Epigenetic Regulation of Stem Cell Maintenance in the *Drosophila* Testis via the Nucleosome Remodeling Factor NURF

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SUMMARY

Regulation of stem cells depends on both tissue-specific transcriptional regulators and changes in chromatin organization, yet the coordination of these events in endogenous niches is poorly understood. In the *Drosophila* testis, local JAK-STAT signaling maintains germline and somatic stem cells (GSCs and cyst progenitor cells, or CPCs) in a single niche. Here we show that epigenetic regulation via the nucleosome remodeling factor (NURF) complex ensures GSC and CPC maintenance by positively regulating JAK-STAT signaling, thereby preventing premature differentiation. Conversely, NURF is not required in early differentiating daughter cells of either lineage. Since three additional ATP-dependent chromatin remodelers (ACF, CHRAC, and dMi-2/NuRD) are dispensable for stem cell maintenance in the testis, epigenetic regulation of stem cells within this niche may rely primarily on NURF. Thus, local signals cooperate with specific chromatin remodeling complexes in intact niches to coordinately regulate a common set of target genes to prevent premature stem cell differentiation.

Keywords

Stem cell niche; Chromatin remodeler; spermatogonial stem cell; niche; NURF, JAK-STAT; *Drosophila* testis

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INTRODUCTON

Stem cells sustain tissues by dividing asymmetrically to generate both stem cells and differentiating daughter cells. Signals from the local microenvironments (niches) where stem cells reside govern the balance between these opposing fates by activating distinct transcriptional programs (Morrison and Spradling, 2008). However, chromatin structure imposes an additional level of regulation during this process (Clapier and Cairns, 2009). While the roles of both cell signaling and chromatin structure in the regulation of cell fate are under intense investigation, little is known of how these events are coordinately regulated in endogenous niches.

The germline stem cells (GSCs) sustaining gametogenesis within *Drosophila* gonads are some of the best-understood adult stem cells, and the importance of local signaling in the

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regulation of stem cell function in these tissues is well established (Fuller and Spradling, 2007; Gregory et al., 2008). Two distinct populations of stem cells reside in the *Drosophila* testis apex: GSCs, which produce differentiating germ cells, and somatic stem cells (cyst progenitor cells or CPCs), which produce daughter cells (cyst cells) that envelop germ cells and ensure their differentiation (Fig. 1A). The hub, a cluster of non-mitotic somatic cells, creates a stem cell niche by secreting the cytokine Unpaired; local activation of the Janus Kinase-Signal Transducer and Activator of Transcription (JAK-STAT) signaling cascade prevents differentiation within adjacent stem cells (Kiger et al., 2001; Tulina and Matunis, 2001). Although the JAK-STAT pathway is a clear example of stem cell regulation via extrinsic signaling, the role of epigenetic regulation, including the state of chromatin, has not been studied in this niche.

Chromatin is highly structured to provide for efficient packaging of DNA and transcriptional regulation (Loden and van Steensel, 2005). Nucleosomes, the fundamental repeating units of chromatin, contain DNA and histones, and are regulated by two main classes of chromatin-remodeling enzymes: those that use ATP hydrolysis to alter histone–DNA contacts, and those that covalently modify histone proteins (Becker and Horz, 2002). Recent work has focused on the role of chromatin in embryonic stem cells (ES cells), which are enriched in euchromatin but accumulate transcriptionally inactive, highly compacted heterochromatin upon differentiation (Arney and Fisher, 2004; Francastel et al., 2000). Consistent with this finding, ATP-dependent chromatin remodelers are found at elevated levels in ES cells (Meshorer and Misteli, 2006). However, the existence of many ATP-dependent chromatin remodelers and few well-characterized niches makes understanding the role of chromatin state in endogenous stem cells challenging.

Nine different ATP-dependent remodelers, grouped into four distinct families, are currently known in *Drosophila* (Bouazoune and Brehm, 2006). Our previous work indicated that components from one of these families (ISWI) have enriched expression in the *Drosophila* testis apex (Terry et al., 2006), providing an opportunity to analyze the role of epigenetic regulation in a well-characterized niche.

The *Drosophila* ISWI ATPase, which is homologous to the yeast SWI2/SNF2 enzyme (Elfring et al., 1998), is found in three distinct chromatin remodeling complexes (Fig. 1B): ACF (ATP-utilizing chromatin assembly and remodeling factor), CHRAC (chromatin accessibility complex), and NURF (nucleosome remodeling factor) (Bouazoune and Brehm, 2006). ACF and CHRAC are involved in chromatin assembly, DNA replication, and transcriptional regulation. NURF regulates higher-order chromatin structure and can act as a transcriptional repressor or activator (Badenhorst et al., 2005). Since expression profiling experiments indicated that three of the four NURF components (ISWI, Nurf55, and Nurf301) are expressed within the testis apex (Terry et al., 2006), we focused on this complex.

Of the four subunits of the *Drosophila* NURF complex, Nurf301 and ISWI are necessary and sufficient for the accurate and efficient sliding of nucleosomes. Nurf301, the only NURF-specific subunit, is well-characterized biochemically: it is essential to the structural integrity of the complex, interacts directly with sequence-specific transcription factors, and binds tri-methylated lysine 4 on histone H3 tails (H3K4me3) (Wysocka et al., 2006), a histone mark typically found in the promoter of actively transcribed genes (Bernstein et al., 2005; Santos-Rosa et al., 2002). Nurf301 has also been well-characterized genetically in *Drosophila*: it is required to maintain homeotic gene expression during development (Badenhorst et al., 2002; Xiao et al., 2001), repress JAK-STAT signaling in the immune system (Badenhorst et al., 2002), and promote ecdysone signaling during metamorphosis

(Badenhorst et al., 2005). Here, we examined the role of NURF in the *Drosophila* testis niche.

RESULTS

nurf301 is required for GSC maintenance in the *Drosophila* testis

To pursue our previous findings that members of the NURF complex are expressed in the *Drosophila* testis apex (Terry et al., 2006), we immunostained testes with antisera specific for the ISWI and Nurf301 subunits of NURF. As expected, both proteins were expressed in all cells within the testis apex including GSCs, CPCs, and the hub, although Nurf301 levels appeared to be slightly lower in the latter (Fig. 1C, D). Both ISWI and Nurf301 displayed nuclear localization consistent with their well-characterized roles in chromatin remodeling. Since these results were consistent with a role for NURF in the germline and/or somatic stem lineages in this tissue, we next analyzed the testis phenotypes from flies with reduced levels of the essential NURF component, Nurf301. Although *nurf301* is required for viability, flies containing a combination of hypomorphic alleles ($nurf301^4/nurf301^{12}$) survive to adulthood (Badenhorst et al., 2005). Testes from 0–3 day old $nurf301^4/nurf301^{12}$ adults contained a wild-type number of GSCs (6.38 ± 0.44, n = 21) compared to heterozygous controls (6.20 ± 0.26, n = 25, p-value > 0.05). However, differentiating spermatogonial cysts were found adjacent to the hub in 25% of $nurf301^4/nurf301^{12}$ testes (n = 20); this phenotype was never observed in heterozygous controls ($nurf301^4/+$, n = 15 and $nurf301^{12}/+$, n = 10) (Fig. 1E, F), suggesting that *nurf301* prevents premature GSC differentiation. Consistent with this finding, larvae with a combination of null alleles ($nurf301^2/nurf301^3$), which survive until the third instar (Badenhorst et al., 2005), contained significantly fewer GSCs (4.65 ± 0.51, n = 17) than heterozygous controls (10.66 ± 0.36, n = 32, p-value < 0.0001). Larval testes lacking Nurf301 also appeared to contain fewer differentiating germline cysts than controls (Fig. 2A, B); this is likely due to reduced GSC numbers rather than a reduction in their division rate, since the frequency of GSC mitoses, as assayed by the mitotic marker phospho-histone H3 (PH3), did not vary significantly from wild-type (0.3% of *nurf301* GSCs were PH3-positive, n=279; 1.5% of wild-type GSCs were PH3-positive, n=482, p-value = 0.270). We conclude that Nurf301 is required for GSC maintenance.

nurf301 is required cell-autonomously for GSC maintenance but is dispensable for spermatogonial differentiation

GSCs could directly or indirectly require *nurf301* for their maintenance. To distinguish between these possibilities we created negatively marked *nurf301* null clones in adult testes using FLP/FRT mediated mitotic recombination (Xu and Rubin, 1993). GSC clones were identified as Vasa-positive cells contacting the hub but lacking GFP. Testes were scored for the presence of one or more negatively marked GSCs at 2, 4, 6, 8, or 10 days after clone induction (ACI). As expected, wild-type control GSC clones were maintained over this time interval. In contrast, GSCs lacking *nurf301* were rapidly lost from the niche, indicating that GSCs directly require *nurf301* for their maintenance (Fig. 2C-F, Table 1). Interestingly, differentiating germline cysts lacking *nurf301* were detected up to the primary spermatocyte stage for several days ACI, suggesting that although *nurf301* is required for GSC maintenance, it is not required for spermatogonial differentiation (Supplemental Fig. 1, Supplemental Table 1).

Since GSCs lacking *nurf301* were depleted from the testis over time, *nurf301* could either maintain GSC viability or prevent GSCs from differentiating. To distinguish between these possibilities, a transcriptional reporter revealing the expression of the differentiation factor *bag of marbles* (*bam*) (Chen and McKearin, 2003) was analyzed in testes containing *nurf301*

null clones. Bam is normally detected in spermatogonia but almost never in GSCs (0.4% of wild-type GSCs express Bam, n=263). In contrast, Bam was expressed in 31.4% (n = 35) of *nurf301²* GSCs and 40.6% (n = 32) of *nurf301³* GSCs at 4 days ACI (Fig. 2G, G'). These results suggest that *nurf301* null GSCs are lost from the niche due to premature differentiation. Consistent with this finding, the number of apoptotic cells in testes containing *nurf301* null clones did not increase compared to testes containing control clones (1.96 ± 0.2 TUNEL-positive cells, n = 66 for *nurf301²*; 2.19 ± 0.2, n = 70 for *nurf301³*; 3.10 ± 0.23, n = 92 for wild-type). Together, these results indicate that *nurf301* is not required generally to sustain germ cell viability or spermatogonial differentiation; rather, it is specifically required within GSCs to prevent them from prematurely entering the differentiation pathway.

nurf301 is required cell-autonomously for CPC maintenance but not for cyst cell differentiation

GSCs cohabit the niche with somatic stem cells called CPCs, and both types of stem cells express NURF components (Figure 1C, D). Therefore, we used mosaic analysis with a repressible cell marker (MARCM) (Lee and Luo, 1999) to determine the requirement for *nurf301* in the CPC lineage. CPC clones were identified as Vasa-negative, GFP-positive cells contacting the hub. Testes were scored for the presence of one or more CPC clones at 2, 4, 6, 8, 10, or 14 days ACI. As expected, wild-type control CPC clones were readily observed throughout the time course (Fig. 3A, B). By 14 days ACI, the percentage of testes with wild-type CPC clones decreased by about half, but CPC clones were still frequently observed (Fig. 3B, Table 2). This moderate loss of wild-type CPC clones likely reflects the relatively short half-life (5–10 days) that has been reported for CPCs (Voog et al., 2008). In contrast, *nurf301* null CPCs were lost much more rapidly than wild-type CPC clones and were rarely detected after 4 days ACI (Fig. 3C, D, Table 2). About 85.5% of *nurf301²* and 100% of *nurf301³* null CPCs were lost after two weeks, indicating that *nurf301* is essential for CPC maintenance (Table 2). The frequency of apoptotic cells did not differ significantly in testes with *nurf301* null clones compared to testes with control clones (assayed via TUNEL staining, as above), suggesting that *nurf301* null CPCs are lost via differentiation, rather than death. Prior to being lost from the niche, *nurf301* null CPCs express the CPC marker zinc-finger-homeodomain protein 1 (Zfh-1) (Leatherman and Dinardo, 2008) (100% of *nurf301* null CPCs at 4 days ACI were Zfh-1-positive, n=34) and produce differentiating CPC daughters (cyst cells) that encyst adjacent spermatogonia (Fig. 3D, Supplemental Table 2). Importantly, cyst cells lacking *nurf301* extinguish Zfh-1 expression (data not shown), and eventually express the late cyst cell marker Eya (Fig. S1). Thus, cyst cells lacking *nurf301* appear to differentiate appropriately. Together, these results indicate that *nurf301* is specifically required to autonomously maintain both germline and somatic stem cells in the testis, yet is dispensable for early daughter cell differentiation in each lineage.

The NURF complex maintains GSCs and CPCs in the *Drosophila* testis

Since *nurf301* is a unique subunit of the NURF complex and is essential to its function, our results suggested that the NURF complex is essential for maintaining stem cell fate in the *Drosophila* testis. Therefore, we analyzed the role of additional members of this complex in GSC maintenance via genetic mosaic analysis as described above. Loss-of-function alleles have not been identified for *nurf55*, but exist for the inorganic pyrophosphatase *nurf38* and the ATPase *iswi*. Therefore, we created *nurf38* and *iswi* loss-of-function clones as described above for *nurf301*. We found that *nurf38^{k16102}* mutant GSCs were completely lost from the testis by 8 days ACI. Similarly, the number of testes containing *iswi²* mutant GSCs declined by about 99% by 10 days ACI (Table 1). Interestingly, the timing of loss of both *nurf38* and *iswi* mutant GSCs was similar to that of *nurf301* mutant GSCs. These results indicate that

Nurf38 and ISWI are required for GSC maintenance, and support the hypothesis that the NURF complex is required for stem cell maintenance in the testis.

We also wanted to determine if CPCs, like GSCs, require ISWI for their maintenance. We successfully reduced ISWI levels in the CPC lineage by expressing an ISWI-RNAi construct specifically in CPCs and their daughters using the *c587-Gal4* driver (Voog et al., 2008). In wild-type testes, ISWI was detected in CPCs and GSCs at comparable levels (Fig. 4A). However, induction of the ISWI-RNAi construct for 7 days at 29°C led to greatly reduced levels of ISWI in CPCs but not GSCs (Fig. 4B). To quantify CPCs before and after ISWI-RNAi induction, we immunostained testes with antibodies against Zfh-1 (Issigonis et al., 2009; Leatherman and Dinardo, 2008). Before RNAi induction, flies carrying the ISWI-RNAi construct contained the same number of CPCs as GFP-RNAi controls (27.8 ± 2.1, n = 8 versus 26.6 ± 1.5, n = 17, p-value > 0.05) (Fig. 4G). However, after RNAi induction, flies carrying the ISWI-RNAi construct contained significantly fewer CPCs (18 ± 1.2, n = 17) (Fig 4D, G) than GFP-RNAi controls (30.4 ± 1.2, n = 22, p-value < 0.0001) (Fig. 4C, G). Thus, ISWI is directly required for CPC maintenance. Following induction of ISWI-RNAi in CPCs and their daughters we also observed a decrease in GSC number (3.80 ± 2.25, n=10) compared to GFP-RNAi controls (8.73 ± 2.66, n=22, p-value 0.0002). This suggests that CPCs with reduced levels of ISWI do not properly signal to the GSCs, thus indirectly causing loss of GSCs from the niche. Signaling between CPCs and GSCs plays an important role in the balance between stem cell self-renewal and differentiation (Kawase et al., 2004; Kiger et al., 2000; Leatherman and Dinardo, 2008), but is poorly understood. It will be interesting to determine if NURF ensures appropriate signaling between stem cell types or if the loss of GSCs following ISWI-RNAi in the CPCs is an indirect effect due to the exit of ISWI-deficient CPCs from the niche. Together our results demonstrate that multiple members of the NURF complex autonomously maintain CPC and GSC fate in the *Drosophila* testis niche.

NURF is one of nine unique chromatin-remodeling complexes currently identified in *Drosophila*, and increasing evidence indicates that chromatin remodelers may play both general and specific roles in regulating cell fate decisions (Clapier and Cairns, 2009). We wondered whether multiple remodelers are required for stem cell maintenance in the testis, or if instead this is a unique feature of NURF. The NURF ATPase ISWI is a component of three distinct remodeling complexes (NURF, ACF and CHRAC), but ACF and CHRAC share a common subunit, ACF1, which is not present in NURF. Therefore, we determined the requirement for ACF and CHRAC in the testis by removing ACF1 function. Two different null alleles of *acf1* exist: *acf1^1* and *acf1^2*. Since *acf1* homozygous null mutants are semi-lethal, but those flies that do survive are fertile (Fyodorov et al., 2004), we asked if *acf1* is necessary for maintaining stem cells in the testis by comparing the number of GSCs and CPCs in surviving *acf1^1/acf1^2* adults to wild-type controls. Testes from *acf1^1/acf1^2* flies had the same number of GSCs as wild-type controls (8.64 ± 0.64, n = 28 versus 9.43 ± 0.34, n = 21, p-value > 0.05) (Fig. 4E, H). The number of CPCs in *acf1^1/acf1^2* testes was also indistinguishable from wild-type controls (26.81 ± 0.78, n = 43 versus 27.60 ± 0.78, n = 33, p-value > 0.05) (Fig. 4F, H). Thus, *acf1* is not required for stem cell maintenance in the *Drosophila* testis. Instead, our results indicate that stem cell maintenance is not a property of ISWI family remodeling complexes in general but can be ascribed specifically to the function of a single ISWI-containing chromatin remodeling complex: NURF.

Although NURF is the sole ISWI family member required in the testis niche, it was not known if members of the other families of chromatin remodelers, which contain different types of ATPase subunits, are also required for stem cell maintenance in this system. We found that a GFP protein trap inserted in the *dMi-2* gene (Buszczak et al., 2007) is broadly expressed throughout the testis apex, indicating that dMi-2 is expressed in this tissue (data

not shown). Since *dMi-2* encodes the core ATPase of the *Drosophila* Mi-2/NuRD complex, which is involved in the repression of homeotic genes during embryogenesis (Kehle et al., 1998), analyzing the role of dMi-2 in the testis enabled us to determine the requirement for the Mi-2/CHD family of remodelers in our system. Although *dMi-2* is essential for viability, 0.1% of *dMi-2* null adults of the genotype *dMi-2⁵/Df(3L)BSC1* survive to adulthood (Yamasaki and Nishida, 2006) (Yamasaki and Nishida, 2006). Immunostaining testes of *dMi-2⁵/Df(3L)BSC1* adults as described above revealed that they contained a similar number of GSCs as heterozygous controls (6.44 ± 0.73, n = 9 versus 5.92 ± 0.30, n = 25, p-value > 0.05). Therefore, dMi-2 is not required for GSC maintenance in the *Drosophila* testis. Thus, the maintenance of *Drosophila* testis stem cells is not dependent on all chromatin remodelers but is a property unique to specific complexes including NURF.

NURF maintains testis stem cells by positively regulating the JAK-STAT pathway

Our data demonstrate that the NURF complex is required to maintain both GSCs and CPCs in the *Drosophila* testis. Since JAK-STAT signaling is also required autonomously to maintain both GSCs and CPCs (Issigonis et al., 2009; Kiger et al., 2001; Leatherman and Dinardo, 2008; Tulina and Matunis, 2001) we postulated that NURF could prevent stem cell differentiation in the testis by promoting the activity of the JAK-STAT pathway within stem cells. To test this hypothesis, we monitored JAK-STAT activity in negatively marked *nurf301* GSC clones by immunostaining for STAT92E, since enrichment of STAT92E indicates pathway activity (Chen et al., 2002). In *nurf301* heterozygous testes before clone induction, STAT92E is enriched in all GSCs surrounding the hub and reduced in gonialblast daughters, in a manner indistinguishable from wild-type (Fig. 5A, A'). At 4 days ACI, GSCs null for either *nurf301²* (n = 24) or *nurf301³* (n = 23) had significantly reduced levels of STAT92E staining relative to neighboring heterozygous GSCs (Fig. 5B, B', E). Instead, the level of STAT92E in GSCs lacking Nurf301 was less than or similar to that typically seen in heterozygous gonialblast daughters (Fig. 5B, B'). This decline in STAT92E enrichment upon loss of Nurf301 suggests that *nurf301* positively regulates the JAK-STAT pathway in GSCs, thus promoting their maintenance in the niche.

To confirm this hypothesis, we asked if *nurf301* genetically interacts with the JAK-STAT pathway in the testis niche. *Suppressor of cytokine signaling 36E* (*socs36E*) is a highly conserved target of the JAK-STAT pathway and functions in a classical negative feedback loop by down-regulating pathway activity in CPCs (Callus and Mathey-Prevot, 2002; Issigonis et al., 2009; Karsten et al., 2002). In *socs36E^PZ1647* homozygous mutant testes, CPCs have aberrantly high JAK-STAT activity and consequently displace neighboring GSCs from the niche, resulting in GSC loss (Issigonis et al., 2009) (Fig. 5C, F). When Stat92E levels were genetically lowered in *socs36E^PZ1647* mutant flies, fewer GSCs were lost (Fig. 5F). Similarly, if Nurf301 levels were genetically reduced in *socs36E^PZ1647* mutant flies, fewer GSCs were lost (Fig. 5D, F). Thus, global reduction of either Stat92E or Nurf301 partially rescues the *socs36E^PZ1647* phenotype. Since *nurf301* genetically interacts with the JAK-STAT pathway member *socs36E* in a manner consistent with that of a positive regulator, our data suggest that both GSCs and CPCs require NURF to effectively activate the JAK-STAT pathway, thus ensuring their maintenance within the testis niche. Considering its role as a chromatin remodeler, we hypothesized that NURF could promote transcription of JAK-STAT pathway activators. To test this hypothesis, we asked if boosting levels of STAT92E specifically within CPCs lacking Nurf301 could overcome the CPC loss phenotype. We found that restoration of STAT92E expression partially rescued *nurf301* null CPC loss at 6 days ACI (Fig. 3E, Table 2). Although it is likely that Nurf301 regulates many genes, our data suggest that a major role of NURF in the maintenance of testis stem cells is to ensure sufficient STAT92E expression. Together these data support the hypothesis that NURF positively regulates JAK-STAT signaling in the testis niche.

DISCUSSION

This work reveals that the ATP-dependent chromatin remodeler NURF cooperates with local JAK-STAT signaling in the *Drosophila* testis niche to ensure stem cell maintenance. This may be a unique feature of NURF as three additional ATP-dependent chromatin remodelers are dispensable for stem cell maintenance in the testis.

The role of NURF in stem cell maintenance

We propose that NURF plays a critical role in maintaining a chromatin configuration that is essential for germline and somatic stem cell maintenance in the *Drosophila* testis. In the germline, NURF promotes expression of the stem cell maintenance factor STAT92E and prevents premature expression of the differentiation factor Bam. STAT92E expression is difficult to detect in CPCs due to inhibition of the JAK-STAT pathway by the suppressor Socs36E (Issigonis et al., 2009); however, expressing STAT92E in *nurf301* null CPCs partially rescues their loss from the niche, suggesting that NURF also promotes JAK-STAT signaling in CPCs. Since both stem cell populations directly require JAK-STAT signaling for their maintenance, identifying targets of NURF in each lineage will be of interest. Interestingly, the JAK-STAT pathway is required for proper integrin expression in CPCs to maintain niche homeostasis (Issigonis et al., 2009), an intriguing possibility is that NURF may directly, or indirectly via regulation of JAK-STAT signaling, control expression of adhesion molecules in testis stem cells to ensure their maintenance in the niche.

Further insights into the epigenetic regulation of stem cells arise when comparing our work to studies of chromatin remodelers in the *Drosophila* ovary. ISWI prevents premature differentiation of testis GSCs, and as a component of the NURF complex, promotes JAK-STAT signaling. Similarly, ISWI prevents differentiation of ovarian GSCs by enabling them to respond to Dpp/Tgfß signals from their niche (Xi and Xie, 2005). This is not likely to involve JAK-STAT signaling, since female GSC maintenance does not require this pathway (Decotto and Spradling, 2005). However, the Dpp/Tgfß signaling pathway maintains GSCs in both the ovary (Xie and Spradling, 1998) and the testis (Kawase et al., 2004). Examining the interactions between Nurf301 and components of the Dpp/Tgfß signaling pathway may reveal whether NURF regulates this signaling pathway in the testis niche. Interestingly, the ability of NURF to interact with the Dpp/Tgfß signaling pathway may be conserved; the mammalian orthologue of *Drosophila* Nurf301 (BPTF) may directly promote Dpp/Tgfß signaling via the NURF remodeling complex by recruiting Smad transcription factors to target sites in mouse ES cells and embryos (Landry et al., 2008). Thus, NURF may have a conserved role in stem cell maintenance.

NURF can positively or negatively regulate JAK-STAT signaling

Our finding that NURF promotes JAK-STAT signaling in the testis niche is surprising, given that it is thought to repress STAT targets during *Drosophila* hematopoiesis by interacting with the transcriptional repressor and JAK-STAT pathway inhibitor Ken and Barbie (Ken) (Arbouzova et al., 2006; Kwon et al., 2008). In contrast, STAT92E expression in GSCs requires NURF, reintroduction of STAT92E into *nurf301* null CPCs partially rescues their loss from the testis niche, and NURF genetically interacts with the JAK-STAT inhibitor SOCS36E in a manner consistent with it being a positive regulator of this pathway in the testis. We propose that *nurf301* likely regulates the JAK-STAT pathway in a tissue-specific manner and it will be important to identify factors that can interact directly with Nurf301 in the testis niche. Furthermore, determining whether Ken plays a role in the testis niche should be informative. Since Nurf301 can both activate and repress the transcription of several hundred genes in *Drosophila* larvae (Badenhorst et al., 2005) and binds to STAT92E binding sites *in vivo* (Kwon et al., 2008), identifying targets of both NURF and

STAT92E in testis stem cells will reveal whether NURF promotes JAK-STAT signaling directly by activating transcription, or indirectly, by prohibiting the expression of JAK-STAT inhibitors.

NURF is the sole ISWI family member needed for testis stem cell maintenance

Although the *Drosophila* ISWI family of chromatin remodelers has three members (NURF, ACF, and CHRAC), NURF alone is required for GSC and CPC maintenance in the testis. Interestingly, germline and follicle stem cells in the ovary use distinct chromatin remodeling factors to control self-renewal; ISWI is required for maintenance of GSCs, but is dispensable in follicle stem cells, while the INO80 family ATPase *Domino* promotes follicle stem cell self-renewal but is not required in GSCs (Xi and Xie, 2005). Thus, within endogenous niches, each type of stem cell requires a unique constellation of genetic and epigenetic regulators. Since NURF is required for GSC maintenance and primary spermatocyte differentiation (Kwon et al., 2009), but is dispensable for spermatogonial differentiation, additional means of epigenetic regulation must exist to support the dramatic changes in chromatin structure that accompany spermatogenesis. The well-characterized polycomb-group (PcG) proteins, which epigenetically silence target genes via covalent histone modification and are essential for maintenance of mammalian hematopoietic and spermatogonial stem cells (Buaas et al., 2004; Lessard and Sauvageau, 2003; Park et al., 2003), are essential regulators of spermatogonial differentiation in *Drosophila* (Chen et al., 2005). It will be interesting to determine if PcG proteins also function in GSCs within the testis. Furthermore, since somatic stem cells also require NURF but their daughter cells do not, it will be interesting to learn whether differentiation in this lineage requires additional chromatin remodelers. Since chromatin immunoprecipitation (ChIP) has been used successfully in the testis (Chen et al., 2005), and we have devised a genetic means to greatly expand the pool of stem cells and identify genes with enriched expression in stem cells (Terry et al., 2006), identification of NURF and STAT92E targets in both testis stem cell lineages is now possible, and should greatly enhance our understanding of how genetic and epigenetic mechanisms coordinately regulate stem cells in an endogenous tissue.

EXPERIMENTAL PROCEDURES

Fly Stocks

w^{1118};; *nurf301⁴/TM6B Hu, w;; nurf301¹²/TM3 Ser* (Badenhorst et al., 2005), *w;; nurf301²/ TM6B Hu*, and *w;; nurf301³/TM3 Ser* (Badenhorst et al., 2002) were from P. Badenhorst. *w; P[w⁺ bamP::GFP] Sp/CyO* (Chen and McKearin, 2003) was from D. McKearin. *y w; acf1¹* and *acf1²* (Fyodorov et al., 2004) were from D. Fyodorov. *w; al b cn ISWI² sp/SM5, sp* (Deuring et al., 2000) was from J. Birchler. *UAS-ISWI-RNAi* (24505) and *UAS-WDS-RNAi* (38926) (Dietzl et al., 2007) were from the Vienna Drosophila RNAi Center. *P[c587-Gal4]* (Kai and Spradling, 2003) and *socs36E^{PZ1647}* (Issigonis et al., 2009) were from A. Spradling. *P[nanos-Gal4-VP16]* (Van Doren et al., 1998) was from M. Van Doren. *UAS-STAT92E* was from D. Montell (Silver et al., 2005). *y w* was used as wild-type; additional fly stocks came from the Bloomington Stock Center and all flies were raised at 25°C on standard molasses/yeast medium unless stated otherwise.

Mosaic Analysis

Negatively marked clones: *nurf301², nurf301³, nurf38^{k16102}, or iswi²* clones were induced using the FLP, FRT-mediated mitotic recombination technique (Xu and Rubin, 1993) in flies of the following genotypes: *y w, P[hs-FLP]/Y;; P[Ubi-GFP] P[neoFRT]80B/ nurf301² or ³ P[neoFRT]80B ry⁵⁰⁶, +/Y; P[neoFRT]42D P[Ubi-GFP.nls]/P[neoFRT]42D nurf38 ^{k16102}; MKRS, P[hs-FLP]/+, +/Y; P[w⁺ FRT]42B P[Ubi-GFP.nls] P[Ubi-GFP.nls]/ P[w⁺ FRT]42B iswi²; MKRS, P[hs-FLP]/+*. Control clones were induced in *P[hs-FLP]/Y;;*

*P[Ubi-GFP] P[neoFRT]80B/P[neoFRT]80B ry[506], +/Y; P[neoFRT]42D P[Ubi-GFP.nls]/
P[neoFRT]42D; MKRS, P[hs-FLP]/+,* or *+/Y; P[w+ FRT]42B P[Ubi-GFP.nls] P[Ubi-
GFP.nls]/P[w+FRT]42B; MKRS, P[hs-FLP]/+.* To induce clones, 0–5 day old adult male
flies were subjected to either two 1 hr. heat shocks at 37°C separated by 5 hrs. at 25°C
(*nurf301 and nurf38* clones) or three 30 min. heat shocks at 37°C separated by 30 min.
intervals at 25°C (*iswi* clones). After the final heat shock, flies were kept at 25°C and
dissected and stained at 2, 4, 6, 8, or 10 days after clone induction (ACI). GSC clones were
identified as Vasa-positive, GFP-negative cells contacting the hub.

Positively marked clones: *nurf301[2]* or *nurf301[3]* were induced using the mosaic analysis with
a repressible cell marker (MARCM) technique (Lee and Luo, 1999) in *y w, P[hs-FLP],
P[tub-Gal4] P[UAS-CD8-GFP];; P[tub-Gal80] P[w+ FRT]2A/nurf301[2 or 3] P[w+ FRT]2A*
flies. Control clones were induced in *y w, P[hs-FLP], P[tub-Gal4] P[UAS-CD8-GFP];;
P[tub-Gal80] P[w+ FRT]2A/P[w+ FRT]2A* (Wang and Struhl, 2004) (a gift from G. Struhl).
For overexpression of STAT92E in *nurf301* null CPCs *y w, P[hs-FLP], P[tub-Gal4]
P[UAS-CD8-GFP];UAS-STAT92E/+; P[tub-Gal80] P[w+ FRT]2A/nurf301[2 or 3] P[w+
FRT]2A* flies were used. Adult males were heat shocked three times for 30 min. at 37°C
separated by 30 min. intervals at 25°C. After the final heat shock, flies were kept at 25°C
and dissected and stained at 2, 4, 6, 8, 10, and 14 days ACI. CPC clones were identified as
Vasa-negative, GFP-positive cells contacting the hub.

RNAi Knockdown

RNA interference (RNAi) knockdown of ISWI in CPCs was accomplished in *P[c587-
Gal4];; UAS-ISWI-RNAi* 24505 flies. Control RNAi was performed with *P[c587-Gal4];
P[UAS-AvicGFP.dsRNA]* flies processed in parallel. 0–3 day old males raised at 18°C were
shifted to 29°C for 7 days to induce robust expression of the RNAi construct. ISWI protein
levels were monitored by staining with rabbit anti-ISWI and comparing the levels of ISWI
in GSCs and CPCs of the same testis.

Genetic Interactions

To assay for genetic interactions between *socs36E* and *stat92E* or *nurf301*, *socs36E[PZ1647];
nurf301[2 or 3]/+* and *socs36E[PZ1647]; stat92E[06346]/+* flies were generated by crossing
socs36E[PZ1647]; nurf301[2 or 3]/TM6B Hu or *socs36E[PZ1647]; stat92E[06346]/TM6B Hu* males to
socs36E[PZ1647] virgins. *socs36E[PZ1647]; +/TM6B Hu* siblings were used as controls. Testes
from 0-3 day old males raised at 25°C were dissected and analyzed as described above.

Additional Loss-of-Function Experiments

To assay GSC number in *nurf301* null larval testes, *w;; nurf301[2]/nurf301[3]* third instar larvae
were generated by crossing *w;; nurf301[2]/TM3, P[Kr-GAL4], P[UAS-GFP], Sb* males to *w;;
nurf301[3]/TM3, P[Kr-GAL4], P[UAS-GFP], Sb* virgins; homozygous mutants were
distinguished from heterozygous siblings by the absence of GFP. *dMi-2* null flies (*dMi-2[5]/
Df(3L)BSC1*) were created by crossing *w;; dMi-2[5]/TM3 Sb* males (Yamasaki and Nishida,
2006) (a gift from Y. Nishida) to *w;; Df(3L)BSC1/TM3 Sb* virgins. *w;; dMi-2[5]/TM3 Sb*
males were used as controls.

Immunostaining

Testes were dissected, fixed and stained as described previously (Matunis et al., 1997),
except testes were incubated with anti-dSTAT92E for 48h at 4°C. The following antibodies
were used: goat anti-Vasa (dC-13) (Santa Cruz Biotechnology; 1:400), rabbit anti-GFP
(Torrey Pines Biolabs; 1:10,000), chicken anti-GFP (Abcam; 1:10,000), mouse anti-β-
Galactosidase (Promega; 1:1000), mouse anti-1B1 (Developmental Studies Hybridoma

Bank; 1:20), mouse anti-Armadillo (Developmental Studies Hybridoma Bank; 1:50), rabbit anti-ISWI (Deuring et al., 2000) (a gift from John Tamkun; 1:100), rabbit anti-Nurf301 (Kwon et al.) (a gift from P. Badenhorst; 1:1000), rabbit anti-dSTAT92E (Flaherty et al., 2010) (a gift from E. Bach; 1:400), rabbit anti-Zfh1 (a gift from R. Lehmann; 1:5000), and rabbit anti-phospho-Histone H3 (Upstate, 1:200). Alexa 488-, Alexa 555-, Alexa 568-, Alexa 633-conjugated (Molecular Probes/Invitrogen) and FITC-conjugated Donkey anti-Chicken (VWR International) secondary antibodies were used at 1:400. DNA was stained with 4,6-diamidino-2-phenylindole (DAPI; Sigma) at 1μg/ml.

Analysis of confocal images

Confocal images were obtained with a Zeiss LSM 5 Pascal or a Zeiss LSM 510 Meta microscope and were collected as serial confocal sections at similar detection settings. Figures were assembled using Adobe Photoshop 7.0 and Adobe Illustrator CS and graphs were created with Prism 5 (GraphPad Software, Inc.). The number of GSCs and CPCs were determined using serial confocal reconstructions of the testis niche. GSCs were scored as Vasa-positive cells (with a spherical fusome where specified) making contact with the hub. CPCs were scored as Zfh1-positive cells with medium to strong staining according to the rainbow indicator in the Zeiss Pascal software. dSTAT92E levels in *nurf301* mutant GSCs were compared to those in *nurf301* heterozygous neighboring GSCs. Pixel intensity was measured using the Zeiss Pascal software in a single confocal section through the middle of the nucleus of the *nurf301* mutant GSC and the nearest heterozygous GSC in the same testis in the most proximal clockwise position. Statistical analysis of stem cell number was performed using Prism 5. Student's T-test was used to compare two groups and ANOVA analysis was used to compare three or more groups.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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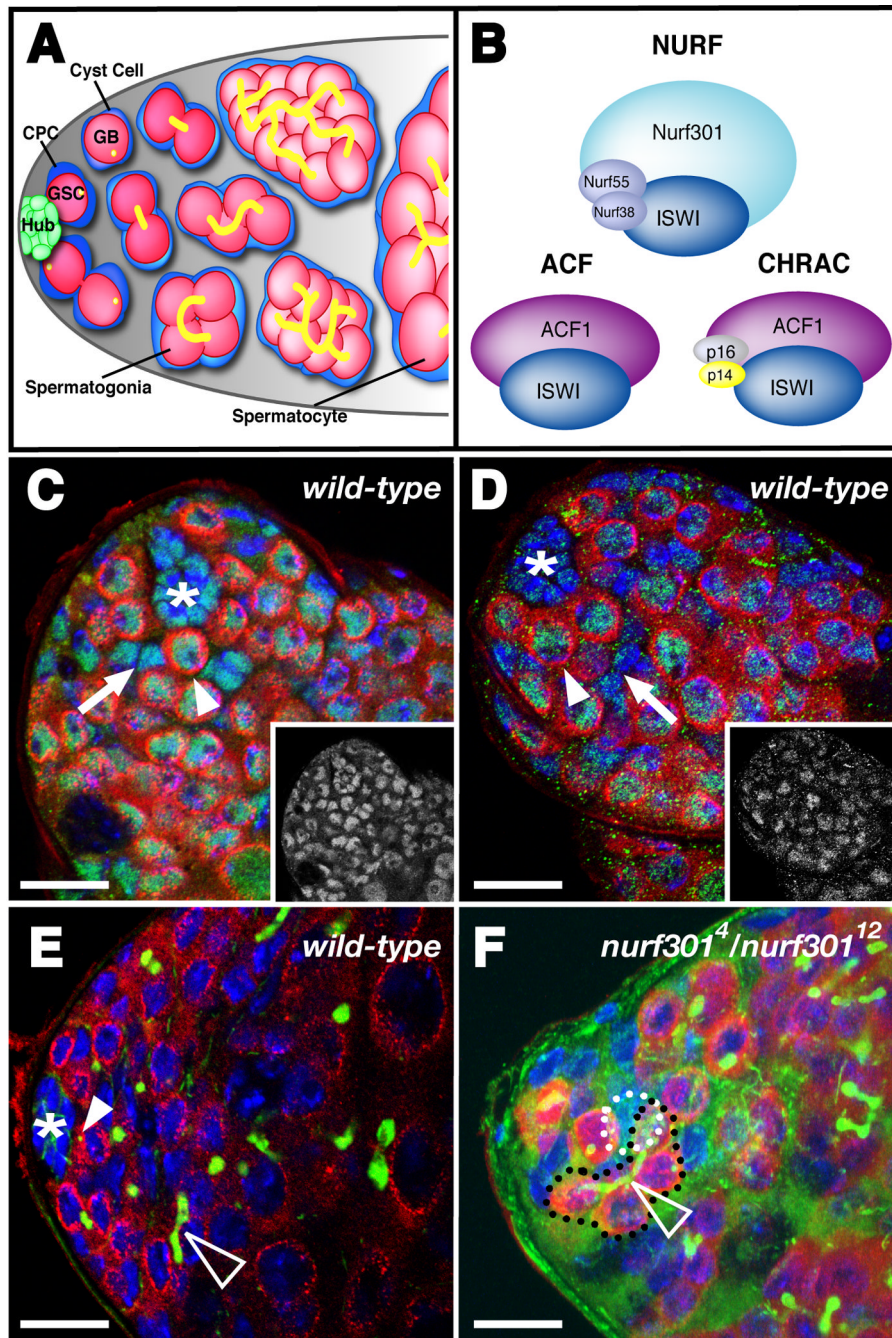
Figure 1. Nurf301 is a unique member of the NURF chromatin remodeling complex and is required for GSC maintenance in the *Drosophila* testis stem cell niche
(**A**) The *Drosophila* testis apex. Approximately 10 GSCs make broad contact with the hub. GSCs divide asymmetrically to produce gonialblast (GB) daughters that are displaced away from the hub. GBs undergo four rounds of mitosis giving rise to 16-interconnected spermatogonia, which further differentiate into spermatocytes. Approximately two CPCs flank each GSC and contact the hub through thin cytoplasmic extensions. CPCs divide asymmetrically, giving rise to non-mitotic somatic cyst cells that encyst differentiating germ cells. (**B**) NURF is one of three ISWI-containing ATP-dependent chromatin remodeling complexes in *Drosophila*. The Nurf301 subunit is unique to the NURF complex and is

essential for its function. ACF and CHRAC are distinct from NURF and share a common subunit: ACF1. (**C–D**) Confocal section of a wild-type testis apex immunostained with germline specific marker anti-Vasa (red), the DNA counterstain DAPI (blue), and (**C**) anti-ISWI (green, inset) or (**D**) anti-Nurf301 (green, inset). (**C**) ISWI is expressed in GSCs (Vasa-positive and contacting the hub, one indicated, arrowhead), CPCs (Vasa-negative and within two cell diameters of the hub, one indicated, arrow) and the hub (*). (**D**) Nurf301 is expressed in all cell types in the testis apex including GSCs (arrowhead), CPCs (arrow), and the hub (*). (**E–F**) Testes immunostained with anti-Vasa (red), the hub marker anti-Armadillo (green), the fusome marker anti-1B1 (green) and DAPI (blue). (**E**) A wild-type testis with a rosette of GSCs surrounding the hub (*). GSCs contain spherical fusomes (green, arrowhead) whereas differentiating spermatogonia contain elongated and branched fusomes (open arrowhead). (**F**) Projection of three confocal sections of a testis with reduced Nurf301 function (*nurf301^4/nurf301^12*) showing differentiating spermatogonia (black outline) containing a branched fusome (open arrowhead) and contacting the hub (white outline). Bars, 10 microns.
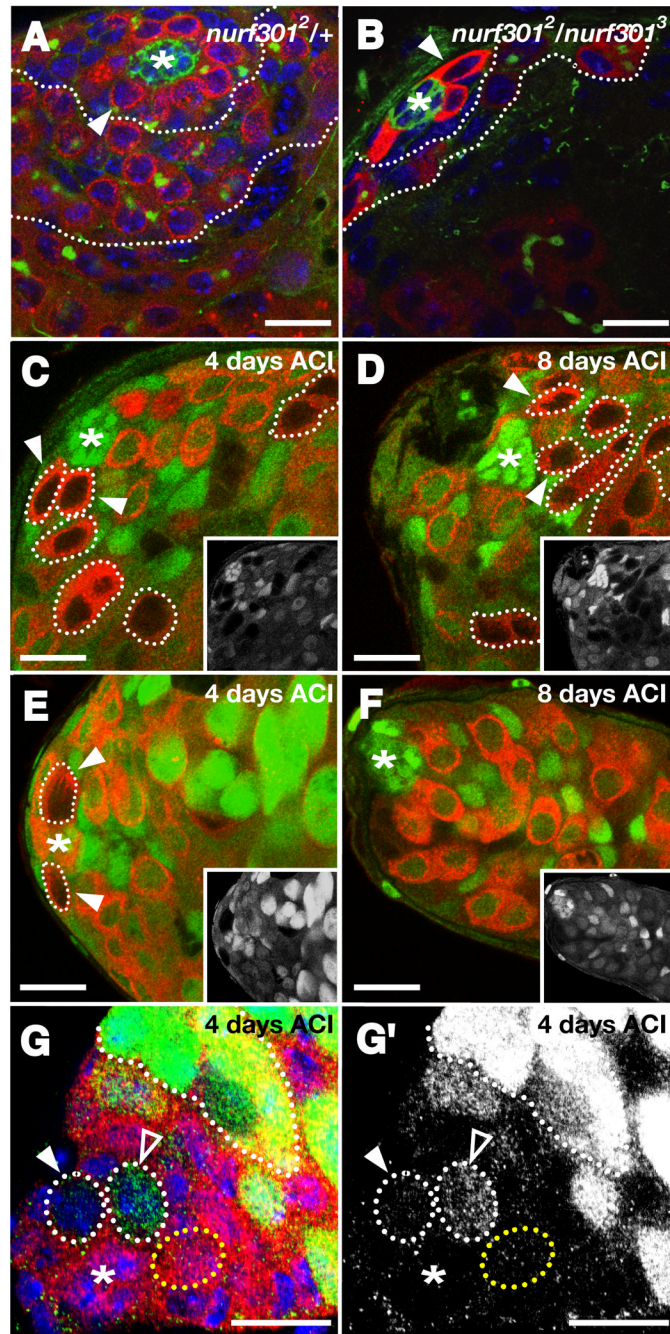
Figure 2. Nurf301 is required cell-autonomously for GSC maintenance. (A–B)
Larval testes immunostained with anti-Vasa (red), anti-Armadillo (green), anti-1B1 (green),
and DAPI (blue). (**A**) Larval testes heterozygous for *nurf301* (*nurf301²* / +) have a wild-type
number of GSCs (one indicated, arrowhead) surrounding the hub (*). Numerous
differentiating daughters (outlined) are displaced away from the hub. (**B**) Larval testes
homozygous mutant for *nurf301* (*nurf301²* / *nurf301³*) have significantly fewer GSCs (one
indicated, arrowhead) surrounding the hub (*) and a smaller zone of differentiating
spermatogonia (outlined). (**C–F**) Confocal sections of testes immunostained with anti-GFP
(green, inset) and anti-Vasa (red); hubs denoted with an asterisk. (**C–D**) Wild-type GSC
clones (arrowheads, outlined) and their differentiating daughters (outlined), identified by the

absence of GFP and the presence of Vasa, are detected (C) 4 days and **(D)** 8 days ACI. (**E**) *nurf301* mutant GSC clones are found at the hub 4 days ACI, but are no longer detected 8 days ACI (**F**). (**G**) Testis immunostained with anti-β-galactosidase (red) and a differentiation marker *bam-GFP* (green). *bam-GFP* is not normally expressed in GSCs (yellow outline) but is detected in differentiating spermatogonia (white outline). Two *nurf301* mutant GSCs (β-galactosidase-negative, white outline, arrowheads) surround the hub (*). One *nurf301* GSC expresses *bam-GFP* (outlined, open arrowhead) while the other is *bam-GFP*-negative (outlined, arrowhead), indicating that *nurf301* null GSCs differentiate prematurely in the niche. (**G'**) *bam-GFP* (green) channel alone. Bars, 10 microns.
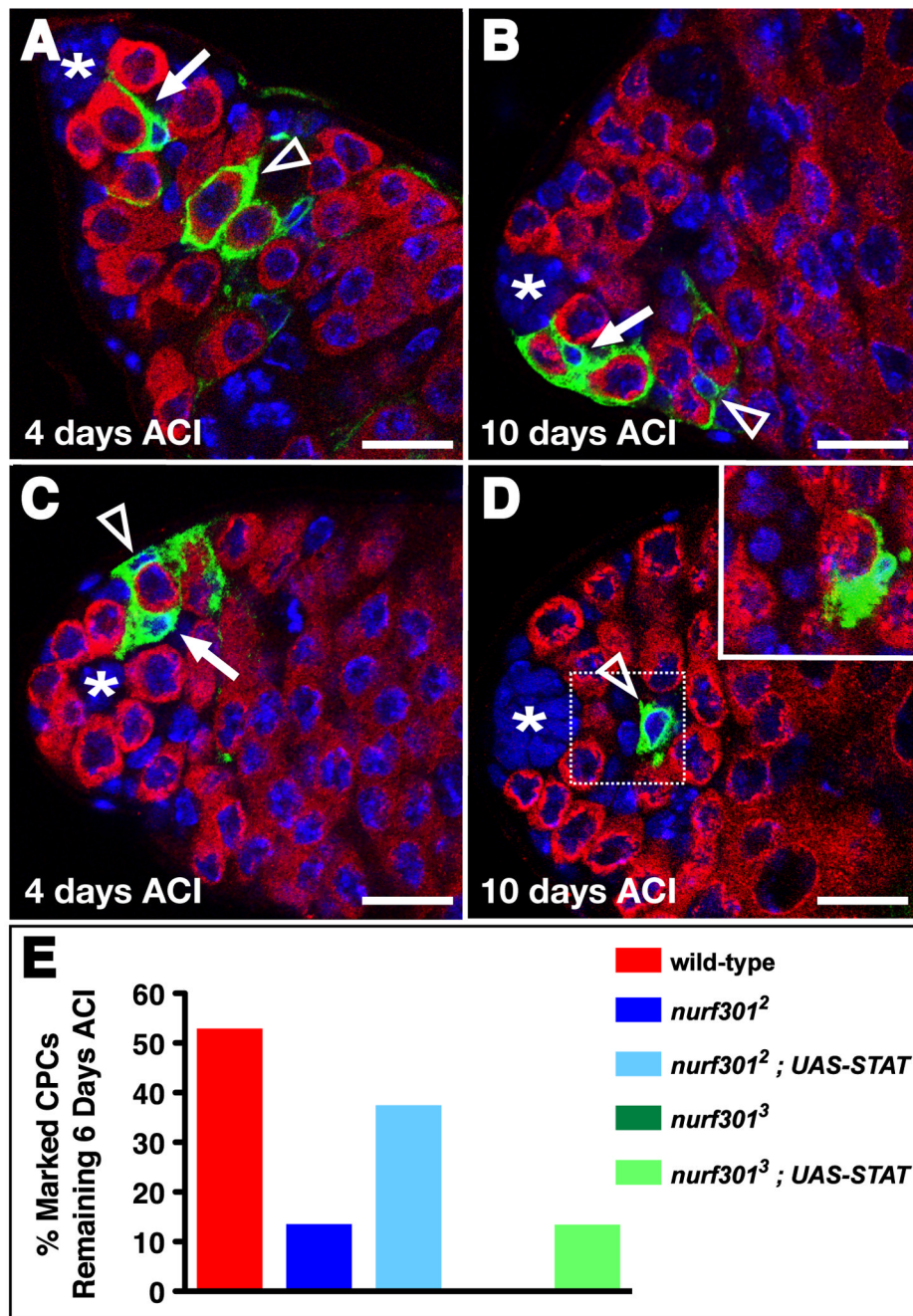
Figure 3. Nurf301 is required cell-autonomously for CPC maintenance. (A-D)
Confocal sections of testes immunostained with anti-GFP (green), anti-Vasa (red), and
DAPI (blue) containing positively-marked wild-type (**A, B**) or *nurf301* mutant (**C, D**) CPC
clones (arrow) and their differentiating daughters (open arrowhead) identified by the
presence of GFP, absence of Vasa, and their position within the tissue. (**A–B**) Wild-type
CPC clones and their daughters are detected at (**A**) 4 and (**B**) 10 days ACI. *nurf301* mutant
CPC clones are detected 4 days ACI (**C**), but are not maintained at 10 days ACI (**D**).
Differentiating mutant CPC daughters are occasionally still found 10 days ACI (open
arrowhead). Inset; higher magnification and adjacent confocal section of the boxed area in D
showing a mutant CPC daughter cell encysting a differentiating germline cell. (**E**) Restoring

STAT92E to *nurf301* null CPCs partially rescues their stem cell loss phenotype. Bars, 10 microns.
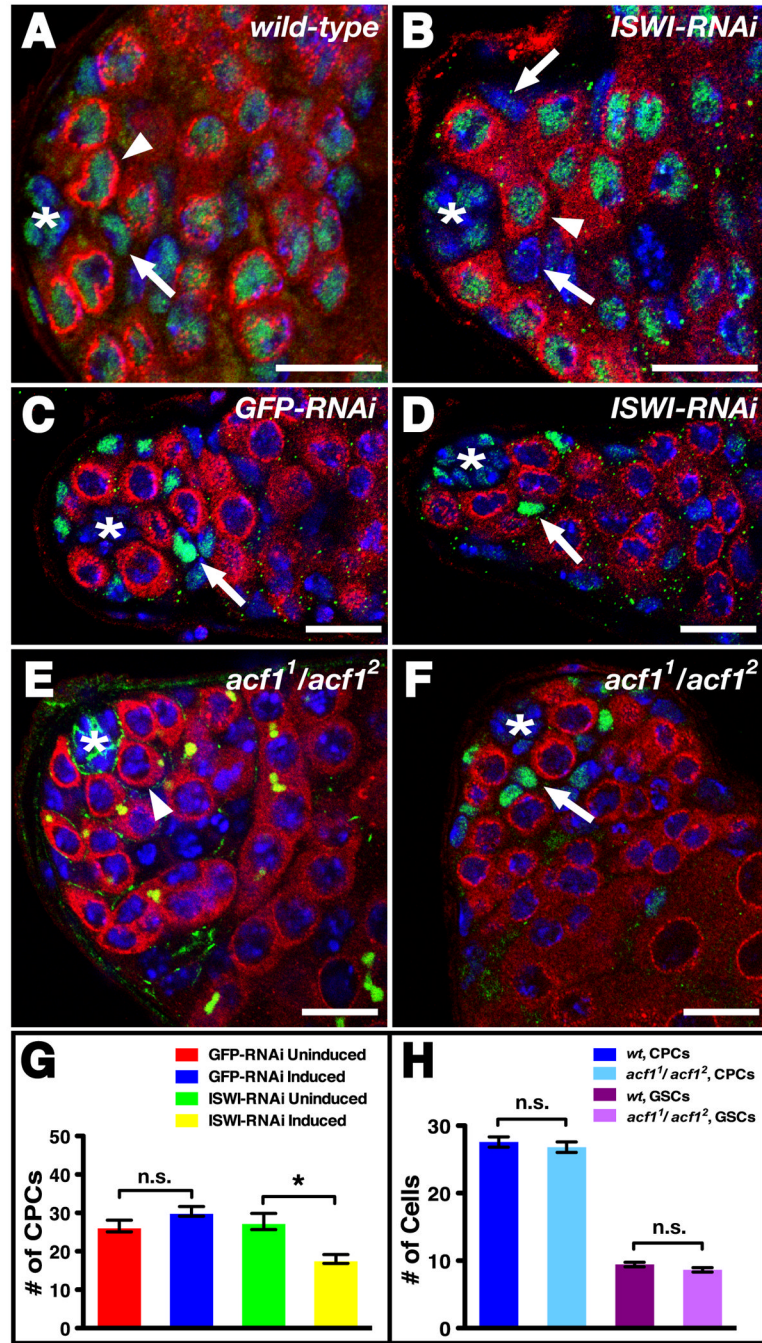
Figure 4. NURF is the sole ISWI-containing chromatin remodeling complex required for stem cell maintenance in the *Drosophila* testis

(**A–B**) Testes immunostained with anti-ISWI (green), anti-Vasa (red), and DAPI (blue). An asterisk denotes the hub. (**A**) In wild-type testes, ISWI is expressed at comparable levels in CPCs (arrows) and GSCs (arrowhead). (**B**) ISWI-RNAi knockdown in CPCs and their daughters reduces ISWI expression in CPCs (arrows) compared to GSCs (arrowhead). (**C–D**) Testes immunostained with anti-Vasa (red), CPC marker anti-Zfh-1 (green), and DAPI (blue) expressing (**C**) GFP-RNAi or (**D**) ISWI-RNAi in CPCs (arrows) and their daughters. When compared to controls (**C**), a reduction in ISWI leads to fewer CPCs around the hub (*) (**D**). (**E**) *acf1* homozygous mutant testis immunostained with anti-Vasa (red), anti-Armadillo

(green), anti-1B1 (green), and DAPI (blue) containing a wild-type number of GSCs (one indicated, arrowhead) surrounding the hub (*). (**F**) *acf1* homozygous mutant testis immunostained with anti-Vasa (red), anti-Zfh-1 (green), and DAPI (blue) containing a wild-type number of CPCs (one indicated, arrow) surrounding the hub (*). (**G**) ISWI-RNAi knockdown in CPCs and their daughters leads to a significant decrease in CPCs [n.s. (not significant), p-value > 0.05; *p-value < 0.001]. (**H**) *acf1* homozygous mutant testes have the same number of GSCs and CPCs as wild-type (n.s., p-value > 0.05). Data are represented as mean +/− SEM. Bars, 10 microns.
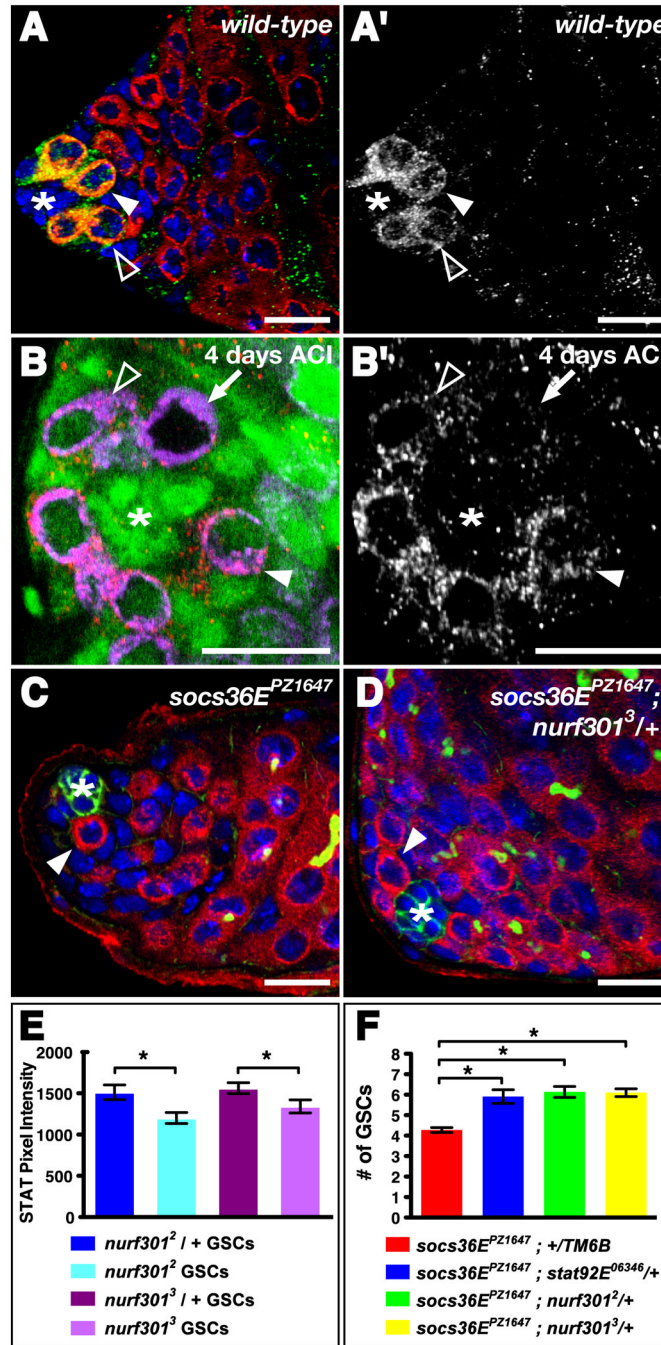
Figure 5. NURF regulates JAK-STAT signaling to maintain GSCs and CPCs in the *Drosophila* testis stem cell niche

(**A**) *nurf301* heterozygous testis immunostained with anti-Vasa (red), anti-dSTAT92E (green), and DAPI (blue). STAT92E is expressed primarily in GSCs (arrowhead) surrounding the hub (*), whereas gonialblasts have reduced STAT92E levels (open arrowhead). (**A**') STAT92E channel alone. (**B**) Testis immunostained with anti-Vasa (magenta), anti-GFP (green), anti-dSTAT92E (red), and DAPI (blue) containing a single negatively marked *nurf301²* homozygous mutant GSC (arrow) indicated by the presence of Vasa and absence of GFP, 4 days ACI. The homozygous *nurf301* GSC has reduced levels of STAT92E protein compared to the wild-type neighboring GSCs (one indicated, arrowhead)

but similar levels to a neighboring wild-type gonialblast (open arrowhead). An asterisk denotes the hub. (**B'**) STAT92E channel alone. (**C–D**) Testes immunostained with anti-Vasa (red), anti-Armadillo (green), anti-1B1 (green), and DAPI (blue). (**C**) *socs36E^{PZ1647}* homozygous mutant testes have a reduced number of GSCs (arrowhead) surrounding the hub (*). (**D**) Testes homozygous for *socs36E^{PZ1647}* and heterozygous for *nurf301³* have a near wild-type number of GSCs (one indicated, arrowhead) surrounding the hub (*). (**E**) *nurf301* null GSCs have significantly less dSTAT92E staining compared to neighboring wild-type GSCs (*, p-value < 0.05). (**F**) Removing one copy of *stat92E* or *nurf301* partially rescues the *socs36E^{PZ1647}* GSC loss phenotype (*, p-value < 0.001). Data are represented as mean +/− SEM. Bars, 10 microns.

Table 1

Nurf301, Nurf38, and ISWI are essential for maintaining GSCs in the *Drosophila* testis.

Genotype	2 days ACI	4 days ACI	6 days ACI	8 days ACI	10 days ACI
Average number of marked GSCs per testis					
Wild-type (*FRT80B*)	0.193 (11/57)	0.395 (32/81)	0.348 (46/132)	0.344 (21/61)	0.471 (16/34)
nurf301²	0.434 (23/53)	0.222 (16/72)	0.151 (16/106)	0 (0/61)	0.12 (3/25)
nurf301³	0.359 (23/64)	0.182 (16/88)	0.029 (4/138)	0.053 (4/76)	0.037 (2/54)
Wild-type (*FRT42D*)	0.06 (3/50)	0.109 (5/46)	ND	0.262 (11/42)	0.279 (12/43)
nurf38^{k16102}	0.308 (8/26)	0.28 (7/25)	ND	0 (0/40)	0 (0/36)
Testes with a marked GSC*					
Wild-type (*FRT42B*)	47.9% (46/96)	58.1% (43/74)	50% (43/86)	51.1% (24/47)	59.7% (46/77)
Iswi²	48.1% (25/52)	16.7% (12/72)	6.8% (4/59)	0% (0/41)	1.7% (1/59)

*The number of testes with one or more marked GSCs/the total number of testes.

Table 2

Nurf301 is essential for maintaining CPCs in the *Drosophila* testis.

Genotype	2 days	4 days	6 days	8 days	10 days	14 days
	Testes with a marked CPC*					
Wild-type (*FRT2A*)	62.3% (66/106)	66.7% (20/30)	33.3% (14/42)	20.9% (9/43)	19.2% (10/52)	33.3% (8/24)
nurf301²	33.3% (14/42)	24.1% (13/54)	4.7% (2/43)	4.7% (2/43)	1.9% (1/54)	4.8% (1/21)
nurf301³	36.4% (16/44)	4.8% (2/42)	0% (0/39)	0% (0/41)	0% (0/38)	0% (0/20)
nurf301² UAS-STAT	34.1% (14/41)	41.0% (9/22)	13.0% (3/23)	12.0% (3/25)	3.4% (2/59)	ND
nurf301³ UAS-STAT	14.3% (6/42)	14.0% (7/50)	2.0% (1/51)	0% (0/51)	0% (0/43)	ND

*The number of testes with one or more marked CPCs divided by the total number of testes.