4 Transcriptional and Post-transcriptional Regulation of *Drosophila* Germline Stem Cells and Their Differentiating Progeny

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Abstract

In this chapter we will concentrate on the transcriptional and translational regulations that govern the development and differentiation of male germline cells. Our focus will be on the processes that occur during differentiation, that distinguish the differentiating population of cells from their stem cell parents. We discuss how these defining features are established as cells transit from a stem cell character to that of a fully committed differentiating cell. The focus will be on how GSCs differentiate, via spermatogonia, to spermatocytes. We will achieve this by first describing the transcriptional activity in the differentiating spermatocytes, cataloguing the known transcriptional regulators in these cells and then investigating how the transcription programme is set up by processes in the progentior cells. This process is particularly interesting to study from a stem cell perspective as the male GSCs are unipotent, so lineage decisions in differentiating progeny of stem cells, which occurs in many other stem cell systems, do not impinge on the behaviour of these cells.

Keywords

Drosophila • Spermatocyte • Spermatogenesis • Testis • TMAC

4.1 Brief Introduction to Anatomy of Drosophila Testes

The ongoing capacity of males of many species to produce sperm throughout adulthood depends upon the presence and normal behaviour of populations of stem cells within the testes. The anatomy of the testis is described in detail in [1–3]. Within the *Drosophila* testes there are two stem cell populations whose function is essential for normal fertility (reviewed in [4, 5]). These stem cells reside in classical niche setting at the apical tip of the blind-ended tubular testis [6]. The testis sheath comprises a layer of pigment cells overlying a muscular layer, supported on a basal lamina. At the testis tip, on the lumenal side of the basal lamina is a tightly clustered group of

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about 20, post-mitotic, hub cells. The two stem cell populations – germline stem cells (GSC) and cyst stem cells (CySC, formerly referred to as cyst progenitor cells (CPCs)) adhere to the hub and thus form a rosette around this structure. Division of a stem cell typically results in displacement of one daughter from the hub, while the other daughter remains hub-associated. The division of a GSC is accompanied by division of CySCs, such that the displaced stem cell daughter can become encysted by two CySC daughters, termed cyst cells, and differentiate into a spermatogonium. Mitotic division of this spermatogonial cell is not accompanied by division of the cyst cells, and results in the formation of a cyst comprising two cyst cells and two spermatogonia. Further spermatogonial divisions amplify the number of germ cells within the cyst, until the transition from spermatogonial to spermatocyte cell identity occurs after the fourth division (i.e. when there are 16 germline cells). These primary spermatocytes initiate a differentiation programme characterised by high levels of tissue-specific gene expression and cell growth. The primary spermatocytes then undergo the meiotic divisions, and the resultant 64 spermatids elongate along the longitudinal axis of the testis, before maturing as sperm and passing into the seminal vesicle at the basal end of the testis tube.

4.2 Transcriptional and Post-transcriptional Regulation in Male Germline Cells

A broad understanding of the gene expression underlying spermatogenesis can be achieved by comparing the transcriptome of whole adult testes to that of the whole fly, or of other specific organs. Microarray datasets have been generated by several groups, but the most comprehensive in terms of analysis of many different organ systems is the FlyAtlas data (www.flyatlas.org) [7–9]. Using Affymetrix microarrays they detected expression of the transcripts corresponding to 85 % of the 18,880 probe sets (representing 18,500 transcripts) on this array in at least one adult tissue. Approximately 60 % of the probe sets were positive with the testis sample (i.e. approx 11,300 transcripts), 1,317 were exclusively detected in testes, and 2,079 were predominantly detected in testes. Thus, about 10 % of the genes expressed in testes are expressed only in testes, and a further 10 % are expressed mostly in testes [8]. Using RNA in situ hybridisation we have determined, rather unsurprisingly, that the vast majority of these testis-specific transcripts are expressed in the male germline cells [10]. The most common pattern is that the transcript is first detected in primary spermatocytes, and then it persists through the meiotic divisions, to be degraded during spermatid elongation [11]. The proteins encoded by these transcripts often encode proteins critical for sperm function, but not needed in other cells, for example the protamines, which replace histones in packaging sperm chromatin. Thus, the key question in understanding the differentiation of stem cells into sperm is understanding what keeps the spermiogenic genes silent in the stem cells and transit amplifying population, and what turns them on in the spermatocytes.

Analysis of dynamic changes in transcriptional patterns during a cellular differentiation programme relies upon being able to compare cells from the relevant populations. There are typically only 5–9 GSCs per testis in adult *Drosophila*. Each testis will contain 8–10 cysts of spermatogonia, and 40 or more cysts of spermatocytes at various stages of maturation. Each testis also contains 40–50 bundles of elongating spermatids. Spermatids are relatively transcriptionally inactive, relying predominantly on stored mRNAs for their protein production [11, 12]. Thus the transcript content of spermatids is very similar to that of spermatocytes. Spermatocytes and spermatids are much larger than spermatogonia. Cytological techniques (such as RNA in situ hybridisation or antibody staining) can reveal the developmental expression profile of genes, one at a time, as mentioned above [10]. Thus the transcriptome deduced from analysis of whole testis samples is strongly biased towards spermatocyte transcripts, with limited input from earlier differentiation stages and from somatic cell population. To analyse and compare gene expression patterns

in stem cells, spermatogonia and spermatocytes with molecular biological assays most researchers have used genetic techniques to enrich for specific cell types in testes. Screens of male sterile mutants over many years have produced a relatively small set of mutant strains in which the stem cell populations of testes are intact, but differentiation is abrogated.

Mutation of either of *bag of marbles* (*bam*) or *benign gonial cell neoplasm* (*bgcn*) results in the absence of differentiated cells in both testes and ovary and an overproliferation of undifferentiated cells, including GSCs and transit-amplifying spermatogonia cells [13, 14]. *bgcn* encodes an RNA binding protein [15], while Bam protein has no motifs indicative of its function [16]. Bam and Bgcn proteins have been shown to be in the same complex in the *Drosophila* ovary, and in this complex they regulate *nos* translation and thus differentiation [17–19]. While there have been no experiments directly assaying whether a similar complex is present in testes, it is likely that they work together to regulate translation of unknown targets in spermatogonia. *bam* and *bgcn* transcription is initiated in spermatogonia, and accumulation of these proteins is critical for promoting the spermatogonial to spermatocyte transition [20]. Thus, in the mutant testes the transition fails, and the predominant cell type in these testes is spermatogonia. Since *Drosophila* spermatogonia are capable, at least under certain conditions, of de-differentiating back to stem cells [21], mutants, such as *bam*, that accumulate stages up to and including spermatogonia are an excellent source of material enriched for cells with stem cell capacity. These testes can then be compared to wild-type testes, which are enriched for differentiating cells, to reveal gene expression profile changes associated with differentiation [13, 22, 23].

A second set of mutants abrogate differentiation at the primary spermatocyte stage. The germ cells in these "meiotic arrest" mutant testes passage normally through the stem cell and spermatogonial phases, and develop into morphologically relatively normal primary spermatocytes [24]. These spermatocytes grow, however they fail to enter the meiotic divisions, and also fail to initiate spermatid differentiation. For most of the characterised meiotic arrest mutants these differentiation defects are due to defects in the spermatocyte-specific transcriptional program [25]. The meiotic arrest genes have been reviewed recently, so we refer the reader to these papers and spare much of the detail here, to concentrate on new findings and integrating information [26, 27].

4.3 Transcription Regulators That Activate Expression of Differentiation Genes

Most of the characterised meiotic arrest genes fall phenotypically into two distinct classes. The *aly*-class, named after its founder member *always early* (*aly*) comprises *aly*, *comr*, *achi/vis* (these genes are a very recent duplicate pair and either one can perform the function), *tomb* and *topi*. These mutants are classified together on the basis of the transcriptional defects seen in mutant primary spermatocytes [25, 28–32]. The mutant cells fail to activate transcription of a large number of genes, predominantly those that are expressed exclusively, or almost exclusively in testes. *aly*-class mutant primary spermatocytes have extremely low, or undetectable, levels of many target transcripts, including *Mst87F*, *dj*, *fzo*, *twe* and *CycB* (although the expression of *CycB* in the mutant spermatogonia is normal). In contrast the *can*-class, comprising *can*, *nht*, *mia*, *rye* and *sa* has a somewhat less severe effect on transcription of target genes [25]. As with the *aly*-class, *can*-class mutant spermatocytes have defects in accumulation of many predominantly testis-specific transcriptions, however they regulate fewer genes, and transcripts from targets are typically detectable, albeit at much lower levels than normal, in mutant testes. *can*-class mutant spermatocytes express very low levels of *Mst87F*, *dj* and *fzo* mRNAs, and normal levels of *twe* and *CycB* mRNAs [25]. Relative expression levels of all the genes discussed here, at various stages of normal differentiation and in various mutant backgrounds is indicated in Table 4.1.

These characteristic gene expression profiles have failed to classify a few more recently identified meiotic arrest mutants. *wuc* deficient

Table 4.1 Relative levels of gene expression in wild type germ line differentiation and mutant classes

	Mst87F	*dj*	*fzo*	*twe*	*CycB*	*LS2*	*Smn*
WT testis	+++++	+++++	+++++	+++++	+++++	+++++	+++++
Spermatogonia	–	–	–	–	++	–	+++++
Spermatocyte	+++++	+++++	+++++	+++++	+++++	+++++	+
bam	–	–	–	–	++	–	+++++
aly-class	–	–	–	–	++[a]	+++++	+++++
can-class	+	+	+	+++++	+++++	+++++	+++++
wuc	+++	+++	+++	+++++	+++++	+++++	+++++
wuc; *aly*	+	+	+	+++++	+++++	+++++	+++++
Nurf301ΔC	+++++	+++++	+	+++++	+++++	ND	ND
thoc5	+++++	+++++	+++++	+++++	+++++	ND	ND

[a]The expression of CycB in aly-class mutant testes is restricted to the spermatogonial cells

spermatocytes, induced by RNAi against the gene, show only a mild reduction in expression of *Mst87F*, *dj* and *fzo*, and normal expression of *twe* and *CycB* [33]. We also see this expression profile in *mip40* mutant spermatocytes (HW-C unpublished data, [34]). Testes from males homozygous for alleles of *Nurf301* which can only produce a C-terminally truncated form of this protein display meiotic arrest, and show a very dramatic defect in *fzo* expression, but no effect on *Mst87F*, *dj*, *twe* and *CycB* [35]. Finally, *thoc5* mutants show fully penetrant meiotic arrest, but no defects in expression of *Mst87F*, *dj*, *fzo*, *twe* or *CycB* [36]. Unlike other meiotic arrest mutants, *thoc5* spermatocytes show defects in nucleolar organisation. The protein products of these meiotic arrest genes are components of four distinct complexes, all acting within the nuclei of primary spermatocytes.

The *aly*-class gene products, along with Wuc and Mip40, assemble into the Testis Meiotic Arrest Complex (TMAC) [34] (see later). This complex is paralogous to the MybMuvB/dREAM complex that has been purified from somatic cells, and that is implicated predominantly in transcriptional repression [37–39]. The complex is conserved in evolution, and orthologous complexes have been purified from *C. elegans* and humans [40–42]. It is likely, but not proven, that several forms of TMAC exist within spermatocytes, and that individual complexes with different subunit compositions have different biochemical functions. At least four TMAC subunits (Topi, Tomb, Comr, Achi/Vis) possess predicted DNA binding motifs, although their DNA binding capacity has not been tested directly. At a gross light microscopy level all the known TMAC subunits co-localise on the chromatin of primary spermatocytes [29–34, 43]. While the net output of TMAC activity is transcriptional activation of testis-specific genes, it has recently been shown that this is not as straightforward as previously thought, [33] see later.

The *can*-class gene products encode paralogues of the TATA-binding protein associated factor (TAF) components basal transcription factor complex $TF_{II}D$, and are sometimes referred to collectively at the testis TAFs (tTAFs) [44, 45]. Although a direct interaction has been detected between Rye and Nht, a complete complex containing all the tTAFs has not yet been reported. However the protein localisations are identical for all those for which data is available. It is most likely that they assemble, with a testis-enriched splice isoform of TAF1, into an alternate form of $TF_{II}D$ [46]. The canonical function for $TF_{II}D$ is recruitment of the pre-initiation complex to the promoter (reviewed in [47]), although this does not seem to be the primary function performed by the tTAFs in spermatocytes (see later).

NURF301 is a subunit of the NURF complex, a chromatin remodelling complex that uses ATP to slide nucleosomes along chromatin. NURF301 is uniquely found in this complex, and is likely to be responsible for its targeting to specific chromatin regions [48]. Three transcript isoforms of NURF301 are produced, one of which lacks the C-terminal region of the protein, including two

PHD fingers and the bromodomain. These motifs are responsible for conferring the ability to bind histone H3 tails that have a tri-methyl modification on lysine 4 (H3K4me3), as well as histone H4 acetylated on lysine 16 (H4K16Ac). Mutants for a specific non-sense allele (*Nurf301ΔC*) produce a truncated protein that is similar to the natural short isoform. These animals are viable, but male and female sterile, with the males displaying meiotic arrest testes, indicating that the ability of the NURF complex to recognise H3K4me3 and H4K16Ac is required only in the germline [35].

Finally, Thoc5 is a subunit of the THO complex, which acts co- and post-transcriptionally to promote transcript elongation and mRNA nuclear export (reviewed in [49]). Thoc5 and other THO subunits localise to a dot adjacent to the primary spermatocyte nucleolus, and display abnormal nucleolar morphology [36]. Given that no defects were reported in accumulation of any of the testis transcripts tested the role, if any, of this complex in transcription or mRNA processing or the testis-specific transcripts is not clear, and so we will not discuss it further. Note that the protein Aly referred to in the RNA export pathway literature is also known as REF, and is not, (in this context) the product of the *aly* (*always early*) meiotic arrest gene.

4.4 Chromatin Architecture at Testis-Specific Promoters in Spermatogonia

A common theme underlying differentiation of cell types is modulation of the epigenetic state of particular chromatin regions, and the *Drosophila* male germline is no exception. Chromatin state comprises two distinct characteristics, namely nucleosome position and histone modification status. Nucleosome position is controlled by chromatin remodelling factors, for example NURF, which position nucleosomes with high precision, while the modification status is determined by the antagonistic actions of histone modifying enzymes, for example histone acetyl transferases and histone deacetylases [50].

High expression levels of a set of chromatin remodelling factors has been detected in *bam* mutant testes, compared to wild type testes, indicative of extensive remodelling in spermatogonia [13]. Specifically spermatogonia show high expression of BAP60 and BAP55 subunits of the BAP complex, which is related to the ATP-dependent SWI/SNF complex. Similarly they also show elevated expression of Nurf-38 when compared to fully differentiated wild type testes. It is likely that this dynamic regulation of chromatin structure probably regulates and maintain the undifferentiated status of male GSC and transit-amplifying spermatogonial cells [13]. Remodelling of chromatin structures would then be implicated in the loss of the undifferentiated cell state, and with commitment to differentiation.

Using *bam* testes and ChIP-seq it has also been possible to determine the chromatin state at promoters of differentiation genes in uncommitted spermatogonia [23]. The key finding was that differentiation genes in these undifferentiated-cell-enriched testis are either marked with the repressive modification, H3K27me3 but not the activation-associated modification H3K4me3, or they lacked both of these marks [23]. This is in contrast to the presence of both these marks at differentiation genes that are poised for expression in other stem cell systems such as ESCs. Previous studies showed that in both Drosophila embryos and mammalian ESCs, differentiation genes can be poised for expression by association of RNA pol II [51, 52]. Thus, these genes are ready for transcription once they receive differentiation stimuli. In contrast to the situation in these other systems, it appears that the most spermatid differentiation genes in undifferentiated-cell-enriched testis of Drosophila are not poised for transcription. They demonstrate no significant binding of Pol II, and carry only repressive chromatin marks [23].

The epigenetic transcriptional silencing mechanism found in many undifferentiated cells is regulated by the Polycomb group (PcG) [53]. PcG involves at least two interacting multiprotein complexes known as Polycomb repressive complex 1 (PRC1), PRC2 [54]. PRC1 complex is composed of a core quartet of PcG proteins,

Pc, RING, Psc and Ph. Pc (Polycomb) contains a chromodomain, which specifically binds to H3k27me3. PRC2 contains E(z), a SET domain protein with histone H3 methytransferase activity [54]. The histone methyl-transferase function of E(z) is activated when is assembled in PRC2 with Su(z)12, p55 CAF1 and Esc (or Escl). PRC2 specifically methylates H3K27, and thus initiates the formation of the repressive chromatin mark to which PRC1 binds. Unsurprisingly, given the presence of the H3K27me3 mark on many differentiation genes in spermatogonia, Pc is also found enriched at these promoters [53].

Thus the promoters of differentiation genes in spermatogonia are in a fully repressed state. The promoters of differentiation genes in primary spermatocytes are obviously highly active. They are associated with RNA polymerase II and H3K4me3, show little association with Pc, and lack H3K27me3. How then is this final active state achieved?

4.5 Stepwise Changes Lead to Activation of Differentiation Genes

In Drosophila testes, PRC2 components E(z) and Su(z)12 are expressed in the GSCs and spermatogonia, and their expression levels decrease dramatically as cells progress into the spermatocyte stage. This decrease in E(z) and Su(z)12 protein levels correlates extremely well with the onset of expression of the tTAFs [53]. The H3K27me3 modification promoted by these factors is detectable by immunostaining in GSCs, spermatogonia and spermatocytes, and declines with much slower dynamics that the PRC2 components [53]. PRC1 components remain detectable at high levels in primary spermatocytes. H3K27me3 immunoreactivity shows a very strong overlap with the DNA staining in the primary spermatocytes, as would be expected for a histone modification in a chromatin context, however the Pc staining is predominantly detected in a subcompartment of the nucleolus, and the signal on the bulk chromatin is relatively weak [55]. This region of the nucleolus is not enriched for H3K27me3 epitopes [55]. The changes that occur at testis-specific promoters as uncommitted spermatogonia progress into spermatocyte differentiation are summarised in Fig. 4.1.

The localisation pattern of Pc in primary spermatocytes is essentially identical to the localisation of the tTAFs, encoded by the *can*-class meiotic arrest genes. Moreover, the tTAFs are required to promote the re-localisation of Pc to the nucleolus in primary spermatocytes [55]. In tTAF mutant testes Pc immunostaining is strongly associated with bulk chromatin, and by ChIP analysis it is apparent that the level of Pc at differentiation gene promoters is increased compared to in wild type testes [55]. Thus one function of the tTAFs is to remove Pc from differentiation gene promoters. This function is likely to be very important, however it is not the only function carried out by tTAFs, since target gene expression is not restored in an *nht; E(z)* double mutant, although H3K27me3 is virtually absent from testes of this genotype [53]. It is interesting to note in this regard that TBP and certain TAFs have been co-purified with PRC1 at sub-stoichiometric levels from *Drosophila* samples [56]. This would infer a direct binding of PRC1 with $TF_{II}D$, suggesting a mechanism for how tTAFs evict Pc from target promoters.

For activation of transcription RNA polymerase II (pol II) needs to be loaded at the target promoters. $TF_{II}D$ has a role in recruitment of Pol II to promoters [57], however it appears that the tTAFs are not required for initial Pol II recruitment to differentiation gene promoters in *Drosophila* primary spermatocytes. Notably, in tTAF mutant testes, and in contrast to *bam* mutant testes, Pol II is found at the target gene promoters, at a level comparable to that of the actively transcribed *CycA* gene [53]. This is consistent with low levels of transcriptional activity from these promoters in mutant testes [25]. More surprisingly, Pol II is better able to load onto target promoters in TMAC (*aly*) mutant testes than in *bam* testes, even though no basal activity is detected from these promoters in TMAC mutant spermatocytes [53]. Thus loading of the pre-initiation complex to differentiation gene promoters is not alone sufficient for activating expression of

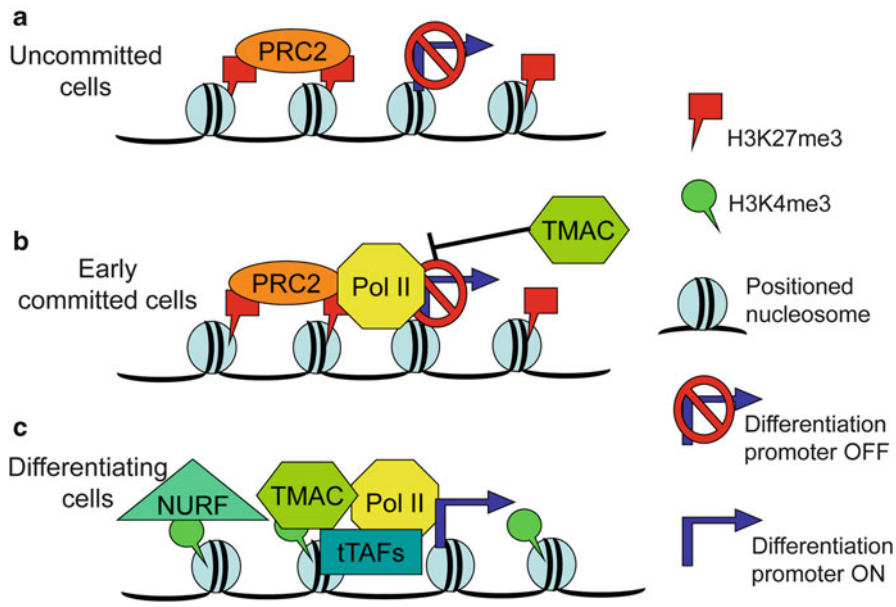

Fig. 4.1 Schematic diagram of some of the processes implicated in repressing activity of differentiation-specific promoters in uncommitted precursor cells (GSCs and spermatogonia). Stepwise changes in the chromatin architecture occur as the cells pass through an early stage in commitment, and then fully activate the expression of differentiation genes

these genes, and TMAC and tTAFS act downstream of the initial loading of Pol II. A factor, as yet unidentified, must be activated during the transition from spermatogonia to spermatocytes that promotes the recruitment of Pol II at differentiation gene promoters. This same factor could also be responsible for activating expression of the meiotic arrest loci, whose function is then to act on the poised promoters. Pol II recruitment to differentiation promoters is not fully independent of the meiotic arrest loci, as the fold enrichment of Pol II at these promoters, compared to control, is much higher in WT testes than in tTAF or TMAC mutant testes [53].

An additional control measure implicated in gene activation is the addition of the activating histone modification H3K4me3, catalysed by the H3K4 methyl-transferase Trx. Levels of this mark are low at all differentiation gene promoters in wild type testes, compared to the levels at the control gene CycA (which is expressed in all the germline cells under discussion), however they are significantly higher than that seen in *bam* mutant testes or tTAF or TMAC mutant testes. Notably there is higher H3K4me3 at the *fzo* promoter in wild type testes than at either dj or Mst87F. Expression of *fzo* at least is also reduced in testes from males mutant for a temperature sensitive *trx* allele after they have been shifted to the restrictive temperature [55].

4.6 TMAC Has a Repressive Activity as well as an Activatory Activity

The TMAC complex purified biochemically from testes comprised Aly, Tomb, Topi, Comr, Mip40 and CAF1 [34]. This purification used Mip40 affinity chromatography to isolate the complex and mass spectroscopy to identify the components, so any TMAC-related complexes that lack Mip40 would not have been detected. Other methods, notably yeast-2-hydrid screening and co-immunoprecipitation followed by immunoblotting, have been used to detect physical interactions between TMAC components and other identified meiotic arrest genes [29, 31–33].

These experiments have confirmed direct interactions between certain TMAC subunits, for example Tomb was identified as a binding partner of Comr [31]. Additionally these approached have revealed a more extended interaction network, for example Achi/Vis co-precipitates with Aly and Comr from testes [32]. The absence of Achi/Vis from the Mip40 affinity purified complex could indicate that distinct variants of TMAC exist, or they could have been lost from the complex as an artefact of the purification procedure. The yeast-2-hybrid approach also identified Wuc as an Aly-binding protein. Paralogy of Wuc to Lin-52, a subunit of the paralogous dREAM complex, supports its inclusion within TMAC [33].

Expression of many differentiation genes is not detected in testes mutant for any one of *aly*, *comr*, *achi/vis*, *tomb* and *topi* [27]. In contrast, mutation of the Wuc or Mip40 TMAC subunits gives only a moderate (approximately twofold) down regulation of genes that are 100-fold or more down regulated in mutants for the other subunits [33]. *CycB*, one of the target genes used to distinguish between *aly*-class and *can*-class mutants is even up-regulated in *wuc* mutant testes. This discrepancy in mutant phenotype could be explained if Wuc and Mip40 are minor players in the complex function, however a genetic interaction between *wuc* and *aly* point to a more complex scenario. Expression of target genes, such as *Mst87F*, *dj*, *fzo*, *twe* or *CycB* is higher in *wuc*; *aly* double mutant testes than it is in testes mutant for *aly* alone [33]. Thus, these genes are only completely dependent on *aly* function in a cell in which *wuc* is present. The restoration of expression in double mutants compared to single mutants varies from gene to gene, for example expression of *Mst87F* is detected at a basal level in *wuc*; *aly* spermatocytes, while *CycB* expression in these cells is similar to wild type. Most interestingly, the expression level of any specific gene in the *wuc*; *aly* double mutant cannot be predicted on the basis of its expression in either of these single mutants, but it does correspond extremely well to the expression level seen in testes mutant for *can* [33].

This interaction, which we also see between *aly* and *mip40* (unpublished data), can be explained by positing a dual function for TMAC in both repressing and activating gene expression. We have proposed that Wuc (and probably Mip40) act to impose a repressive effect on differentiation genes in early primary spermatocytes [33]. This repression actively prevents the transition of differentiation promoters from a silent state (as seen in spermatogonia) to a poised state capable of supporting basal transcriptional activity (as seen in tTAF testes). This repressive step must be pre-requisite for full transcriptional activity at most differentiation gene promoters, since full activation is not achieved in *wuc* or *mip40* mutant testes. The activatory subunits of TMAC must then act on the target genes, in a tTAF-independent step, to first relieve the repression. Then, in conjunction with tTAFs, the TMAC activatory complex must promote full activity of the differentiation gene promoters.

This model fully explains the genetic data, however we have no direct experimental insights into the molecular nature of the repressive function for *wuc* and *mip40*. The complex homologous to TMAC in *C. elegans*, DRM, interacts genetically with a histone deacetylase and nucleosome remodelling complex, NURD [41]. By analogy, it is possible that the repressive function of TMAC is mediated via an interaction with the NURD complex in *Drosophila* testes. Notably CAF1, a subunit of TMAC (at least when purified by Mip40 affinity chromatography) also purifies as subunit of NURD [58]. However the interaction could instead be with a distinct nucleosome remodelling complex, NURF, which comprises Nurf301, Nurf38, Iswi and CAF1 [48]. Transheterozygoes for hypomorphic alleles of Nurf301, which reduce the level of the functional protein are viable, but reveal a requirement for Nurf301 in maintenance of GSCs [59]. In contrast mutant alleles of Nurf301 which can produce normal levels of a truncated form of the protein lacking the ability to bind to H3K4me3 and H4K16Ac (Nurf301ΔC), display meiotic arrest testes, and have no obvious defects in GSC maintenance [35]. Like mutation of *wuc* alone, there is only a mild effect on most differentiation

gene transcripts tested, with the exception of *fzo*. A genome scale analysis of gene expression changes in the *Nurf301* mutant testes has not been reported. Intriguingly, the gene that showed the highest requirement for full length Nurf301, *fzo*, also demonstrated the highest level of H3K4me3 at its promoter in wild type testes [35, 53]. Direct interaction of Nurf301 with the *fzo* promoter has been demonstrated, and moreover this interaction correlates with the highest levels of both H3K4me3 and H4K16Ac in this genomic region [35]. The C-terminal region of Nurf301 is clearly critical for its bulk localisation to chromatin in primary spermatocytes, as the Nurf301ΔC protein fails to accumulate on chromatin in mutant spermatocytes. Nurf301ΔC also fails to accumulate substantially on chromatin in *aly* or tTAF mutant testes, indicating that the activity of these transcription complexes is implicated in setting up the active chromatin state in primary spermatocytes to which the C-terminus of Nurf301 binds [35]. This correlates with the molecular analysis revealing that H3K4me3 is low at target promoters in both *aly* and tTAF testes.

4.7 Chromosomal Territories and Testis Gene Expression

In the preceding discussion of the mechanism underlying the activation of testis-specific promoters as male germline cells progress into spermatocyte development we have considered each promoter to be an independently functioning unit. However, it is clear that the chromosomal context of genes with testis-biased expression needs to be taken into account. The organization of genes within the genome is non-random, and there is significant clustering of genes with similar expression patterns. These clusters can be detected with stringent methods, that require contiguous genes with similar expression, or with more relaxed algorithms, which allow clusters to contain interspersed genes with dissimilar expression from the bulk of the cluster [60–62]. Such clusters or gene neighbourhoods must be advantageous to the organism, otherwise they would not have evolved, and an attractive explanation for the clustering would be that the genes share transcriptional control elements. This sharing could be at the level of shared enhancer element(s), or a shared chromatin environment. Testing this involved disruption of three different clusters, via precisely targeted inversions [63]. These inversions did not alter the expression level of the testis genes analysed. This indicates that clustering of genes with testis-enriched expression is not implicated in regulating the expression level in testis of these genes, at least for the clusters tested. If the clusters are not essential for setting up testis expression perhaps instead they are important in the repression of expression of these testis-enriched genes in other tissues? Notably, the genes within testis-enriched clusters are repressed in somatic cells, in part by association with the nuclear lamina [64]. This association would place all the genes in a contiguous cluster into a transcriptionally inactive region of the nuclear periphery. Clusters of testis-differentiation genes have been shown to be associated with the nuclear lamina in spermatogonia, and displaced from the lamina in spermatocytes. Indeed, ectopic activation of testis differentiation genes in somatic cells can be induced by depletion of laminB0 [64].

4.8 Influence of Chromosomal Position on Gene Expression as Male Germline Cells Differentiate

At the broadest genome scale genes are organised onto chromosomes. *Drosophila melanogaster* has just three autosomes (of which one is very small) and a pair of sex chromosomes. The male is the heterogametic sex, possessing an X and a Y chromosome. The Y chromosome is not essential for viability, but is required for male fertility. A small number of genes have been localised to this chromosome, and all are expressed exclusively in primary spermatocytes. Extensive transcription of the Y-chromosome in primary spermatocytes leads to formation of specific structures within the nuclei – the Y-loops [65]. The mechanism by

which the transcription of these Y-linked genes is activated in primary spermatocytes is not fully elucidated, although the Y-loops are disrupted in several meiotic arrest mutants (R. White, pers. comm.). More intriguing is the role of chromosomal location on expression of genes from the X chromosome.

Microarray analyses of gene expression have revealed that there is a paucity of X-linked genes with male-specific expression. Since the most sexually dimorphic organ is the gonad, this correlates with a significant reduction in the number of testis-specifically expressed genes located on the X chromosome. Most testis-specific transcripts are produced in primary spermatocytes, and so this effect could be caused by a general inactivation of the X chromosome during the meiotic programme. Support for the idea that the X chromosome is transcriptionally less active than the autosomes comes from the finding that new genes generated by retroposition show a trend consistent with escape from the X chromosome [66]. I.e. the parental gene will be on the X and the daughter gene will insert on an autosome. Frequently the retroposed copy also acquires testis-specific expression while the parental gene has a broader expression domain [67]. However, none of these observations show conclusively that the X chromosome is inactive in primary spermatocytes, and if it is generally inactivated there are many loci which are X-linked and highly active in these cells. Transgenes inserted on the X chromosome are expressed at lower levels in the germline than identical transgenes inserted on the autosomes [68]. Three approaches have been used to quantify the activity of genes on the X vs Autosomes as germ line cells differentiate. Firstly *bam* mutant testes have been compared to wild type testes, secondly testes have been manually dissected and samples enriched for spermatogonis and early spermatocytes have been compared to pure spermatocyte samples and finally testes from larvae at various stages of development have been analysed [12, 13, 22]. Unfortunately contamination of the samples, particularly the early cell population with both later cells and somatic cells complicates the analysis. Initial analysis of the microdissected samples suggested a lower level of expression of X-linked genes in primary spermatocytes, correlating with the documented lower level of testis-biased genes on the X [12]. However, reanalysis, taking into account the sample complexity, fails to support a model of meiotic X-inactivation [69, 70]. Indeed the finding that testis-enriched genes are less likely to be on the X-chromosome does not reveal anything special about this chromosome in the male germline since somatically-expressed male-biased genes are also less likely to be on the X, as are genes with no sexually dimorphic pattern, but with a highly restricted gene expression pattern [70]. This has recently been refuted in a reanalysis, leaving the question of gene expression from the X in spermatocytes still open [71].

In somatic cells the level of expression of X-linked genes in males is doubled compared to females via dosage compensation [72]. The dosage compensation mechanism is not active in the male germline. Specifically, of the known dosage compensation genes, only *mle* is expressed in these cells [73]. Mle protein is abundant in spermatocytes, however it is not strongly chromatin associated, and is definitely not specifically found on the X chromosome as it is in the soma. Moreover the histone mark promoted by the dosage compensation machinery, H4K16Ac is uniform on chromatin in early-mid primary spermatocytes, and is weak and predominantly nucleolar, in later primary spermatocytes [73].

What is special about the X chromosome in primary spermatocyte? The balance of evidence suggests that the X chromosome is less conducive for high expression levels in primary spermatocytes than the autosomes [69, 70]. It might also be less good for expression in earlier male germline cells, due to the lack of dosage compensation. It is intriguing to note that there are a few chromatin associated factors that differentiate between the XY bivalent and the autosomes in primary spermatocytes. Nurf301, discussed earlier, accumulates preferentially on the autosomes, and is much less associated with the XY bivalent [35]. Similarly, Mtor, a nuclear scaffold protein, specifically associates with the autosomes, as well as with the nuclear lamina, in primary spermatocytes (HW-C unpublished). Borr, a

chromosomal passenger protein implicating in regulating cytokinesis, has a testis-specific paralogue, Aust [74]. Notably, Aust protein appears just before the meiotic divisions, binds chromosomes, and promotes meiotic cytokinesis. Borr functions in the mitotic divisions of spermatogonia, however the protein remains highly expressed in spermatocytes, and labels the two autosomal bivalents, but not the XY [74]. Thus at least three markers indicate that during the transition from spematogonia to spermatocytes there is a dramatic change in the XY bivalent's chromatin environment. Further investigation into the functions of these proteins in spermatocytes could reveal mechanisms underling some of the differences in expression seen for X-linked genes compared to autosomal genes.

4.9 Alternative Splicing of Transcripts Is Prevalent in Undifferentiated Cells

The majority of genes within metazoan genomes contain multiple exons, and thus the mRNAs are produced as a result of splicing. For many genes this can be used to generate alternative mRNA products, with different properties and functions via alternative splicing [75]. These products can differ in terms of RNA sequence, for example use of RNA localisation signals, or can result in production of variant proteins. About 78 % of all predicted coding genes in *Drosophila* are spliced and about 40 % are alternatively spliced [76]. Within mammalian testes there is an increase in alternative splicing compared to many other adult tissues [77]. In contrast, alternative splicing seems to decrease in *Drosophila* male germ cells as they differentiate from stem cells into spermatocytes [13]. Gan et al. used RNA-seq to determine the expression of all genes in normal testes, and compared this to the expression seen in *bam* mutant testes. Thus genes whose transcription is enriched in spermatogonia and spermatocytes can be distinguished from the differentiation genes. Notably, they detected a significant enrichment for expression of known splicing regulators in *bam* mutant testes compared to whole testes. Indeed over half of all annotated splicing factors were enriched in *bam* testes, while only 8.4 % were relatively depleted in *bam* testes. This correlates well with their finding that the proportion of differentiation genes (defined as genes not expressed in *bam* testes) with multiple annotated isoforms is significantly lower than the proportion of genes with multiple isoforms in the whole genome annotation. It is not clear from these pair-wise comparisons if the *bam* sample represents elevated alternative splicing relative to other tissues, or if the WT sample represents lower levels of splicing in general, and alternative splicing in particular. Many retroposed genes, which naturally lack introns and therefore cannot be subject to alternative splicing, are expressed exclusively in testes [78]. Indeed, analysis of the expression of splicing factors in testis compared to other adult tissues, using FlyAtlas microarray data [8], would suggest that there is a general down-regulation of ubiquitous splicing factor expression in testis (i.e. differentiating spermatocytes) compared to other tissues. A small number of annotated splicing genes are up-regulated in WT testes compared to *bam* testes. Analysis of these genes indicates that they are predominantly testis-specifically expressed. Most have not been studied in detail, however one, LS2 (CG3162), a retroposed duplicate of $U2AF^{50}$, has been analysed [79]. Consistent with the expression in WT testes, but not *bam* testes, LS2 protein is detected exclusively in nuclei of primary spermatocytes, and not in spermatogonia. In our microarray analysis of gene expression in WT and meiotic arrest mutant testes we find that LS2 expression, and expression of most of the other WT-testis enriched splicing factors from [13] is not dependent on the function of TMAC or tTAFs. Thus LS2 transcription is probably initiated during the spermatogonia-spermatocyte transition, potentially using the same activator as the meiotic arrest genes. $U2AF^{50}$ is the large subunit of U2-associated factor, which interacts with the 3′ end of the intron to be spliced and promotes spliceosome assembly (reviewed in [80]). LS2 has diverged considerably in sequence from its parent gene, controls splicing of a distinct transcript pool, and has a distinct sequence preference.

Unexpectedly, LS2 acts as a splicing repressor rather than an enhancer [79].

Analysis of the splicing factor SMN in the male germline has recently shed some light on the importance of regulation of splicing in the stem cells. Loss of SMN activity in humans leads to the disease spinal muscular atrophy, in which there is a progressive loss of specific motor neurons, leading to paralysis, muscle wasting, and in severe cases, death. In *Drosophila*, *Smn* expression is ubiquitous, but the highest expression is detected in larval central nervous system and in gonads. Smn protein has been shown to be high in GSCs and spermatogonia, and the concentration of the protein declines dramatically in early spermatocytes [81]. Thus there is a gradient of Smn protein in differentiation, with a peak in the undifferentiated cells. *Smn* mutants are lethal as larvae, precluding analysis of homozygous adults, but mitotic recombination techniques allowed generation of *Smn* mutant GSCs in testes. The mutant GSCs were inefficiently maintained, indicating that Smn function is important for GSC survival or for maintenance of the stem cell fate. Moreover, analysis of the testes of mutant larvae indicate a critical role for *Smn* in regulating the differentiation of germline cells. WT late larval testes contain stages of spermatogenesis up to meiotic spermatocytes, or occasionally early spermatids, *Smn* mutant testes in contrast contain elongated spermatids, and many fewer early germ cells. In contrast, ectopic expression of Smn expanded the early germ cell population in the testes [81].

It appears that alternative splicing, and perhaps splicing in general, is down-regulated as stem cells and spermatogonia transit to the differentiating fate by both reduction in expression of core splicing proteins, and by activation of expression of a variant core splicing factor that has evolved a splicing repression function. This would result in suppression of the broad repertoire of alternative spliced mRNA variants seen in the undifferentiated cells, pushing the differentiating cells towards production of a more refined, cell type specific, transcriptome. The high expression of splicing factors in the undifferentiated cells in the testis is likely to be critical in maintaining their state, and the reduction in expression of these factors as the cells progress into differentiation could be fundamental to restricting their ability to de-differentiate.

4.10 An Integrated View of Activation of Gene Expression as Cells Lose Stem Cell Properties

Taken together these data indicate a succession of changes at differentiation gene promoters as cells transition from a stem cell identity into differentiation. In cells with stem cell capacity these genes are fully repressed, with H3K27me3, and no RNA polymerase associated. Exit from the mitotic amplification programme results in a change in the chromatin at these promoters so that RNA polymerase is able to load, but not begin transcription elongation. This is co-incident with a repositioning of these loci within the nucleoplasm to place them in a less repressive environment. An early spermatocyte transcriptional repertoire is induced, including activation of expression of the testis-specific meiotic arrest genes, and testis-specific splicing factors. The RNA pol II at differentiation gene promoters could be kept inactive via the function of *wuc* and *mip40*, via an unknown mechanism. The function of the meiotic arrest genes is then essential to promote the activity and further recruitment of RNA pol II at the differentiation promoters, and to relieve the repression imposed by *wuc* and *mip40*. H3K27me3 declines as PRC2 is removed from target promoters by tTAFs, while H3K4me3 increases, presumably as a result of Trx activity. Recruitment of NURF, via Nurf301, to the H3K4me3 would then allow sliding of nucleosomes and remodelling of chromatin as the differentiation promoters become fully active.

This leaves a few critical questions still unanswered: how are the differentiation genes recognised as targets for repression in stem cells? what factors promote RNA pol II recruitment to promoters? what is the transcriptional profile underlying transition of spermatogonia to spermatocytes, particularly what genes are responsible

for activating transcription of the meiotic arrest genes and other genes that are activated as cells commit to differentiation?

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