactions per genotype were averaged. ChIP values for antibodies against modified histone H3 in A (H3K4me3) and D (H3K27me3) were further normalized to values from ChIP with anti-H3 to control for possible variation in nucleosomal distribution. **, $P<0.01$; *, $P<0.05$; #, $P>0.1$ (one sample *t*-test). Error bars indicate s.d. from three independent biological replicates.

active markers Pol II and H3K4me3, but show markers of action by PcG components in precursor cells, consistent with ChIP-seq results (Gan et al., 2010a) (see Fig. S4 in the supplementary material).

Pol II is recruited to differentiation genes in spermatocytes, but full levels of transcription await tMAC and tTAF function

As male germ cells proceed along the differentiation pathway, a Bam-dependent switch allows exit from the transit-amplifying spermatogonial stage and entry into the spermatocyte differentiation program (Insco et al., 2009). Once this switch occurs, the cells turn on expression of a number of spermatocyte-specific transcripts, including several testis-specific components of the tMAC complex and the five tTAFs. In a second transcriptional wave, tMAC and tTAFs then activate the expression of many spermatid differentiation genes, including *Mst87F*, *dj* and *fzo* (White-Cooper et al., 1998).

The switch from spermatogonial proliferation to the spermatocyte differentiation program appears to trigger the recruitment of Pol II to terminal differentiation gene promoters, perhaps poising these genes for robust expression. *Mst87F*, *dj* and *fzo* were still largely transcriptionally silent in *aly* mutant testes, which have large numbers of spermatocytes (Fig. 2). ChIP analysis indicated that H3K4me3 levels near the *Mst87F*, *dj* and *fzo* promoters were extremely low in *aly* mutant testes (Fig. 3A and see Fig. S3A in the supplementary material), comparable to the level in *bam* mutant testes. However, the relative enrichment of the *Mst87F*, *dj* and *fzo* promoter regions by ChIP with antibodies specific for the unphosphorylated form of Pol II (8WG16) increased in *aly* mutant compared with *bam* mutant testes (Fig. 3B and see Fig. S3B in the supplementary material). Indeed, in *aly* mutant testes, the relative enrichment of *Mst87F*, *dj* and *fzo* by ChIP with antibodies against the unphosphorylated form of Pol II was similar to the enrichment of *CycA*, which is actively expressed (Fig. 3B and see Fig. S3B in the supplementary material).

The unphosphorylated form of Pol II was also detected as associated with the differentiation gene promoters by ChIP from tTAF mutant testes, in which *Mst87F*, *dj* and *fzo* transcripts are detectable but still very low (Fig. 2). Upon ChIP from *can* mutant testes with antibodies against the unphosphorylated form of Pol II, enrichment of *Mst87F* was slightly higher than that of *CycA* ($P<0.05$, Fig. 3B) and enrichment of *dj* and *fzo* was similar to that of *CycA* (Fig. 3B). Enrichment of the differentiation gene promoters by ChIP with anti-Pol II was higher in wild-type than in *can* testes (Fig. 3B,C). By contrast, in *bam* mutant testes, enrichment of the three terminal differentiation genes by ChIP with anti-unphosphorylated Pol II (8WG16) was substantially lower than that of *CycA* ($P<0.01$, Fig. 3B). ChIP using the antibody (4H8) that recognizes both unphosphorylated and Ser5 phosphorylated forms of Pol II gave similar results. Enrichment of the three terminal differentiation genes by ChIP with the 4H8 anti-Pol II antibody in *aly* or *can* mutant testes was similar to that of the active *CycA* gene promoter (Fig. 3C), with the exception that enrichment of *Mst87F* in *aly* mutant testes was slightly lower than that of *CycA*. By contrast, enrichment of the differentiation gene promoters by ChIP with the 4H8 anti-Pol II antibody was significantly lower than that of *CycA* in *bam* mutant testes ($P<0.01$ for *Mst87F* and *dj*, $P<0.05$ for *fzo*, Fig. 3C). The presence of unphosphorylated Pol II (recognized by the 8WG16 antibody) or Ser5 phosphorylated Pol II (recognized by the 4H8 antibody) at the differentiation genes in *aly* mutant testes, in which the genes are

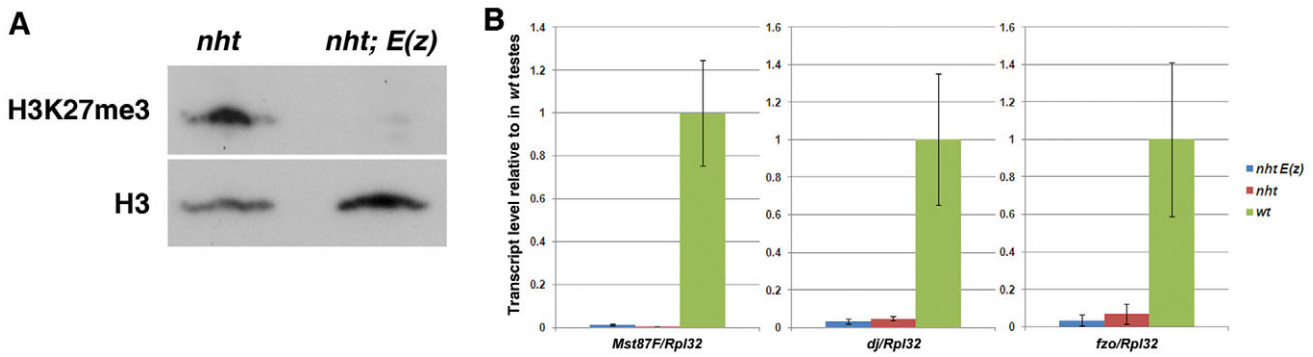

Fig. 4. Inactivation of E(z) is insufficient to turn on terminal differentiation genes in a tTAF mutant background. (**A**) Immunoblot analysis showing the H3K27me3 band in *nht* testes and the drastic reduction of H3K27me3 in *nht; E(z)* double-mutant testes. H3 provided a loading control. (**B**) Quantitative RT-PCR analysis of *dj*, *fzo* and *Mst87F* transcript levels in *nht; E(z)*, *nht* and wild-type (*wt*) testes, normalized to *Rpl32* from the same sample. The *nht; E(z)*, *nht* and wild-type flies were all raised to adulthood at permissive temperature and then shifted to 29°C (restrictive temperature) for 7 days. The relative level of each gene in the wild type was set to 1 to allow comparison. Error bars indicate s.d. from three independent biological replicates.

still largely silent, is reminiscent of the stalled Pol II observed at many transcriptionally quiescent genes in ESCs (Guenther et al., 2007; Stock et al., 2007), as well as in *Drosophila* embryos (Muse et al., 2007; Zeitlinger et al., 2007). Enrichment of the differentiation gene promoters by ChIP with anti-Pol II was still higher in wild-type than in *can* mutant testes (Fig. 3B,C).

Presence of the PRC1 complex near the promoter region of the terminal differentiation genes might contribute to their transcriptionally silent state even in the presence of Pol II in *aly* or tTAF mutant testes. ChIP with antibodies against the PRC1 component Polycomb enriched for the promoter regions of the three terminal differentiation genes 3- to 4-fold relative to the *CycA* promoter in *bam* testes and by 2- to 3-fold relative to *CycA* in *aly* or *can* mutant testes. By contrast, in wild-type testes, where these genes are fully expressed, enrichment for the three differentiation genes was comparable to that of *CycA* (Fig. 3E and see Fig. S3E in the supplementary material). The level of Polycomb at the differentiation genes in spermatocytes might be even lower than that indicated by the ChIP results using entire wild-type testes (Fig. 3E and see Fig. S3E in the supplementary material), as wild-type testes also contain precursor cells, in which the genes are highly occupied by Polycomb (see *bam* data in Fig. 3E and Fig. S3E in the supplementary material). However, because spermatocytes significantly outnumber precursor cells in wild-type testes, we can still gain information on the chromatin state of differentiation genes using whole testes, especially for changes in which a particular chromatin mark or protein occupancy appears or turns on as cells switch from precursor to spermatocyte. The relative enrichment of Polycomb at the differentiation genes in this study (~3- to 4-fold enrichment of *Mst87F* in *can* mutant compared with wild-type testes) was comparable to that reported in a previous study (Chen et al., 2005), when the results were plotted in the same way (see Fig. S5 in the supplementary material).

Inactivating the key PRC2 component E(z) in the absence of tTAF is insufficient to turn on terminal differentiation genes

The wild-type function of tTAFs is required to reduce the occupancy of Polycomb at the differentiation gene promoters (Fig. 3E) and to turn on robust expression of the differentiation genes (Fig. 2) (Chen et al., 2005). Analysis of a double-mutant strain generated by combining the tTAF mutant *no hitter* (*nht*, *TAF4L*) with an *E(z)* conditional allele, $E(z)^{61}$, which was used previously to inactivate E(z) activity at restrictive temperature (Wang et al., 2004b), indicated that the action of tTAFs is required for more than just abolishing silencing by PcG. When *nht;* $E(z)^{61}$ double-mutant flies were grown to adulthood at permissive temperature and then shifted to 29°C (the restrictive temperature) for 7 days, little E(z) function remained, as H3K27me3 was almost undetectable in the double-mutant testes extracts, as compared with the robust levels in testes from sibling control *nht* mutant males that were raised under exactly the same conditions (Fig. 4A). However, no significant change in transcript levels for *Mst87F*, *dj* or *fzo* was detected by quantitative RT-PCR analyses in *nht; E(z)* double-mutant testes as compared with *nht* single-mutant sibling controls (Fig. 4B). In both *nht* and *nht; E(z)* mutant testes, transcript levels for the differentiation genes were very much reduced compared with those of wild-type testes shifted through the same temperature regimen in parallel, suggesting that inactivation of E(z) is insufficient to turn on differentiation gene expression without tTAF function.

tMAC function is required for the recruitment of tTAFs to target genes

A number of spermatid differentiation genes require wild-type function of both tMAC and the tTAFs for normal expression in spermatocytes (White-Cooper et al., 1998). Results from ChIP analysis of testis extracts with antibodies directed against the TAF8L Sa indicated that the proper function of spermatocyte-specific components of tMAC is required for the tTAFs to properly associate with the promoter regions of these differentiation genes. ChIP of wild-type testes with antibodies against Sa enriched for *Mst87F*, *dj* and *fzo* ~3- to 6-fold over the level of the control gene *CycA*, which is transcribed in spermatocytes independently of tTAFs (Fig. 5A). By contrast, although Sa protein was present in *aly* mutant spermatocytes (Fig. 5C′), ChIP of *aly* mutant testes with anti-Sa did not significantly enrich for any of these three target genes compared with *CycA* (Fig. 5A).

The proper localization of tTAFs and Polycomb depends on tMAC action

The wild-type function of tMAC is also required for proper subnuclear localization of tTAFs and PRC1 proteins in spermatocytes. In wild-type spermatocytes, immunofluorescence

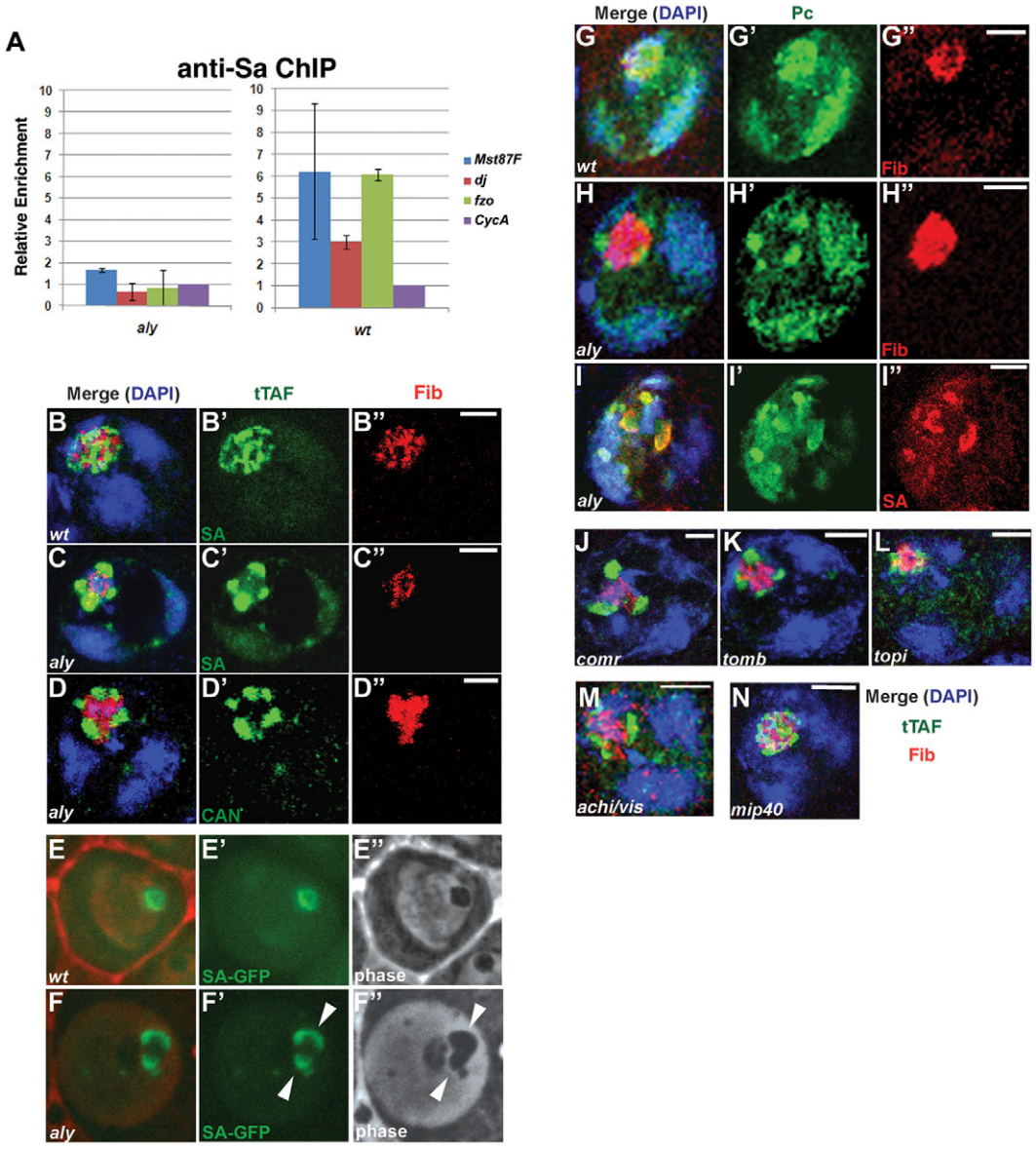

Fig. 5. tMAC function is required for the recruitment of tTAFs to target genes and for proper subnuclear localization of tTAFs and Polycomb. (**A**) Real-time PCR analysis of ChIP using anti-Sa from *aly* and wild-type (*wt*) *Drosophila* testes. Relative enrichment by ChIP was computed as the ratio of ChIP DNA/input at the spermatid differentiation genes *Mst87F*, *dj* and *fzo* to ChIP DNA/input for the control gene *CycA*, which is expressed in both spermatogonia and spermatocytes independently of tMAC and tTAFs. Error bars indicate s.d. from three independent biological replicates). (**B-I″**) Localization of (B-F″) tTAFs and (G-I″) Polycomb (Pc) in wild-type and *aly* mutant spermatocytes by immunostaining of fixed cells (B-D″,G-I″) or in epifluorescent images of live cells (E-F″). Blue, DAPI; green, tTAF (Sa-GFP in B′,C′,E′,F′ or anti-Can in D′) or Pc-GFP (G′,H′,I′); red, the nucleolar marker anti-Fibrillarin (Fib in B″,C″,D″,G″,H″) or pseudo-color of the phase (E,F) or anti-Sa (I″). (**J-N**) Localization of Sa protein in spermatocytes mutant for other tMAC components: (J) *comr*, (K) *tomb*, (L) *topi*, (M) *achi/vis*, (N) *mip40*. Blue, DAPI; green, tTAF; red, anti-Fib. Scale bars: 4 μm.

analysis revealed colocalization of tTAF proteins (e.g. Sa and Can) with Polycomb and other PRC1 components to a subdomain within the nucleolus, interdigitated with the nucleolar marker Fibrillarin (Fib) (Fig. 5B-B″) (Chen et al., 2005). By contrast, in *aly* mutant spermatocytes, the tTAFs were concentrated in lobe-shaped domains outside of, but next to, the Fib-enriched nucleolar region (Fig. 5C-C″,D-D″). Similar results were observed for the TAF4L Nht (data not shown) and for the TAF6L Meiosis I arrest (Mia) (Metcalf and Wassarman, 2007). The perinucleolar lobes containing mislocalized tTAFs were also apparent in freshly squashed live samples viewed by phase contrast microscopy, in which they appeared as phase-dark structures extending from the nucleolar periphery (Fig. 5F-F″, compare with the wild-type spermatocyte in Fig. 5E-E″). A substantial amount of the Polycomb protein in *aly* mutant spermatocytes was also localized to the same perinucleolar domains (Fig. 5H-H″, Fig. 4I-I″). Some Polycomb still retained association with condensing chromatin in *aly* mutant spermatocytes (Fig. 5H,H′,I,I′), as in wild-type spermatocytes (Fig. 5G,G′).

The localization of tTAFs to the perinucleolar lobes in *aly* mutant spermatocytes was not due to total disruption of the nucleolus, as phase contrast microscopy (Fig. 5F″) and staining for the nucleolar marker Fib (Fig. 5C″,D″) indicated that the nucleolus

was present. It is possible that the wild-type function of Aly is required to maintain proper nucleolar architecture in spermatocytes (Metcalf and Wassarman, 2007), and that collapse of nucleolar architecture in *aly* mutant spermatocytes squeezed the tTAF-containing subcompartment to the perinuclear lobes.

Similar mislocalization of tTAFs to the lobed perinucleolar domains was also observed in spermatocytes mutant for other testis-specific components of the tMAC complex, including *cookie monster* (*comr*), *tombola* (*tomb*), *matotopetli* (*topi*) and *achintya/vismay* (*achi/vis*; *achi* – FlyBase) (Fig. 5J-M). However, even though Myb-interacting protein 40 (Mip40) has been identified biochemically as a component of tMAC (Beall et al., 2007), tTAFs were localized within the nucleolus, interdigitated with the Fib-positive subcompartment, in *mip40* mutant spermatocytes, similar to in the wild type (Fig. 5N), suggesting that Mip40 has different role(s) than the other tMAC components in spermatocytes.

DISCUSSION

Sequential developmentally regulated steps lead to the activation of terminal differentiation genes

Our results suggest a stepwise series of developmentally programmed events as terminal differentiation genes convert from a transcriptionally silent state in precursor cells to full expression in differentiating spermatocytes (Fig. 6). In precursor cells, differentiation genes are repressed and associated with background levels of hypophosphorylated Pol II and H3K4me3. These genes also display elevated levels of H3K27me3 and Polycomb at the promoter region, suggesting that they are acted upon by the PcG transcriptional silencing machinery. Notably, the differentiation genes studied in precursor cells here did not show the hallmark bivalent chromatin domains enriched for both the repressive H3K27me3 mark and the active H3K4me3 mark that have been characterized for a cohort of differentiation genes in mammalian ESCs (Bernstein et al., 2006).

The cell fate switch from proliferating spermatogonia to the spermatocyte differentiation program initiates both global and local changes in the transcriptional regulatory landscape, starting a cell type-specific gene expression cascade that eventually leads to robust transcription of the terminal differentiation genes. Globally, soon after the switch from spermatogonia to spermatocytes, core subunits of the PRC2 complex are downregulated, including E(z), the enzyme that generates the H3K27me3 mark. Locally, after male germ cells become spermatocytes, Pol II accumulates at the terminal differentiation gene promoters, although these genes still remain transcriptionally silent, with low H3K4me3 and high Polycomb protein levels near their promoters.

The next step awaits the expression of spermatocyte-specific forms of core transcription machinery and chromatin-associated regulators, including homologs of subunits of both the general transcription factor $TF_{II}D$ (tTAFs) and the MIP/dREAM complex (Aly and other testis-specific components of tMAC) (Fig. 6B). The tMAC complex acts either locally or globally, perhaps at the level of chromatin or directly through interaction with tTAFs, to allow recruitment of tTAFs to promoters of target terminal differentiation genes. The action of tTAFs then allows full and robust transcription of the terminal differentiation genes, partly by displacing Polycomb from their promoters.

Strikingly, the two major PcG protein complexes appear to be regulated differently by the germ cell developmental program: whereas the PRC2 components E(z) and Su(z)12 are downregulated, the PRC1 components Polycomb, Polyhomeotic and dRing continue to be expressed in spermatocytes. The global downregulation of the epigenetic 'writer' E(z) in spermatocytes

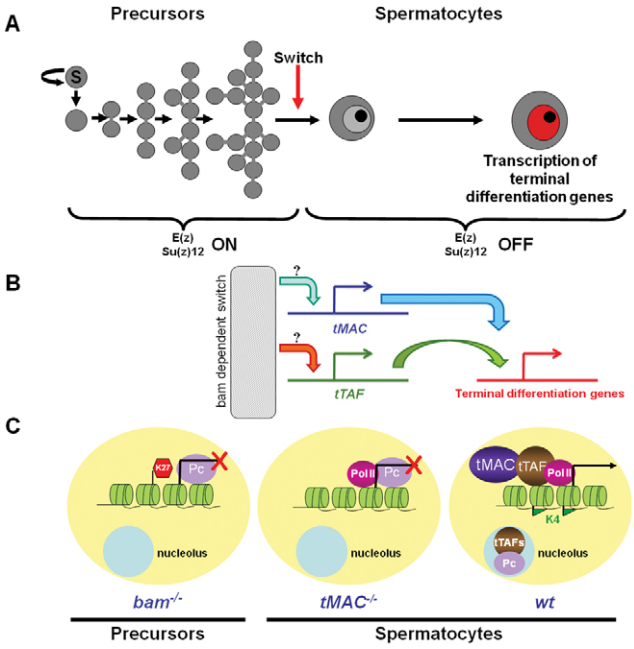

Fig. 6. Model for the developmentally programmed steps that oppose PcG repression and turn on terminal differentiation gene expression. (**A**) The early stages of spermatogenesis in *Drosophila*. Precursors include stem cells (S) and mitotically dividing spermatogonia. Spermatocytes include early spermatocytes (light gray nucleus) prior to the expression of the terminal differentiation genes (red nucleus). The PRC2 components E(z) and Su(z)12 are highly expressed in precursor cells, including germline stem cells (S) and spermatogonial cells. (**B**) Potential transcription waves in developing spermatocytes. Upon the Bam-dependent switch from spermatogonia to spermatocytes, unknown factor(s) (question marks) turn on testis-specific tMAC and tTAF components in early spermatocytes. The action of tMAC and tTAFs is subsequently required for robust transcription of spermatid differentiation genes. (**C**) Potential chromatin states in precursor cells (left; analyzed with *bam* mutant testes), spermatocytes lacking tMAC function (middle; analyzed with *aly* mutant testes) and mature spermatocytes (right; analyzed with wild-type testis). K4, H3K4me3; K27, H3K27me3.

might facilitate displacement of the epigenetic 'reader', the PRC1 complex, from the differentiation genes, with the local action of tTAFs at promoters serving to select which genes are relieved of PRC1. In addition, the tTAFs act at a second level to regulate Polycomb by recruiting and accompanying Polycomb and several other PRC1 components to a particular subnucleolar domain in spermatocytes (Fig. 6C) (Chen et al., 2005). It is not yet known whether sequestering of PRC1 to the nucleolus by tTAFs plays a role in the activation of terminal differentiation genes, perhaps by lowering the level of PRC1 that is available to exchange back on to differentiation gene promoters. Conversely, recruitment of PRC1 to the nucleolar region might have a separate function, such as in chromatin silencing in the XY body as observed in mammalian spermatocytes (Baarends et al., 2005; Takada et al., 2007).

Stalled Pol II and the developmental control of gene expression

Our findings indicate that, upon the switch from spermatogonia to spermatocytes, the terminal differentiation genes go through a poised state, marked by presence of both active Pol II and repressive Polycomb, before the genes are actively transcribed. Stalled Pol II

and abortive transcript initiation are emerging as a common feature in stem/progenitor cells. This mechanism may prime genes to rapidly respond to developmental cues or environmental stimuli (Muse et al., 2007; Zeitlinger et al., 2007). Stalled Pol II could represent transcription events that have initiated elongation but then pause and await further signals, as in the regulation of gene expression by the androgen receptor (Zhao et al., 2008) or by heat shock (Lis, 1998; Rasmussen and Lis, 1995; Rougvie and Lis, 1988). Alternatively, Pol II might be trapped at a nascent preinitiation complex, without melting open the DNA, as found in some instances of transcriptional repression by Polycomb (Dellino et al., 2004). Although our ChIP analyses did not have the resolution to distinguish whether Pol II was stalled at the promoter or had already initiated a short transcript, the results with antibodies specific for unphosphorylated Pol II suggest that Pol II is trapped in a nascent preinitiation complex. The PRC1 component dRing has been shown to monoubiquitylate histone H2A on Lys119 near or just downstream of the transcription start site (Wang et al., 2004a). We propose that in early spermatocytes, before expression of the tTAFs and tMAC, the local action of PRC1 in causing H2AK119ub at the terminal differentiation gene promoters might block efficient clearing of Pol II from the preinitiation complex and prevent transcription elongation.

Gene-selective transcriptional regulation by cell type-specific forms of the core transcription machinery

Removal of PRC1 from the promoter and full expression of the terminal differentiation genes in spermatocytes require the expression and action of tMAC and tTAFs. Cell type-specific homologs of $TF_{II}D$ subunits have been shown to act gene-selectively to control developmentally programmed gene expression. For example, incorporation of one subunit of the mammalian TAF4b variant into $TF_{II}D$ strongly influences transcriptional activation at selected promoters, directing a generally expressed transcriptional activator to turn on tissue-specific gene expression (Liu et al., 2008).

The local action of the tTAFs to relieve repression by Polycomb at target gene promoters provides a mechanism that is both cell type specific and gene selective, allowing expression of some Polycomb-repressed genes while keeping others silent. Similar developmentally programmed mechanisms may also reverse PcG-mediated epigenetic silencing in other stem cell systems. Indeed, striking parallels between our findings and recent results from mammalian epidermis (Ezhkova et al., 2009; Sen et al., 2008) suggest that molecular strategies are conserved from flies to mammals. In mouse epidermis, the mammalian *E(z)* homolog *Ezh2* is expressed in stem/precursor cells at the basal layer of the skin. Strikingly, as we observed for *E(z)* and *Su(z)12* in the *Drosophila* male GSC lineage, the *Ezh2* level declines sharply as cells cease DNA replication and the epidermal differentiation program is turned on. Overexpression of *Ezh2* in epidermal precursor cells delays the onset of terminal differentiation gene expression (Ezhkova et al., 2009), and removal of the Ezh2-generated H3K27me3 mark by the Jmjd3 (Kdm6b) demethylase is required for epidermal differentiation (Sen et al., 2008).

In particular, our results suggest a possible explanation for the conundrum that, although PcG components are bound at many transcriptionally silent differentiation genes in mammalian ESCs, loss of function of PcG components does not cause loss of pluripotency but instead causes defects during early embryonic differentiation (Boyer et al., 2006; Chamberlain et al., 2008; Surface et al., 2010). In *Drosophila* male germ cells, events during the switch from precursor cell proliferation to differentiation are required to recruit Pol II to the promoters of differentiation genes. Without this differentiation-dependent recruitment of Pol II, loss of Polycomb is not sufficient to precociously turn on terminal differentiation genes in precursor cells. Rather, Polycomb that is pre-bound at the differentiation gene promoters might serve to delay the onset of their transcription after the mitosis-to-differentiation switch. Robust transcription must await the expression of cell type- and stage-specific components of the transcription machinery. These might in turn guide gene-selective reversal of Polycomb repression to facilitate appropriate differentiation gene expression in specific cell types.

Acknowledgements

We thank P. Dimario and M. Pollard for anti-Fibrillarin; J. Muller for $E(z)^{731}$ FRT2A flies and anti-Su(z)12 antibody; R. Jones for anti-E(z) antibody; R. Paro for Pc-GFP flies and anti-Pc; R. Kingston and A. Saurin for anti-Pc; H. White Cooper for tMAC mutant strains and Q. Gan for analyzing and depositing microarray data. C.L. was supported in part by a Stanford Dean's Fellowship and J.R.M.P. by an NSF Predoctoral Fellowship. This work was supported by NIH K99/R00 HD055052 Pathway to Independence Award and Research Grant No. 05-FY09-88 from the March of Dimes Foundation to X.C. and by NIH 3 R01 GM061986 to M.T.F. Deposited in PMC for release after 12 months.

Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.056572/-/DC1

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