RESEARCH ARTICLE

**DREF Genetically Counteracts Mi-2 and Caf1 to Regulate Adult Stem Cell Maintenance**

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Abstract

Active adult stem cells maintain a bipotential state with progeny able to either self-renew or initiate differentiation depending on extrinsic signals from the surrounding microenvironment. However, the intrinsic gene regulatory networks and chromatin states that allow adult stem cells to make these cell fate choices are not entirely understood. Here we show that the transcription factor *DNA Replication-related Element Factor* (*DREF*) regulates adult stem cell maintenance in the Drosophila male germline. A temperature-sensitive allele of *DREF* described in this study genetically separated a role for *DREF* in germline stem cell self-renewal from the general roles of *DREF* in cell proliferation. The *DREF* temperature-sensitive allele caused defects in germline stem cell self-renewal but allowed viability and division of germline stem cells as well as cell viability, growth and division of somatic cyst stem cells in the testes and cells in the Drosophila eye. Germline stem cells mutant for the temperature sensitive *DREF* allele exhibited lower activation of a TGF-beta reporter, and their progeny turned on expression of the differentiation factor Bam prematurely. Results of genetic interaction analyses revealed that *Mi-2* and *Caf1/p55*, components of the Nucleosome Remodeling and Deacetylase (NuRD) complex, genetically antagonize the role of *DREF* in germline stem cell maintenance. Taken together, these data suggest that *DREF* contributes to intrinsic components of the germline stem cell regulatory network that maintains competence to self-renew.

Author summary

Many adult tissues are maintained throughout life by the dual ability of adult stem cells to produce progeny that either self-renew or differentiate to replace specialized cells lost to turnover or damage. Although signals from the surrounding microenvironment have been shown to regulate the choice between self-renewal and onset of differentiation, the intrinsic gene regulatory programs that set up and maintain this bipotential state are not
well understood. In this report we describe antagonistic components of an intrinsic stem cell program important for maintaining the balance between self-renewal and differentiation in Drosophila male germline adult stem cell lineage. We identified a temperature-sensitive mutant in the transcription factor DNA Replication-related Element Factor (DREF) gene that disrupts the ability of germline stem cells to self-renew, but not stem cell viability, ability to divide or differentiate under the same conditions. DREF mutant germ-line stem cells showed defects in the TGF-beta signaling pathway, a pathway that is critical for maintaining the stem cell population. Genetic interaction analyses revealed that Mi-2 and Caf1/p55, components of the Nucleosome Remodeling and Deacetylase complex genetically antagonize the role of DREF in germline stem cell maintenance. We propose that DREF contributes to a transcriptional environment necessary for maintaining a bi-potential stem cell state able to properly respond to extrinsic niche signals.

Introduction

Adult stem cells maintain tissues during the lifetime of an organism by replenishing short-lived differentiated cells such as in the skin, intestinal epithelium and blood. Adult stem cells also give rise to differentiated cells upon injury in tissues such as skeletal muscle and lung. To maintain tissue homeostasis, daughter cells produced by adult stem cell divisions must make the critical cell fate decision between self-renewal and the onset of differentiation. Deviation from the tightly regulated balance between these alternate fates may result in poor tissue maintenance or cancerous growth of poorly differentiated precursor cells[1]. Adult stem cells are thus in a bi-potential state, able to self-renew or to initiate differentiation in response to extrinsic signals from the surrounding microenvironment[2,3]. This bi-potential state relies on intrinsic transcriptional and chromatin programs that dictate how stem cells respond to external signals from the niche.

Here we show that in Drosophila male germline adult stem cells, the transcription factor DNA Replication-Related Element Factor (DREF) and members of the Nucleosome Remodeling (NuRD) complex, Mi-2 and Chromatin Assembly Factor 1 (Caf1, also known as p55), act antagonistically to regulate the balance between germline stem cell self-renewal and differentiation. In the adult testis, two populations of stem cells, germline stem cells (GSCs) and somatic cyst stem cells (CySCs), reside adjacent to a group of post-mitotic somatic cells called the hub. The hub cells and the CySCs provide a microenvironment for GSC maintenance[4]. Both the germline and the somatic cyst stem cells divide asymmetrically: after division, one daughter remains in contact with the hub and self-renews while the other daughter is displaced away from the hub and initiates differentiation[5,6]. In the germline stem cell lineage, the differentiating daughter, termed the gonialblast, initiates four rounds of transit amplifying mitotic divisions with incomplete cytokinesis. The resulting 16 interconnected germ cells undergo premeiotic S phase in synchrony, become spermatocytes, and commit to terminal differentiation[7]. In the somatic cyst cell lineage, the differentiating daughter cell becomes a post-mitotic cyst cell, two of which enclose each gonialblast and its progeny, providing a supportive microenvironment necessary for the proper differentiation of the germ cells [8–10].

We identified a missense allele of DREF (DREF<sup>ts</sup>) that revealed a role for DREF in the maintenance of Drosophila male GSCs. DREF is known to function in cell growth, cell division, and DNA replication[11–13]. However, its role in these housekeeping processes has masked identification of other biological functions of DREF. The protein encoded by DREF<sup>ts</sup> is able to
function in cell division and cell survival but is defective for maintenance of GSCs in the testes. Analysis of mutant germline stem cells demonstrated defects in downstream targets of TGF-beta signaling. Genetic interactions with this allele of DREF suggested that DREF functions antagonistically to the chromatin regulators Caf1/p55 and Mi-2 to maintain GSCs. We propose that DREF may promote expression of self-renewal genes by overcoming transcriptional repression by a Mi-2 containing chromatin-remodeling complex.

Results

DREF function is required for male germline stem cell maintenance

A temperature-sensitive allele of DREF (DREFts) was discovered in an EMS-mutagenesis screen to identify genes required cell-autonomously for GSC maintenance in the Drosophila male germline. When GSCs homozygous for DREFts and negatively marked for GFP were generated using the FLP/FRT system [14] and grown at 25°C for 3 days post clone induction, 84.2 ±3.0% of the testes scored (n = 49) had at least one marked DREFts GSC adjacent to the hub. This is comparable to 95±3.0% of control testes (n = 38) with marked GSCs wild-type for DREF adjacent to the hub. However, the percentage of testes with at least one marked homozygous DREFts mutant GSC steadily decreased over time, so that by day 12 post clone induction, only 16.7±4.7% of testes (n = 91) contained one or more marked homozygous DREFts mutant GSCs, compared to 82.0±9.1% of testes (n = 64) in control flies (Fig 1A) (p<0.001). Surprisingly, DREFts did not have the same effect on CySC maintenance. The percentage of testes with marked CySCs in DREFts/+ and wild-type controls were comparable at day 3 post clone induction (75.6% of DREFts/+ (n = 38) to 78.3% (n = 49) of control testes) and also at day 12 post clone induction (39.2±14.5% of DREFts/+ (n = 41) to 44.3±6.6% (n = 62) of control testes) (Fig 1B).

Complementation analysis indicated that the mutation responsible for the failure to maintain GSCs mapped to the DREF locus. The DREFts allele failed to complement Df(2L)BSC17 [Df(DREF)], which deletes the DREF locus, as well as two independently-generated alleles, DREFkg9294 (referred to here as DREFnull) and DREFNP4719 for early germ cell loss. Analysis of testes by phase contrast microscopy revealed that an average of 94.6± 5.6% testes (n = 58, p<0.0001) from newly eclosed DREFts/Df(DREF) males grown at 25°C had late-stage germ cells (elongating spermatids) but lacked early germ cells (spermatogonia and primary spermatocytes), while 0% of testes from sibling DREF mutant/+ males lacked early germ cells (Fig 1C and 1D). The presence of elongating spermatids in testes from newly eclosed DREFts/Df (DREF) males suggests that GSCs and early germ cells were initially present during development but were eventually lost due to differentiation. Immunofluorescence analysis of mutant testes confirmed the absence of GSCs and early germ cells; testes from newly eclosed DREFts/Df(DREF) males grown at 25°C lacked Vasa-positive germ cells at the apical tip (Fig 1F), while testes from sibling controls had an abundance of Vasa-positive germ cells at the apical tip (Fig 1G). DREF function is also required for maintenance of female GSCs/early germ cells. In ovaries isolated from DREFts/Df(DREF) mutant adult females grown at 25°C and examined three days after eclosion, 26.9% (n = 26) of ovarioles contained developing germ cell cysts and egg chambers but empty geraria (S1 Fig).

The loss of early germ cells in DREFts/Df(DREF) males reared at 25°C was rescued by expression of a UAS-DREF cDNA transgene in early germ cells, confirming that the mutation causing GSC loss is in the DREF locus and that DREF function is required in germ cells for GSC maintenance. As assessed by phase contrast microscopy, only 5.37± 5.56% (n = 58) of testes from DREFts/Df(DREF) males grown at 25°C contained early germ cells, while 100% (n = 57) of testes from DREFts/Df(DREF) males expressing UAS-DREF cDNA under control of
Fig 1. DREF function is required cell-autonomously for male GSC maintenance. (A, B) Percentage of testes with at least one marked DREF<sup>ts</sup> mutant GSC (A, red), or DREF<sup>ts</sup> mutant CySC (B, red) or marked control GSCs (A, blue) or DREF<sup>ts</sup> function required for stem cell maintenance.
control CySCs (B, blue) at the indicated time points post clone induction. (C-H) Phase contrast (C-E) and immunofluorescence (F-H) images of a DREF<sup>ts</sup>/Df(DREF) mutant testis (C, F), a sibling control testis (D, G), and a DREF<sup>ts</sup>/Df(DREF) testis expressing UAS-DREF under the control of the germ cell driver nanos-Gal4VP16 (E, H). Apical tip of testis (denoted by the star) immunostained for vasa (green), FasIII (red), and DAPI (blue) is shown in (F-H). (I) Loss of DREF<sup>ts</sup>/Df(DREF) mutant GSCs grown at 22°C and then shifted to 30°C, compared with sibling controls. Scalebar: 50µm.

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nanos-Gal4-VP16 (Fig 1E) and 93.3 ± 8.80% (n = 59) of testes from DREF<sup>ts</sup>/Df(DREF) males expressing UAS-DREF cDNA under control of Vasa-Gal4 (Fig 1E and Table 1) contained early germ cells. Immunofluorescence analysis revealed the presence of GSCs adjacent to the hub in DREF<sup>ts</sup>/Df(DREF) mutant testes expressing UAS-DREF cDNA under control of nanos-Gal4-VP16 (Fig 1H).

The GSC loss phenotype of DREF<sup>ts</sup>/DREF<sup>null</sup> flies was temperature-dependent. As assayed by phase contrast microscopy, 100 ± 0% (n = 82) of testes isolated from DREF<sup>ts</sup>/DREF<sup>null</sup> trans-heterozygous flies grown at 22°C had early germ cells present on the day of eclosion. However, when DREF<sup>ts</sup>/DREF<sup>null</sup> flies were grown at 25°C, only 20.2 ± 12.9% (n = 375) of testes scored contained early germ cells on the day of eclosion. DREF<sup>ts</sup>/DREF<sup>null</sup> flies grown at 30°C failed to survive to adulthood and died in late third instar larval and/or pupal stages (Table 2).

Sequencing of the DREF coding region revealed that the DREF<sup>ts</sup> allele had two amino acid substitutions (Methionine 651 to Leucine and Glycine 652 to Alanine), which occur in a domain that has been shown to be responsible for co-factor binding in Drosophila DREF [15,16]. Germ cells homozygous mutant for DREF<sup>ts</sup> still expressed DREF protein as assayed by immunostaining 3 days and 6 days post clone induction. In contrast, at day 3 post clone induction, DREF protein expression was not detected in DREF<sup>null</sup> mutant germ cells by immunofluorescence using anti-DREF antibodies (S2 Fig). Furthermore, GSCs in testes from DREF<sup>ts</sup>/ DREF<sup>null</sup> versus DREF<sup>null</sup>/+ flies grown at 22°C until eclosion then shifted to 30°C for 2 days failed to show significantly different levels of DREF protein (0.63±0.15 n = 18 GSCs versus 0.72±0.21 n = 24 GSCs, Materials and Methods).

**DREF<sup>ts</sup> cells proliferate and are able to differentiate**

The DREF<sup>ts</sup> allele allowed separation of the role of DREF in stem cell maintenance from a general role of DREF in cell survival and proliferation. Male GSCs homozygous mutant for the DREF<sup>ts</sup> allele appeared to be lost because they differentiate more often than self-renew. While homozygous DREF<sup>ts</sup> mutant GSCs were rapidly lost from the apical hub region of the testis at 25°C, their marked clonal progeny appeared to differentiate normally to at least the spermatocyte stage, as assessed by phase contrast microscopy. Cysts of spermatocyte clones homozygous for the DREF<sup>ts</sup> allele examined on day 4 post clone induction contained 16 germ cells per spermatocyte cyst, as expected after 4 rounds of transit amplification divisions, and were comparable in size to neighboring wild-type spermatocytes (Fig 2A, 2A’ and 2C). In contrast, loss of DREF function in germline clones homozygous for the DREF<sup>null</sup> allele or due to expression in early germ cells of an RNAi hairpin directed against DREF mRNA under control of nanos-

**Table 1. Rescue of early germ cell loss in DREF mutants by different transgenes.**

| Genotype                                      | Average Percentage of testes with early germ cells | SD    | P-value       |
|-----------------------------------------------|----------------------------------------------------|-------|---------------|
| DREF<sup>ts</sup>, UAS-DREF/Df(2L)BSC17       | 4                                                  | 4.87  |               |
| DREF<sup>ts</sup>, UAS-DREF/Df(2L)BSC17; nanos-Gal4VP16/+ | 100                                                | 0     | 1.05E-10      |
| DREF<sup>ts</sup>, UAS-DREF/Df(2L)BSC17; Vasa-Gal4/+ | 91.67                                              | 11.78 | 9.44E-08      |
| DREF<sup>ts</sup>/Df(2L)BSC17; UAS-E-Cadherin/nanos-Gal4VP16 | 2.67                                               | 3.37  | 0.77          |

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Gal4-VP16 resulted in defects in germ cell survival, growth, proliferation, and/or differentiation. At day 4 post induction of marked clones homozygous for DREF\textsuperscript{null}, 32.6% of the testes scored contained no DREF\textsuperscript{null} clones, 24.5% had marked GSCs and/or spermatogonia but no late spermatocyte cysts homozygous for DREF\textsuperscript{null}, and only 42.9% contained cysts with recognizable spermatocytes at 25˚C (Fig 2D). The cysts of DREF\textsuperscript{null}/DREF\textsuperscript{null} mutant spermatocytes in the clones appeared smaller in size compared to neighboring DREF\textsuperscript{null}/+ germ cell cysts (Fig 2B and 2B'). In addition, about 25% of the germ cell cysts that made it to the spermatocyte stage exhibited fewer than 16 cells per cyst (Fig 2C). Together, these observations suggest that lack of DREF function may cause developing germ cell cysts to either die, grow slowly, or fail to initiate the differentiation program. Similarly, knockdown of DREF function in late spermatogonia and spermatocytes by expressing UAS-DREF-RNAi under the control of a Bam-Gal4 driver resulted in extensive cell death and absence of meiotic and post-meiotic stages (S3 Fig).

Consistent with the finding that homozygous DREF\textsuperscript{ts} germ cells proliferate and differentiate normally, cells homozygous for DREF\textsuperscript{ts} in the eye were able to proliferate and differentiate, unlike cells homozygous for DREF\textsuperscript{null}. Flies with eyes entirely composed of cells homozygous mutant for DREF\textsuperscript{null} generated using the EGUF-hid method \cite{17} and reared at 25˚C had very small eyes (Fig 2G). In contrast, eyes entirely homozygous mutant for DREF\textsuperscript{ts} appeared similar in size and morphology to wild type controls, whether grown at 25˚C (Fig 2E) or 30˚C (Fig 2F). Additionally, eyes in DREF\textsuperscript{ts}/DREF\textsuperscript{null} transheterozygous flies grown at 25˚C were wild type in size and appearance (Fig 2H).

The temperature-sensitivity of the DREF\textsuperscript{ts} allele allowed analysis of how GSCs are lost when shifted from permissive to non-permissive temperature. GSCs present when DREF\textsuperscript{ts}/DREF\textsuperscript{null} males were grown at 22˚C until eclosion were lost within 2–3 days upon shifting the males to 30˚C (Fig 1I). On the day of eclosion, DREF\textsuperscript{ts}/DREF\textsuperscript{null} males grown at 22˚C had an average of 6.6± 0.9 GSCs around the hub (n = 22 testes), compared to 8.2± 0.6 GSCs in heterozygous control testes (n = 27 testes). In contrast, testes from DREF\textsuperscript{ts}/DREF\textsuperscript{null} males grown at 22˚C then shifted to 30˚C for 3 days after eclosion had an average of 0.8± 0.7 GSCs (n = 69 testes), while sibling control testes from DREF mutant/+ males had an average of 8.19±1.4 GSCs per testis (n = 59 testes) (p <0.0001) (Fig 11).

The loss of DREF function when DREF\textsuperscript{ts}/DREF\textsuperscript{null} males were shifted to 30˚C did not appear to affect the rate of GSC division or survival. The percentage of GSCs in mitosis scored by immunostaining for phosphorylated-Threonine 3 of histone H3 (PH3) was similar in testes isolated from DREF\textsuperscript{ts}/DREF\textsuperscript{null} transheterozygotes (3.90 ± 0.65%, n = 154 GSCs) and sibling control flies (4.49±2.84%, n = 178 GSCs) that were grown at 22˚C, shifted to 30˚C at eclosion

Table 2. Viability of DREF mutants and different temperatures.

| Genotype                  | 22˚C          | 25˚C          | 30˚C          |
|---------------------------|---------------|---------------|---------------|
| DREF\textsuperscript{ts}/DREF\textsuperscript{ts} | Viable        | Viable, early germ cell loss (−25%) | lethal (3rd instar-pupal stages) |
| DREF\textsuperscript{ts}/DREF\textsuperscript{KGO9294} | Viable        | Viable, early germ cell loss (−75%) | lethal (3rd instar-pupal stages) |
| DREF\textsuperscript{ts}/DREF\textsuperscript{NP4719} | viable        | Viable, early germ cell loss (−80%) | lethal (3rd instar-pupal stages) |
| DREF\textsuperscript{ts}/Df(2L)BSC17 | viable        | Viable, early germ cell loss (−95%) | lethal (3rd instar-pupal stages) |
| DREF\textsuperscript{KGO9294}/DREF\textsuperscript{KGO9294} | lethal (embryonic/first instar) | lethal (embryonic/first instar) | lethal (embryonic/first instar) |
| DREF\textsuperscript{KGO9294}/DREF\textsuperscript{NP4719} | lethal (embryonic/first instar) | lethal (embryonic/first instar) | lethal (embryonic/first instar) |
| DREF\textsuperscript{NP4719}/DREF\textsuperscript{NP4719} | lethal (embryonic/first instar) | lethal (embryonic/first instar) | lethal (embryonic/first instar) |
| DREF\textsuperscript{NP4719}/Df(2L)BSC17 | lethal (embryonic/first instar) | lethal (embryonic/first instar) | lethal (embryonic/first instar) |
| DREF\textsuperscript{KGO9294}/Df(2L)BSC17 | lethal (embryonic) | lethal (embryonic) | lethal (embryonic) |
| Df(2L)BSC17/Df(2L)BSC17 | lethal (embryonic) | lethal (embryonic) | lethal (embryonic) |

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and then held at 30˚C for two days (Fig 3A). Additionally, TUNEL assays failed to detect dying GSCs in testes from either DREF\textsubscript{ts}/DREF\textsuperscript{null} transheterozygotes or control flies (n = 83 and n = 94 GSCs, respectively), while in both cases some dying spermatogonial cysts were detected.

The loss of GSCs in DREF\textsubscript{ts}/DREF\textsuperscript{null} testes did not appear to be due to loss of hub-GSC adhesion. Expression of UAS-E-Cadherin-GFP specifically in early germ cells using the nanos-Gal4-VP16 driver resulted in localization of E-Cadherin-GFP protein to the hub-GSC interface in both DREF\textsubscript{ts}/DREF\textsuperscript{null} (Fig 3C–3C''') and DREF\textsuperscript{ts}/+ (Fig 3D–3D''') GSCs in testes isolated from males grown at 22˚C until eclosion then shifted to 30˚C for two days. Expression of E-Cadherin-GFP using the UAS-GAL4 system in early germ cells did not rescue the loss of GSCs in DREF\textsuperscript{ts}/DREF\textsuperscript{null} temperature-shifted flies, as testes from DREF\textsuperscript{ts}/DREF\textsuperscript{null} males shifted to 30˚C for two days contained similar numbers of GSCs per testis whether or not they

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Fig 2. DREF\textsubscript{ts} does not affect germ cell differentiation in the testis and cell proliferation in the testis and eye. (A-B) DREF\textsubscript{ts} mutant spermatocyte clones (dashed-outline in A, A') are comparable in size to neighboring spermatocytes with wild-type DREF, while DREF\textsuperscript{null} mutant spermatocyte clones (dashed-outline B, B') show a significant reduction in cell size compared to neighboring wild-type spermatocytes. (C) DREF\textsuperscript{ts} and control spermatocytes contain the proper number of germ cells per cyst (16) whereas DREF\textsuperscript{null} mutant clones have cysts containing less than 16 cells. (D) Percentage of testes that contain marked spermatocyte clones (blue), marked early germ cell clones (red), or neither (green). (E-G) Analysis of the role of DREF in eye tissue using the EGUF/hid method to induce clones. DREF\textsuperscript{ts} mutant clones induced in the eye appear comparable to wild type whether grown at 25˚C (E) or 30˚C (F), while DREF\textsuperscript{null} clones cause cell death and display smaller eyes compared to controls (G). (H) Eyes from DREF\textsuperscript{ts}/DREF\textsuperscript{null} transheterozygous flies grown at 25˚C also have a wildtype appearance. Scalebar: 10 μm for (A and B).

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DREF function required for stem cell maintenance
expressed UAS-E-Cadherin-GFP under control of nanos-Gal4-VP16 (average of 0.9±0.2 GSCs per testis (n = 52 testes) and 0.8±0.7 GSCs per testis (n = 59 testes), respectively).

DREF<sup>ts</sup>/DREF<sup>null</sup> germ cells adjacent to the hub also oriented their centrosomes as in wild-type GSCs, where one centrosome is positioned adjacent to the hub-GSC interface throughout the cell cycle, resulting in oriented GSC division[5]. In testes isolated from DREF<sup>ts</sup>/DREF<sup>null</sup>...
males grown at 22˚C then shifted to 30˚C for two days after eclosion, an average of 92.78 ± 3.76% (n = 201 from 62 testes) of GSCs that remained next to the hub and contained two centrosomes had one centrosome adjacent to the hub-GSC interface, similar to control DREFts/+ GSCs (94.1 ± 1.07%, n = 239 GSCs from 30 testes) (Fig 3B).

Hub-GSC adhesion and oriented centrosome positioning are two features of GSCs that depend on activation of the JAK-STAT pathway in response to Unpaired (Upd) ligand secreted from the hub[10,18]. Consistent with intact hub-GSC attachment and correct positioning of centrosomes, GSCs in testes from DREFts/DREFnull males grown at 22˚C then shifted to 30˚C for two days post-eclosion expressed STAT92E protein, a downstream target of JAK-STAT signaling, at levels comparable to sibling control GSCs (Fig 3E–3E’ and 3F–3F’).

Impaired TGF-beta signal transduction pathway in DREFts/DREFnull GSCs

Many GSCs homozygous mutant for DREFts showed reduced expression of a reporter of TGF-beta signaling, Dad-LacZ, compared to their DREFts/+ neighbors next to the hub. In wildtype, Dad-LacZ is primarily expressed in GSCs, the gonialblast, and in later stage somatic cyst cells [19]. In many germ cells next to the hub made homozygous for DREFts by FLP induced mitotic recombination, LacZ staining was often drastically reduced compared to neighboring DREFts/+ + GSCs (Fig 4A–4A’). The effect was variable, however, with some GSCs homozygous for DREFts appearing to have normal levels of LacZ, likely leading to the observed gradual loss of DREFts mutant GSCs. As a population, mutant GSCs showed a 30.3% reduction (P < 0.05, n = 14 GSCs in 11 testes, Fig 4E) in Dad-LacZ staining intensity relative to control GSCs (n = 17 GSCs in 11 testes) within the same testes as quantified by ImageJ (Materials and Methods).

Consistent with a reduced response to TGF-beta signaling, the differentiation marker Bag of Marbles (Bam) was expressed earlier than normal in germ cells in DREFts/DREFnull testes. Bam protein is normally detected by antibody staining in 4- to 16-cell spermatogonial cysts [20]. Likewise, using a reporter line driving GFP-tagged Bam protein expressed from its own promoter and regulatory elements[21], Bam-GFP expression, as detected by anti-GFP immunostaining, was first detected in 4 cell cysts, with very few 2 cell cysts scoring positive. GSCs from DREFts/DREFnull males grown at 22˚C until eclosion then shifted to 30˚C for two days did not express Bam-GFP in GSCs adjacent to the hub (n = 36 GSCs from 14 testes), similar to GSCs from sibling controls that were either DREFts/+ or DREFnull/+ (n = 100 GSCs from 12 testes) (Fig 4B–4B’). Likewise, gonialblasts, defined as single germ cells away from the hub containing a dot-fusome, from DREFts/DREFnull temperature shifted males also did not express Bam-GFP (n = 23 gonialblasts from 14 testes), similar to the gonialblasts in heterozygous controls, in which Bam-GFP was not detected (n = 31 gonialblasts from 12 testes). However, there was a marked increase in the percentage of Bam-GFP positive two-cell cysts in DREFts/DREFnull mutant testes, where 35.1% of the two-cell cysts analyzed (n = 37 counted in 14 testes) were positive for Bam-GFP, compared with only 4.6% of two-cell cysts scored as positive for Bam-GFP in testes from DREFts/+ or DREFnull/+ sibling controls (n = 44 cysts counted in 12 testes)(Fig 4C’–4C’’’ and 4D).

Forced overactivation of the JAK-STAT signaling pathway did not rescue loss of GSCs in DREFts mutants

Function of DREF appeared to be required for maintenance of germline stem cell state even under the condition of forced ectopic expression of the ligand Upd, which activates the JAK-STAT signal transduction pathway. In control DREFts/+ or Df(DREF)+/+ flies, ectopic expression of UAS-Upd in early germ cells under the control of nanos-Gal4-VP16 at 25˚C resulted in
100% (n = 68 testes) of testes examined containing an overabundance of GSC-like Vasa-positive cells with dot spectrosomes, as well as many CySC-like cells positive for Zfh-1 (Fig 5A–5A’’').
Under the same conditions, in contrast, only 4.98±5.64% (n = 66 testes) of DREF<sup>ts</sup>/Df(DREF) testes ectopically expressing UAS-Upd under control of nanos-Gal4-VP16 exhibited an overabundance of GSC and CySC-like cells. Rather, the vast majority of testes (95.02%, n = 66) from DREF<sup>ts</sup>/Df(DREF) males carrying nanos-Gal4-VP16; UAS-Upd grown at 25˚C had an abundance of CySC-like cells but few or no germ cells as assayed by immunostaining for Vasa (Fig 5B–5B’’’). The abundance of Zfh-1 positive CySC-like cells in many of the DREF<sup>ts</sup>/Df(DREF) testes suggested that sufficient Upd was expressed early, prior to the GSC loss (Fig 5C–5C’’’).

Fig 5. DREF is required independently of the JAK-STAT pathway. (A, B, C) Ectopic expression of Upd in the germline leads to an overproliferation of GSC-like cells in heterozygous control testes (A-A’’’), but not in DREF<sup>ts</sup>/Df(DREF) testes (B-B’’’). DREF<sup>ts</sup>/Df(DREF) testes ectopically expressing Upd in the germline still contain an overabundance of Zfh-1 positive CySC-like cells (C-C’’’). Scalebar: 10μm.

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Under the same conditions, in contrast, only 4.98±5.64% (n = 66 testes) of DREF<sup>ts</sup>/Df(DREF) testes ectopically expressing UAS-Upd under control of nanos-Gal4-VP16 exhibited an overabundance of GSC and CySC-like cells. Rather, the vast majority of testes (95.02%, n = 66) from DREF<sup>ts</sup>/Df(DREF) males carrying nanos-Gal4-VP16; UAS-Upd grown at 25˚C had an abundance of CySC-like cells but few or no germ cells as assayed by immunostaining for Vasa (Fig 5B–5B’’’). The abundance of Zfh-1 positive CySC-like cells in many of the DREF<sup>ts</sup>/Df(DREF) testes suggested that sufficient Upd was expressed early, prior to the GSC loss (Fig 5C–5C’’’).

Caf1 and Mi-2 genetically antagonize DREF to inhibit GSC Self-Renewal

The DREF<sup>ts</sup> allele provided a sensitized background in which to screen for genetic interactors important for male GSC differentiation vs. maintenance. When DREF<sup>ts</sup>/DREF<sub>mut</sub> flies were raised at 25˚C, 79.8±12.9% of testes (n = 375) from newly eclosed males had few or no early germ cells and displayed elongating spermatid bundles close to the apical tip (Fig 6A).
Fig 6. Mi-2 and Caf1 genetically antagonize DREF. (A-F) Phase contrast images and immunostaining images of (A, D) DREF<sup>ts</sup>/DREF<sup>null</sup> testes; (B, E) DREF<sup>ts</sup>/DREF<sup>null</sup>; Caf<sup>9-2</sup>/+ testes, and (C, F) DREF<sup>ts</sup>/DREF<sup>null</sup>; Df(Mi-2)/+ testes, respectively. Half-dose reduction of Caf1 and Mi-2 resulted in DREF function required for stem cell maintenance.
Strikingly, reducing the gene dosage of either Chromatin assembly factor 1, p55 subunit (Caf-1/p55), a subunit of multiple chromatin-modifying complexes including the NuRD complex, or Mi-2, a subunit of the Nucleosome Remodeling and Deacetylase (NuRD) complex and the dMEC complex, rescued the DREF early germ cell loss phenotype. While only 20.2 ±12.9% (n = 375) of testes from newly eclosed DREFts/DREFnull males had early germ cells (Fig 6A), 93.9±5.35% (n = 62) of testes from DREFts/DREFnull; Caf1ts/+ males contained both plentiful early germ cells and abundant spermatocytes as assessed by phase contrast microscopy (Fig 6B). Similarly, 91.8±7.5% (n = 76) of testes from DREFts/DREFnull; Df(Mi-2)/+ males contained many early germ cells and spermatocytes (Fig 6C). Immunofluorescence analysis revealed that reducing Caf1 or Mi-2 dosage by half in a DREFts/DREFnull background restored the presence of GSCs next to the hub (Fig 6D–6F). The suppression of the DREF/DREFnull GSC loss phenotype by lowering the dosage of either Mi-2 or Caf1, both of which are components of the NuRD complex, suggests that DREF and the NuRD complex may act antagonistically to influence GSC maintenance. However, lowering the dose of Rpd3 or Mbd-like, two other subunits of the NuRD complex, did not suppress the early germ cell loss phenotype in DREF mutant testes as assessed by phase contrast microscopy (Fig 6G). Similarly, lowering the dose of XNP, a member of the DREF-containing XNP/dATRX repression complex, or Putzig, a member of the DREF-TRF2 complex, did not affect the germ cell loss phenotype, with only 29.1±10.9% (n = 74) and 23.3±10.4% (n = 41), respectively, of testes scored by phase contrast microscopy containing visible early germ cells (Fig 6G). TRF2, which is male lethal, could not be tested in the same manner.

**Mi-2 and Caf1 are required for GSC maintenance**

Mi-2 is required cell-autonomously for GSC maintenance in the testis. GSCs made homozygous mutant for either the Mi-2^4^ (frameshift) or the Mi-2^6^ (premature stop codon) allele using the Flp-FRT system were lost over time. At day 3 post clone induction, 65.9±16.1% of testes scored (n = 33) for Mi-2^4^ and 70.7±11.5% of testes scored (n = 66) for Mi-2^6^ contained marked homozygous mutant GSCs, compared to control clones, for which 82.5±5.9% of testes scored (n = 55) contained marked GSCs. By day 8 post clone induction, however, only 14.3±15.2% (n = 44) for Mi-2^4^ and 17.2±7.9% (n = 70) for Mi-2^6^ of testes scored contained marked homozygous mutant GSCs, while the percentage of control testes with marked GSCs was significantly higher (72.3±9.1%, n = 76, p<0.001) (Fig 7A). Germ cells homozygous mutant for Mi-2 were able to differentiate into spermatocytes. Expression of RNAi directed against Mi-2 using the nanos-Gal4-VP16 driver also resulted in early germ cell loss, confirming a role for Mi-2 in GSC maintenance (Fig 7B and 7C). Similarly, RNAi targeting Caf1 expressed using the nanos-Gal4-VP16 driver also resulted in GSC loss, demonstrating a requirement for Caf1 for GSC maintenance (S4 Fig). Homozygous Mi-2^4^ or Mi-2^6^ mutant GSCs remaining at day 5 post clone induction expressed DREF protein at levels comparable to neighboring Mi-2/+ heterozygous GSCs, as detected by immunostaining (Fig 7D). However, later stage germ cells lacking Mi-2 function demonstrated prolonged DREF expression, visible as persistent high levels of DREF protein in spermatocyte cysts in germline clones homozygous mutant for Mi-2, compared to neighboring Mi-2/+ spermatocytes (Fig 7E–7E”).
The DREFs allele demonstrates a specific requirement for DREF in adult stem cell self-renewal

Drosophila DREF was initially isolated based on its ability to bind the DNA-Replication Related Element (DRE), an 8bp sequence 5’TATCGATA’3 located upstream of many genes related to DNA replication. Binding of DREF protein to the DRE has been shown to activate expression of genes regulating cell division, including DNA polymerase, E2F, and Cyclin A[22–24]. In addition, Drosophila DREF has been shown to act downstream of the TOR [13] signaling pathway and Drosophila DREF and its human homolog, hDREF, have also been shown to...
control cell growth by regulating the expression of ribosomal genes and histone H1[13,25,26]. The DRE bound by DREF is known to be a key cis-regulatory component of a class of core promoters different from the canonical TATA box containing promoters[27]. Binding of DREF protein to the DRE recruits TRF2, a transcription factor related to TATA-box-binding protein (TBP), directing recognition of these alternate core promoters, regulating, for example expression of the proliferating cell nuclear antigen (PCNA)[28]. DREF Protein binds to and potentially regulates 1,961 distinct loci in the genome[29]. For example, DREF has been shown to regulate chromatin by: 1) by activating transcriptional expression of chromatin regulators such as brahma, moira and osa[22,30], Mes4[31], and HP6[32], 2) physically interacting with XNP/dATRX and potentially targeting them to regions in the genome[16], 3) competing with the chromatin insulator Boundary Element Association Factor 32 (BEAF32) for a mutual binding site[29,33], and 4) regulating the HET-A, TART, TAHER array (HTT) array in Drosofila[34]. Previous reports, and our work here, showed that null alleles of DREF have defects in cell division and cell growth in tissues ranging from the eye[12,22], salivary glands[12], the imaginal wing discs[35], and the testis (this report).

The DREFts allele we identified a role for DREF in self-renewal of germ line stem cells, genetically separable from the previously-defined role of DREF in cell proliferation. The DREFts allele did not strongly affect cell viability and division in the eye, and somatic cyst stem cells (CySCs) homozygous mutant for the DREFts allele were able to self-renew and differentiate normally. However, in flies homozygous mutant for the DREFts allele, GSCs were not maintained, although the progeny of mutant GSCs were able to differentiate into spermatocytes. It is possible that the DREFts allele might affect specific physical interactions between DREF and particular binding partner(s) required in GSCs but not in many other cell types, resulting in stem cell loss.

Intriguingly, previous research suggests that the domain mutated in DREFts (the CR3 domain) does not contribute to the DNA binding or dimerization functions of DREF, but may play a role in the ability of DREF to bind and interact with different cofactors[36]. Overexpression of the CR3 domain of DREF has been shown to have a dominant-negative effect on DREF function, possibly by competing for normal binding partners of endogenous DREF[36]. In support of this view, DREF has been shown to bind to the chromatin remodeling factor XNP through its CR3 domain[16]. When bound to XNP, DREF can function as a transcriptional repressor, in contrast to its typical role as a transcriptional activator when bound to TRF2[16]. However, XNP showed no genetic interaction with the DREFts/DREFnull mutant phenotype, suggesting that either XNP is not dosage sensitive or that the role of DREF in GSC maintenance is not mediated through the DREF-XNP complex.

Although an RNAi screen by Yan et al. [37] showed a requirement for DREF in the female germline, it is important to note that in our hands complete loss of function of DREF (through either RNAi or null alleles) causes severe phenotypes likely due to the role of DREF in housekeeping functions required for cell growth and division, which parallels the multiple, complex defects Yan et al. [37] notes in knockdown of DREF in the female germline.

**DREF is required for GSC maintenance independently of the JAK-STAT signaling pathway**

The JAK-STAT signaling pathway plays pivotal roles in regulating the two adult stem cell populations in the testis. In male GSCs, the JAK-STAT signal transduction pathway is required cell autonomously for adhesion to the hub and oriented divisions, but not for self-renewal[18]. Many mutants that result in GSC loss, such as NURF301 [38] have reduced JAK-STAT signaling, possibly resulting in loss of GSC adhesion to the hub and subsequent differentiation. In
contrast, DREF\textsuperscript{ts}/DREF\textsuperscript{null} GSCs had normal levels of STAT protein and did not appear to be defective in hub-GSC adhesion, as evidenced by localization of E-Cadherin and proper centrosome orientation, suggesting that they are not likely lost due to defects in JAK-STAT signaling.

JAK-STAT activation is also required in CySCs for self-renewal\cite{18,39}. Forced activation of JAK-STAT signaling in the testes, either by expressing constitutively active JAK in CySCs or by forced ectopic expression of the activating ligand Upd in germ cells, results in an apparent failure of CySCs to differentiate\cite{18}. As a consequence of the early CySC-like state, the neighboring germ cells fail to differentiate and the testis is filled with GSC-like and CySC-like cells. This overproliferation of GSC-like cells due to forced activation of the JAK-STAT pathway can mask or override the GSC-loss phenotype in his2Av or GEF26 mutants\cite{40,41}. Although, like his2Av and GEF26 mutant GSCs, DREF\textsuperscript{ts} GSCs are lost to differentiation, the outcome of combining DREF\textsuperscript{ts} with forced expression of Upd ligand in germ cells was strikingly different, with the DREF\textsuperscript{ts}/Df(DREF) germ line stem cell loss phenotype predominating even with forced activation of JAK-STAT signaling due to ectopic expression of Upd. Thus, while his2av and GEF26 may be important for fine-tuning the balance between self-renewal and differentiation, the function of DREF altered by the temperature-sensitive mutation may be intrinsically required for maintaining the GSC state.

**DREF may act in concert with Mi-2/NuRD to regulate chromatin structure and gene expression**

Genetic interaction studies uncovered a novel role for Caf1 and Mi-2, components of the Nucleosome Remodeling and Deacetylase (NuRD) chromatin-modifying complex in repressing DREF-mediated self-renewal. Reducing the gene dosage of either Caf1 or Mi-2 function by half was able to rescue the GSC-loss phenotype in a DREF\textsuperscript{ts}/DREF\textsuperscript{null} mutant background. Our results are consistent with previous studies indicating an antagonistic relationship between DREF and Mi-2. Reduction of Mi-2 gene dosage by half had been shown previously to enhance defects caused by DREF overexpression in the eye, consistent with Mi-2 antagonizing DREF\cite{42}. Yeast two-hybrid screening identified the human homolog of Mi-2, CHD4, as a binding partner of human DREF and pull-down assays confirmed this association in Drosophila, showing that Mi-2 physically associates with DREF\cite{42}. Mi-2 has been shown to interact with the DNA-binding domain of DREF, thereby inhibiting the ability of DREF to bind DNA in vitro\cite{42}. Additionally, recent work with hDREF has shown that the reciprocal regulation as well: hDREF has been shown to increase SUMOylation of Mi-2 protein, thereby increasing dissociation of Mi-2 from chromatin\cite{43}.

Our data also indicated an inhibitory interaction between DREF and Caf1, raising the possibility that Mi-2 might act as a part of the NuRD complex to inhibit DREF function in male germ line stem cells. The genetic interactions between Mi-2 or Caf1 and the DREF\textsuperscript{ts} allele suggest that Mi-2 and Caf1 can act as repressors of GSC self-renewal. Interestingly, we also found that Mi-2 and Caf1 are required for GSC maintenance in a genetic background wild-type for DREF function. The Mi-2/NuRD complex is known to play broad roles in reorganizing chromatin architecture to promote silencing\cite{44}, and Mi-2/NuRD has been shown to localize to hundreds of regions in the genome of Drosophila\cite{42}. One possibility is that complete loss of Mi-2 function may cause general de-repression of genes important for cell-identity, self-renewal, and differentiation. Indeed, loss of Mi-2 in spermatocytes causes activation cryptic promoters at many sites in the genome, leading to massive misexpression\cite{45}. It may be that Mi-2 plays a similar role in restraining misexpression during the early stages of germ cell development as well.

Previous studies suggest that Drosophila male GSCs are sensitive to changes in the transcript levels of genes required for self-renewal versus differentiation\cite{41}. One role of DREF in
Drosophila male GSCs may be to exclude Mi-2 from promoters of self-renewal genes, thereby allowing higher levels of expression at these loci. Under conditions of reduced DREF function in the DREFts mutant flies, Mi-2 may gain abnormal access to self-renewal genes, dialing down their expression and tilting the balance of GSC fate towards differentiation. We propose that in the context of DREFts/DREFnull background, a half-dose reduction of Mi-2 or CAF1 function is sufficient to allow the partially functional DREF protein expressed in the DREFts mutant to overcome Mi-2-mediated repression of self-renewal genes and tilt the balance back towards GSC maintenance.

**Materials and methods**

**Fly stocks and husbandry**

All crosses were grown at 25˚C on standard molasses media unless otherwise stated. DREF mutant alleles used in this study include 1) alts, dpov1, DREFts, bts, prts, FRT40A/SM6a (derived from an EMS mutagenesis screen), 2) DREFKG09294 (from BDSC); this allele is a P-element insertion into the 5’UTR of DREF and has been previously reported to be a null allele that expresses little to no DREF protein[13], 3) DREFKG09294, FRT40A (from DGRC), 4) Df(2L) BSC17 (referred to as Df(DREF), from BDSC) is a deletion that spans from pelota to DREF, and 5) DREFNP4719 (from DGRC), a P-element insertion in the 5’ UTR of DREF. Other mutant alleles used in this study include 1) Caf1ts-4, a deletion allele of Caf1 (gift from Joseph Lipsick, Stanford University[46]), 2) Df(3R) BSC471 (referred to as Df(Caf1), from BDSC), 3) Mi-2ts and Mi-2al alleles (from BDSC and gift from J. Müllerr[47]), 4) Df(3L) BSC445 (referred to as Df(Mi-2), from BDSC), 5) Df(3R) BSC471 (referred to as Df(Putzig), from BDSC), 6) Df(3R) XNPts (referred to as Df(XNP1), from BDSC), 7) Df(3L) Exel7208 (referred to as Df(Rpd3), from BDSC), 8) Df(3R) Exel6153 (referred to as Df(Mbd1), from BDSC) and 9) Nurf301ts, a deletion allele of Nurf301 (from BDSC). Other fly stocks used include 1) alts, dpov1, bts, prts, FRT40A (an isogenized version of FRT40A from BDSC), 2) yw, hs-flp122; FRT40A, Ubi-GFP, 3) eyeless-Gal4, UAS-Flp; GMR-hid, 2LCL FRT40A(17), 3) UAS-DEFL #6–1 [48] 4) bam::bam-GFP, a transgenic line driving Bam-GFP under the control of bam promoter (a gift from D. McKearin (21)) 5) UAS-Upd [49], and 6) UAS-DREF[22]. alts, dp, DREFts, bts, prts, FRT40A, UAS-DREF/SM6a and DREFts, UAS-Upd were generated by recombining DREFts chromosome onto the UAS-DREF or UAS-Upd chromosome, respectively. The following Gal4 drivers were used to drive UAS transgen in a cell-type specific manner, 1) yw; nanos-Gal4VP16 (a gift from R. Lehmann[50]), 2) UAS-dicer2; nanos-Gal4VP16, 3) yw; Vasa-Gal4, 4) yw; Bam-Gal4, UAS-dicer2, (Bam-Gal4 lines was a gift from D. McKearin[21]) and 5) CS87-Gal4; tub-Gal80ts; UAS-dicer2 (CS87-Gal4 line was a gift from S. Hou). RNAi lines for DREF (BDSC#35962), Caf-1 (BDSC#34069 and VDRC#105838) and Mi-2 (VDRC#107204 and 10766) were used in this study.

Temperature shift experiments for germ cell loss analysis in DREF transheterozygotes were performed by growing flies at 22˚C until eclosion, and then shifting to 30˚C on the day of eclosion. Dissections were performed on day of eclosion, or on one, two, or three days post eclosion.

For analysis of DREF function in the eye, clones were generated by crossing DREF alleles on FRT40A chromosomes to eyeless-Gal4, UAS-Flp; GMR-hid, 2LCL FRT40A/SM6a. These crosses were grown at either 25˚C, or at 30˚C one day after the cross was set up.

**Immunofluorescence**

Testes were dissected in 1X phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde diluted in PBS for 20 minutes at room temperature, washed twice in PBS with 0.1%
TritonX-100, permeabilized in PBS with 0.3% TritonX-100 and 0.6% sodium deoxycholate for 30 minutes and blocked in PBS with 0.1% TritonX-100 and 3% bovine serum albumin for 30 minutes. Testes were incubated overnight at 4°C in primary antibodies against DREF (mouse, 1:100; gift from Dr. Andreas Hochheimer[28]), Vasa (goat, 1:50; Santa Cruz Biotechnology), Zfh-1 (rabbit, 1:5000; gift from R. Lehman), Traffic Jam (guinea pig, 1:5000; gift from D. Godt) LI ET AL NAT CELL BIOL 2003, Armadillo (mouse, 1:10; Developmental Studies Hybridoma Bank (DSHB)), DE-Cadherin (rat, 1:10; DSHB), FasIII (mouse, 1:10; DSHB), alpha-spectrin (mouse, 1:10; DSHB), Green Fluorescent protein (rabbit 1:400–1:1000; Invitrogen and Sheep 1:100; Abd-Serotec), gamma-tubulin (mouse, 1:50; Sigma), phosphor-Histone3 Threonine3 (rabbit, 1:100; Upstate Biotechnology/Millipore), and STAT92E (rabbit, 1:100; gift from E. Bach[51]). Testes were incubated in appropriate secondary antibodies were from the Alexa Fluor-conjugated series (1:500; Molecular Probes) and mounted in VECTASHIELD medium containing DAPI to visualize DNA (Vector Labs). Tunel Assays were performed using the In Situ Cell Death Detection Kit, TMR red by Roche. Immunofluorescence images were taken using the Leica SP2 Confocal Laser scanning microscope. Phase and clonal analysis images were captured using a Zeiss Axioskop microscope and SPOT RT3 camera by Diagnostic Instruments or CoolSNAP camera by Phomometrics. Images were processed using Adobe Photoshop and Illustrator CS6. Nuclear protein quantification was performed using ImageJ software and comparing the relative levels of the stained protein of interest (standardized to DAPI to control for sample depth) between experimental and control GSCs.

**RNAi protocol**

To perform RNAi knockdown in the germline, males from strains containing the RNAi hairpin of interest were crossed to UAS-dicer2;; nanos-Gal4VP16 virgins and grown at 25˚C for 4 days after which the progeny of the cross were shifted to 30˚C. Testes were isolated from males of the cross on the day of eclosion and 7 days post eclosion. In other cases, to follow the time course of stem cell loss, the progeny of crosses were grown at 18˚C until eclosion, shifted to 30˚C on the day of eclosion and males from the cross were dissected at different days post-shift to 30˚C.

Somatic RNAi knockdown was performed by crossing the RNAi hairpin strains to virgin females containing the somatic lineage driver C587-Gal4;tub-Gal80ts;UAS-dicer2. Crosses were performed at 18˚C until eclosion, when adults were shifted to 30˚C and dissected 3- and 7-days post temperature shift.

Knockdown in transit amplifying cells and later stages was performed by crossing the RNAi hairpin to;; BamGal4, UAS-dicer2. Crosses were grown at 25˚C for 4 days and then shifted to 29˚C until eclosion and then they were dissected.

**Supporting information**

**S1 Fig. DREF is required in the female germline.** (A) Immunostaining of DREFts/Df(DREF) mutant germaria (denoted by the bracket) which lacks vasa-positive germ cells. (B) DREF heterozygous germaria (bracket) contains vasa-positive germ cells. (C). Percentage of ovarioles containing empty germaria in DREFts/Df(DREF) mutant germaria and sibling controls. Scale-bar: 10 μm. (TIF)

**S2 Fig. DREFts vs DREFnull protein Expression.** (A-A’’) DREFnull mutant GSCs (arrow) do not express DREF protein. (B-B’’) DREFts mutant GSCs (arrow) still express DREF protein. Scalebar: 10 μm. (TIF)
S3 Fig. DREF RNAi using bam-gal4 driver. (A) DREF RNAi driven under the BamGal4 driver in transit amplifying cells show dying cysts and an absence of meiotic and post-meiotic cell types (bracket). (B) Sibling hairpin only controls show meiotic and post-meiotic cell types (bracket). Scalebar: 50μm. (TIF)

S4 Fig. CAF1 RNAi using nanos-gal4vp16 driver. (A-A’’) Caf1 RNAi driven in germline stem cells using NG4VP16 results in a loss of early germ cells after 5 days of RNAi expression at 30°C. (B-B’’) Sibling hairpin only controls still retain GSCs under the same conditions. Scalebar: 20μm. (TIF)

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References
1. Clarke MF, Fuller M. Stem Cells and Cancer: Two Faces of Eve. Cell [Internet]. 2006 Mar 24 [cited 2019 Mar 17]; 124(6):1111–5. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16564000 https://doi.org/10.1016/j.cell.2006.03.011 PMID: 16564000
2. Jones DL, Wagers AJ. No place like home: anatomy and function of the stem cell niche. Nat Rev Mol Cell Biol [Internet]. 2008 Jan [cited 2019 Mar 17]; 9(1):11–21. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18097443 https://doi.org/10.1038/nrm2319 PMID: 18097443
3. Morrison SJ, Spradling AC. Stem Cells and Niches: Mechanisms That Promote Stem Cell Maintenance throughout Life. Cell [Internet]. 2008 Feb 22 [cited 2019 Mar 17]; 132(4):598–611. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18295578 https://doi.org/10.1016/j.cell.2008.01.038 PMID: 18295578

4. de Cuevas M, Matunis EL. The stem cell niche: lessons from the Drosophila testes. Development [Internet]. 2011 Jul 15 [cited 2019 Mar 17]; 138(14):2861–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21693509 https://doi.org/10.1242/dev.056242 PMID: 21693509

5. Yamashita YM, Jones DL, Fuller MT. Orientation of Asymmetric Stem Cell Division by the APC Tumor Suppressor and Centrosome. Science (80-) [Internet]. 2003 Sep 12 [cited 2019 Mar 19]; 301(5639):1547–50. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12970569 https://doi.org/10.1126/science.1087795 PMID: 12970569

6. Cheng J, Tiyaboonchai A, Yamashita YM, Hunt AJ. Asymmetric division of cyst stem cells in Drosophila testis is ensured by anaphase spindle repositioning. Development [Internet]. 2011 Mar 1 [cited 2019 Mar 19]; 138(5):831–7. Available from: https://doi.org/10.1242/dev.057901 PMID: 21303845

7. Davies EL, Fuller MT. Regulation of self-renewal and differentiation in adult stem cell lineages: lessons from the Drosophila male germ line. Cold Spring Harb Symp Quant Biol [Internet]. 2008 Jan 1 [cited 2019 Mar 19]; 73(0):137–45. Available from: http://symposium.cshlp.org/cgi/doi/10.1101/sqb.2008.73.063

8. Lim JGY, Fuller MT. Somatic cell lineage is required for differentiation and not maintenance of germline stem cells in Drosophila testes. Proc Natl Acad Sci [Internet]. 2012 Nov 6 [cited 2019 Mar 19]; 109(45):18477–81. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23091022 https://doi.org/10.1073/pnas.1215516109 PMID: 23091022

9. Tran J, Brenner TJ, DiNardo S. Somatic control over the germline stem cell lineage during Drosophila spermatogenesis. Nature [Internet]. 2000 Oct 12 [cited 2019 Mar 19]; 407(6805):754–7. Available from: http://www.nature.com/articles/35037613 https://doi.org/10.1038/35037613 PMID: 11048723

10. Kiger AA, Jones DL, Schulz C, Rogers MB, Fuller MT. Stem cell self-renewal specified by JAK-STAT activation in response to a support cell cue. Science [Internet]. 2001 Dec 21 [cited 2019 Mar 19]; 294(5551):2542–5. Available from: https://doi.org/10.1126/science.1067070 PMID: 11732574

11. Hirose F, Yamaguchi M, Kuroda K, Oomori A, Hachiya T, Ikeda M, et al. Isolation and characterization of cDNA for DREF, a promoter-activating factor for Drosophila DNA replication-related genes. J Biol Chem [Internet]. 1996 Feb 16 [cited 2019 Mar 19]; 271(7):3930–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8632015

12. Hirose F, Yamaguchi M, Matsukage A. Targeted expression of the DNA binding domain of DRE-binding factor, a Drosophila transcription factor, attenuates DNA replication of the salivary gland and eye imaginal disc. Mol Cell Biol [Internet]. 1999 Sep [cited 2019 Mar 19]; 19(9):6020–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10454549 https://doi.org/10.1074/mcb.19.9.6020 PMID: 10454549

13. Killip LE, Grewal SS. DREF is required for cell and organismal growth in Drosophila and functions downstream of the nutrition/TOR pathway. Dev Biol [Internet]. 2012 Nov 15 [cited 2019 Mar 19]; 371(2):191–202. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0012160612004605 https://doi.org/10.1016/j.ydbio.2012.08.020 PMID: 22960233

14. Xu T, Rubin GM. Analysis of genetic mosaics in developing and adult Drosophila tissues. Development [Internet]. 1993 Apr [cited 2019 Mar 19]; 117(4):1223–37. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8404527 PMID: 8404527

15. Yamashita D, Komori H, Higuchi Y, Yamaguchi T, Osumi T, Hirose F. Human DNA replication-related element binding factor (hDREF) self-association via hATC domain is necessary for its nuclear accumulation and DNA binding. J Biol Chem [Internet]. 2007 Mar 9 [cited 2019 Mar 19]; 282(10):7563–75. Available from: https://doi.org/10.1074/jbc.M607180200 PMID: 17209048

16. Valadez-Graham V, Yoshioka Y, Velazquez O, Kawamori A, Vazquez M, Neumann A, et al. XNP/dATRX interacts with DREF in the chromatin to regulate gene expression. Nucleic Acids Res [Internet]. 2012 Feb [cited 2019 Mar 19]; 40(4):1460–74. Available from: https://doi.org/10.1093/nar/gkr865 PMID: 22021382

17. Stowers RS, Schwarz TL. A genetic method for generating Drosophila eyes composed exclusively of mitotic clones of a single genotype. Genetics [Internet]. 1999 Aug [cited 2019 Mar 19]; 152(4):1631–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10430588 PMID: 10430588

18. Leatherman JL, Dinardo S. Germline self-renewal requires cyst stem cells and Stat regulates niche adhesion in Drosophila testes. Nat Cell Biol [Internet]. 2010 Aug 11 [cited 2019 Mar 19]; 12(8):806–11. Available from: http://www.nature.com/articles/ncb2086 https://doi.org/10.1038/ncb2086 PMID: 20622868

19. Kawase E, Wong MD, Ding BC, Xie T. Gbb/Bmp signaling is essential for maintaining germline stem cells and for repressing bam transcription in the Drosophila testes. Development [Internet]. 2004 Mar 18
Hirose F, Ohshima N, Shiraki M, Inoue YH, Taguchi O, Nishi Y, et al. Ectopic expression of DREF

Yamashita D, Sano Y, Adachi Y, Okamoto Y, Osada H, Takahashi T, et al. hDREF regulates cell prolif-

Ohshima N, Takahas hi M, Hirose F. Identificati on of a human homolog ue of the DREF transcrip tion fac-

Chen D, McKearin DM. A discrete transcript ional silencer in the bam gene determines asymmetr ic divi-

Ohler U, Liao G, Niemann H, Rubin GM. Comput ational analysis of core promoter s in the Drosophil a

Insco ML, Leon A, Tam CH, McKearin DM, Fuller MT. Accum ulation of a differentiation regulator speci-

Hochheim er A, Zhou S, Zheng S, Holmes MC, Tjian R. TRF2 associates with DREF and directs pro-

Suyari O, Ida H, Yoshioka Y, Kato Y, Hashimoto R, Yamaguchi M. Identification of the Drosophil a Mes4

Nakamura K, Ida H, Yamaguchi M. Transcriptional regulation of the Drosophila moira and osa genes by

Nakamura K, Ida H, Yamaguchi M. Transcriptional regulation of the Drosophila genome. Genome Biol [Interne[t]. 2002 [cited 2019 Mar 20];3(12):RESEARCH0087. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12537576

Hochheimer A, Zhou S, Zheng S, Holmes MC, Tjian R. TRF2 associates with DREF and directs pro-

Gurudatta B V, Yang J, Van Bortle K, Donlin-A sp PG, Corces VG. Dynami c changes in the genomic

Ida H, Suzusho N, Suyari O, Yoshida H, Ohno K, Hirose F, et al. Genetic screening for modifiers of the

Iida K, Ohno K, Hirose F, Sakaguchi K, Nishida Y, Matsukage A. Transcriptional regulation of the Drosophila

Ohno K, Hirose F, Sakaguchi K, Nishida Y, Matsukage A. Transcriptional regulation of the Drosophila

Sawado T, Hirose F, Takahashi Y, Sasaki T, Shinomiya T, Sakagu chi K, et al. The DNA replication-

Ohno K, Hirose F, Sakaguchi K, Nishida Y, Matsukage A. Transcriptional regulation of the Drosophila

Chen D, McKearin DM. A discrete transcript ional silencer in the bam gene determines asymmetr ic divi-

Suyari O, Ida H, Yoshioka Y, Kato Y, Hashimot o R, Yamagu chi M. Identificati on of the Drosophil a Mes4

Hirose F, Ohshima N, Shiraki M, Inoue YH, Taguchi O, Nishi Y, et al. Ectopic expression of DREF

Yamashita D, Sano Y, Adachi Y, Okamoto Y, Osada H, Takahashi T, et al. hDREF regulates cell prolif-

Ohno K, Hirose F, Sakaguchi K, Nishida Y, Matsukage A. Transcriptional regulation of the Drosophila

Sawado T, Hirose F, Takahashi Y, Sasaki T, Shinomiya T, Sakaguchi K, et al. The DNA replication-

Ohno K, Hirose F, Sakaguchi K, Nishida Y, Matsukage A. Transcriptional regulation of the Drosophila

Sawado T, Hirose F, Takahashi Y, Sasaki T, Shinomiya T, Sakaguchi K, et al. The DNA replication-

Ohno K, Hirose F, Sakaguchi K, Nishida Y, Matsukage A. Transcriptional regulation of the Drosophila

Sawado T, Hirose F, Takahashi Y, Sasaki T, Shinomiya T, Sakaguchi K, et al. The DNA replication-

Ohno K, Hirose F, Sakaguchi K, Nishida Y, Matsukage A. Transcriptional regulation of the Drosophila
35. Hyun J, Jasper H, Bohmann D. DREF is required for efficient growth and cell cycle progression in Drosophila imaginal discs. Mol Cell Biol [Internet]. 2005 Jul 1 [cited 2019 Mar 19]; 25(13):5590–8. Available from: https://doi.org/10.1128/MCB.25.13.5590-5598.2005 PMID: 15968414

36. Takahashi Y, Hirose F, Matsukage A, Yamaguchi M. Identification of three conserved regions in the DREF transcription factors from Drosophila melanogaster and Drosophila virilis. Nucleic Acids Res [Internet]. 1999 Jan 15 [cited 2019 Mar 19]; 27(2):510–6. Available from: http://www.ncbi.nlm.nih.gov/pubmed/9862973 PMID: 9862973

37. Yan D, Neumüller RA, Buckner M, Ayers K, Li H, Hu Y, et al. A Regulatory Network of Drosophila Germline Stem Cell Self-Renewal. Dev Cell [Internet]. 2014 Feb 24 [cited 2019 Mar 19]; 28(4):459–73. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24576427 https://doi.org/10.1016/j.devcel.2014.01.020 PMID: 24576427

38. Cherry CM, Matunis EL. Epigenetic regulation of stem cell maintenance in the Drosophila testis via the nucleosome-remodeling factor NURF. Cell Stem Cell [Internet]. 2010 Jun 4 [cited 2019 Mar 19]; 6 (6):557–67. Available from: https://linkinghub.elsevier.com/retrieve/pii/S1934590910002092 https://doi.org/10.1016/j.stem.2010.04.018 PMID: 20569893

39. Leatherman JL, Dinardo S. Zfh-1 controls somatic stem cell self-renewal in the Drosophila testis and nonautonomously influences germline stem cell self-renewal. Cell Stem Cell [Internet]. 2008 Jul 3 [cited 2019 Mar 19]; 3(1):44–54. Available from: https://linkinghub.elsevier.com/retrieve/pii/S1934590908002154 https://doi.org/10.1016/j.stem.2008.05.001 PMID: 18593558

40. Wang H, Singh SR, Zheng Z, Oh S-W, Chen X, Edwards K, et al. Rap-GEF signaling controls stem cell anchoring to their niche through regulating DE-cadherin-mediated cell adhesion in the Drosophila testis. Dev Cell [Internet]. 2006 Jan [cited 2019 Mar 19]; 10(1):117–26. Available from: https://linkinghub.elsevier.com/retrieve/pii/S1534580705004260 https://doi.org/10.1016/j.devcel.2005.11.004 PMID: 16399083

41. Morillo Prado JR, Srinivasan S, Fuller MT. The histone variant His2Av is required for adult stem cell maintenance in the Drosophila testis. White RAH, editor. PLoS Genet [Internet]. 2013 Nov 7 [cited 2019 Mar 19]; 9(11):e1003903. Available from: https://doi.org/10.1371/journal.pgen.1003903 PMID: 24244183

42. Hirose F, Ohshima N, Kwon E-J, Yoshida H, Yamaguchi M. Drosophila Mi-2 negatively regulates dDREF by inhibiting its DNA-binding activity. Mol Cell Biol [Internet]. 2002 Jul [cited 2019 Mar 19]; 22 (14):5182–93. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12077345 https://doi.org/10.1128/MCB.22.14.5182-5193.2002 PMID: 12077345

43. Yamashita D, Moruchi T, Osumi T, Hirose F. Transcription Factor hDREF Is a Novel SUMO E3 Ligase of Mi2α. J Biol Chem [Internet]. 2016 May 27 [cited 2019 Mar 19]; 291(22):11619–34. Available from: https://doi.org/10.1074/jbc.M115.713370 PMID: 27068747

44. Bowen NJ, Fujita N, Kajita M, Wade PA. Mi-2/NuRD: multiple complexes for many purposes. Biochim Biophys Acta [Internet]. 2004 Mar 15 [cited 2019 Mar 19]; 1677(1–3):52–7. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0005276X0300276X https://doi.org/10.1016/j.bbadis.2003.10.010 PMID: 15020045

45. Kim J, Lu C, Srinivasan S, Awe S, Brehm A, Fuller MT. Blocking promiscuous activation at cryptic promoters directs cell type-specific gene expression. Science [Internet]. 2017 May 19 [cited 2019 Mar 19]; 356(6339):717–21. Available from: https://doi.org/10.1126/science.aal3096 PMID: 28522526

46. DeBruhl H, Wen H, Lipsick JS. The complex containing Drosophila Myb and RB/E2F2 regulates cytokinesis in a histone H2Av-dependent manner. Mol Cell Biol [Internet]. 2013 May 1 [cited 2019 Mar 19]; 33 (9):1809–18. Available from: https://doi.org/10.1128/MCB.01401-12 PMID: 23438598

47. Kehle J, Beuchle D, Treueh S, Christen B, Kennison JA, Bienz M, et al. dMr-2, a hunchback-interacting protein that functions in polycistrn repression. Science [Internet]. 1998 Dec 4 [cited 2019 Mar 19]; 282 (5395):1897–900. Available from: http://www.ncbi.nlm.nih.gov/pubmed/9836641 https://doi.org/10.1126/science.282.5395.1897 PMID: 9836641

48. Oda H, Tsukita S. Nonchordate classic cadherins have a structurally and functionally unique domain that is absent from chordate classic cadherins. Dev Biol [Internet]. 1999 Dec 1 [cited 2019 Mar 19]; 216 (1):406–22. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0012160699994940 https://doi.org/10.1006/dbio.1999.9494 PMID: 10588889

49. Zeidler MP, Perrimon N, Strutt DI. Polarity determination in the Drosophila eye: a novel role for unpaired and JAK/STAT signaling. Genes Dev [Internet]. 1999 May 15 [cited 2019 Mar 20]; 13(10):1342–53. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10346822 https://doi.org/10.1101/gad.13.10.1342 PMID: 10346822

50. Van Doren M, Williamson AL, Lehmann R. Regulation of zygotic gene expression in Drosophila primordial germ cells. Curr Biol [Internet]. 1998 Feb 12 [cited 2019 Mar 19]; 8(4):243–6. Available from: http://www.ncbi.nlm.nih.gov/pubmed/9501989 PMID: 9501989
51. Flaherty MS, Salis P, Evans CJ, Ekas LA, Marouf A, Zavadil J, et al. chinmo is a functional effector of the JAK/STAT pathway that regulates eye development, tumor formation, and stem cell self-renewal in Drosophila. Dev Cell [Internet]. 2010 Apr 20 [cited 2019 Mar 19]; 18(4):556–68. Available from: https://linkinghub.elsevier.com/retrieve/pii/S1534580710001048 https://doi.org/10.1016/j.devcel.2010.02.006 PMID: 20412771