The *Drosophila BCL6* homolog *ken and barbie* promotes somatic stem cell self-renewal in the testis niche

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Abstract

Stem cells sustain tissue regeneration by their remarkable ability to replenish the stem cell pool and to generate differentiating progeny. Signals from local microenvironments, or niches, control stem cell behavior. In the *Drosophila* testis, a group of somatic support cells called the hub creates a stem cell niche by locally activating the Janus Kinase-Signal Transducer and Activator of Transcription (JAK-STAT) pathway in two adjacent types of stem cells: germline stem cells (GSCs) and somatic cyst stem cells (CySCs). Here, we find that *ken and barbie* (*ken*) is autonomously required for the self-renewal of CySCs but not GSCs. Furthermore, Ken misexpression in the CySC lineage induces the cell-autonomous self-renewal of somatic cells as well as the nonautonomous self-renewal of germ cells outside the niche. Thus, Ken, like Stat92E and its targets ZFH1 (Leatherman and Dinardo, 2008) and Chinmo (Flaherty et al., 2010), is necessary and sufficient for CySC renewal. However, *ken* is not a JAK-STAT target in the testis, but instead acts in parallel to Stat92E to ensure CySC self-renewal. Ken represses a subset of Stat92E targets in the embryo (Arbouzova et al., 2006) suggesting that Ken maintains CySCs by repressing differentiation factors. In support of this hypothesis, we find that the global JAKSTAT inhibitor *Protein tyrosine phosphatase 61F* (*Ptp61F*) is a JAK-STAT target in the testis that is repressed by Ken. Together, our work demonstrates that Ken has an important role in the in the inhibition of CySC differentiation. Studies of *ken* may inform our understanding of its vertebrate orthologue *B-Cell Lymphoma 6* (*BCL6*) and how misregulation of this oncogene leads to human lymphomas.

Keywords

ken and barbie; *Drosophila*; JAK-STAT; stem cell; niche

Introduction

Stem cells divide asymmetrically to give rise to one daughter that remains a stem cell (a process known as self-renewal) and another daughter that commits to differentiation. In this way, stem cells are able to provide a continuous source of differentiating cells for tissue

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regeneration while sustaining the original stem cell population. Signals from niches, or local microenvironments that regulate stem cell behavior, regulate the decision between stem cell fate and differentiation (Lin, 2002). Some of the best-characterized stem cell niches are found in the *Drosophila* gonads (Fuller and Spradling, 2007). The stem cells found in these tissues can be identified at single-cell resolution with markers that easily distinguish them from their differentiating progeny as well as from neighboring niche-generating cells. Furthermore, stem cells and their niches can be genetically manipulated in vivo in order to investigate the molecular requirements for stem cell maintenance (Morrison and Spradling, 2008). Studies using *Drosophila* spermatogenesis as a model system have shown that multiple conserved signaling pathways regulate stem cell self-renewal and differentiation in the testis niche (de Cuevas and Matunis, 2011).

Two populations of stem cells reside in the apex of the *Drosophila* testis: germline stem cells (GSCs) which produce sperm, and somatic stem cells known as cyst stem cells (CySCs) which produce support cells. Both types of stem cells are anchored around a cluster of somatic support cells known as the hub. The hub specifically expresses the secreted glycoprotein Upd, which activates the highly conserved Janus Kinase-Signal Transducer and Activator of Transcription (JAK-STAT) signaling pathway in adjacent stem cells via the transmembrane receptor Domeless. JAK-STAT signaling is required for the maintenance of both GSCs (Kiger et al., 2001; Tulina and Matunis, 2001) and CySCs (Issigonis et al., 2009; Leatherman and Dinardo, 2008). In CySCs, activation of JAK-STAT signaling leads to the expression of the Stat92E target Zinc finger homeodomain 1 (ZFH1), which is highly expressed in CySCs and quickly downregulated in cyst cell daughters (Leatherman and Dinardo, 2008). Similar to Stat92E, ZFH1 is required intrinsically for CySC maintenance; *zfh1* or *Stat92E* mutant CySCs differentiate within 2-3 days (Issigonis et al., 2009; Leatherman and Dinardo, 2008). Furthermore, sustained activation of the JAK-STAT signaling pathway or its target *zfh1* in the CySCs and cyst cells is sufficient to cause CySC-like cells to accumulate throughout the testis, far outside of the normal niche region. A striking consequence of this phenotype is that the excess CySCs nonautonomously promote the accumulation of GSCs throughout the testis. This is remarkable considering that ectopic activation of the JAK-STAT pathway throughout the germline is not sufficient to prevent differentiation of the germ cells (Leatherman and Dinardo, 2008). However, a yet unidentified signal from CySCs which activates the BMP pathway in neighboring GSCs may be partially responsible for the maintenance of GSCs in a GSC-like state (Leatherman and Dinardo, 2010). Therefore, the GSC niche is made up not only of hub cells, but CySCs as well.

GSCs and CySCs typically divide asymmetrically, such that one daughter cell remains adjacent to the hub while the other one gets pushed away from the niche (Cheng et al., 2011; Sheng and Matunis, 2011; Yamashita et al., 2003). Since Upd appears to act over a short distance, the GSC and CySC daughters (known as gonialblasts and cyst cells, respectively) that are displaced from the hub no longer receive the signals that specify stem cell identity and begin to differentiate. The gonialblast daughter undergoes four mitotic divisions with incomplete cytokinesis resulting in 16 interconnected spermatogonia, which further differentiate, undergoing meiosis and spermiogenesis to form sperm. Cyst cell daughters exit the mitotic cycle, but increase in size as they differentiate. Pairs of cyst cells continue to envelop each gonialblast and its descendants throughout spermatogenesis. In fact, encystment of the germline by the cyst cells is essential for their proper differentiation (Kiger et al., 2001; Matunis et al., 1997; Sarkar et al., 2007; Schulz et al., 2002; Tran et al., 2000).

Several negative regulators of the JAK-STAT pathway have been characterized. These include proteins of the Suppressor of Cytokine Signaling (SOCS) family; all contain an SH2

domain and a SOCS box (Hilton et al., 1998), and bind to phosphorylated tyrosines on receptors and/or JAKs to attenuate signaling by recruiting the proteasomal degradation machinery to these targets (Alexander, 2002; Alexander and Hilton, 2004). *Socs36E*, the best characterized *Drosophila* SOCS protein, is a known target of JAK-STAT signaling and behaves in a classic negative feedback loop to attenuate the pathway (Callus and Mathey-Prevot, 2002; Issigonis et al., 2009; Karsten et al., 2002). STAT itself can also be regulated by several different mechanisms. Phosphorylated STAT molecules can be dephosphorylated and thereby deactivated by protein tyrosine phosphatases (PTPases), leading to the global downregulation of STAT targets. Ptp61F is the *Drosophila* homologue of the human phosphotyrosine phosphatase B1 (PTPB1) and is one of 28 predicted PTPs in the fly genome. The expression pattern of *Ptp61F* during embryogenesis mirrors that of *upd*, suggesting that Ptp61F may be a target of JAK-STAT signaling (Baeg et al., 2005). Depletion of Ptp61F leads to increase JAK-STAT pathway activity. The precise mechanism of Ptp61F remains unclear but potentially involves the dephosphorylation of Stat92E (Baeg et al., 2005; Muller et al., 2005).

SOCS proteins and PTPases cause global downregulation of the JAK-STAT pathway by inhibition of the receptor/JAK complex in the cytoplasm or phosphorylated STATs in the nucleus, respectively. Recently, a JAK-STAT inhibitor was found in *Drosophila* that did not act in this global fashion. The *ken & barbie* (*ken*) gene was originally identified in a P-element mutagenesis screen for male sterility, and mutants of this gene lacked external genitalia (Castrillon et al., 1993). *ken* was later implicated to be a novel interactor of the JAK-STAT pathway (Arbouzova et al., 2006). In a genetic screen designed to uncover modifiers of the adult eye overgrowth phenotype caused by Upd overexpression in the developing eye imaginal disc, *ken* enhanced the eye overgrowth phenotype suggesting that, in this tissue, it normally inhibited the JAK-STAT signaling pathway (Arbouzova et al., 2006). Ken (and its vertebrate orthologue B-Cell Lymphoma 6, BCL6) is characterized by an N-terminal Broad complex, tramtrack, brica-brac (BTB) domain and C-terminal zinc finger (ZF) motifs, a domain structure shared by known transcriptional repressors. Ken was found to bind the sequence GAAA, which overlaps with a subset of Stat92E consensus binding sites (TTCNNNGAA). Furthermore, ectopic expression of Ken in the embryo inhibits the expression of known JAK-STAT target genes *ventral veins lacking* (*vvl*), *tracheales* (*trh*), and *knirps* (*kni*). In contrast, misexpression of Ken does not affect the expression of the JAK-STAT target *Socs36E* (Arbouzova et al., 2006). Therefore, Ken behaves as a selective inhibitor of a subset of JAK-STAT targets that contain DNA binding sites that accommodate both Stat92E and Ken (Stat92E^{+}/Ken[+]) binding sites (TTCNNNGAAA).

Here, we investigate the role of Ken in the *Drosophila* testis niche. Although *ken* is expressed throughout the testis apex, it is cell-autonomously required in CySCs but not GSCs for their maintenance. Furthermore, expression of Ken in the CySC lineage is sufficient to cause CySCs as well as GSCs to self-renew outside of their normal niche.

Materials and methods

Fly stocks and culture

Flies were raised on standard yeast/molasses medium at 25°C unless otherwise stated. The following stocks were used: *y w* (wild-type), ken alleles (which also serve as enhancer trap lines): ken[1] (hypomorphic allele; Bloomington Stock Center), *ken[02970]* (hypomorphic allele; Bloomington Stock Center), *ken[k11035]* (strongest hypomorphic allele; M. Zeidler), *UAS-ken* (M. Zeidler), *UAS-zfh1* (Bloomington Stock Center), *UAS-hop[TumL]* (D. Harrison), *UAS-stat92E-RNAi* (Transformants 43866 and 106980; Vienna Drosophila RNAi Center), *UAS-zfh1-RNAi* (Transformants 42856 and 42857; Vienna Drosophila RNAi Center), *c587-*

GAL4 (A. Spradling), *nanos-GAL4, hs-upd* (D. Harrison), and *hs-ken* (*Drosophila* Genetic Resource Center, Kyoto).

Induction of ectopic ken, zfh1, hop^{TumL}, upd, and RNAi constructs

Ectopic Ken, Zfh1, or Hop^{TumL} was induced in *c587-GAL4/Y;; UAS-ken/tub-GAL80^{ts}*, *c587-GAL4/Y; UAS-zfh1/+; UAS/tub-GAL80^{ts}/+*, or *c587-GAL4/Y; UAS-hop^{TumL} +; UAS/tub-GAL80^{ts}/+* males by setting up crosses at 18°C to permit survival until adulthood. Newly eclosed males were then shifted to 31°C (for Ken) or 29°C (for Zfh1 and Hop^{TumL}) for two weeks before dissection. *Stat92E*-RNAi and *Zfh1*-RNAi were induced in *c587-GAL4/Y; UAS-stat92ERNAi/+; tub-GAL80^{ts}/+* or *c587-GAL4/Y; UAS-zfh1-RNAi/+; tub-GAL80^{ts}/+* males by shifting newly eclosed males raised at 18°C to 31°C for one week before dissection. For in situ hybridization and qPCR experiments, newly eclosed *hs-upd* or *hs-ken* males were heat-shocked for 45 minutes at 37°C and then allowed to recover for 1 hour at 25°C.

Mosaic analysis

ken mutant alleles *ken^1*, *ken^{02970}*, and *ken^{k11035}* were recombined onto *FRT42B* chromosomes and crossed to *FRT42B Ubi-GFP::nls; hsFLP* flies. The FLP-mediated mitotic recombination technique (Xu and Rubin, 1993) was used to generate negatively marked *ken* homozygous mutant GSC and/or CySC clones. Newly eclosed males of the genotype +/Y; P{FRT(w[hs])}G13 ken*/P{FRT(w[hs])}G13 P{GFP::nls}; MKRS, P[hsFLP]/+ (experimental) and +/Y; P{FRT(w[hs])}G13/P{FRT(w[hs])}G13 P{GFP::nls}; MKRS, P[hsFLP]/+ (control) were heat-shocked 3 times for 30 minutes at 37°C, then dissected 2, 6, 10, and 14 days after clone induction (ACI). Negatively marked GSC clones were identified by their absence of GFP and the somatic markers ZFH1 or Traffic jam (Tj) and by their position adjacent to the hub. Negatively marked CySC clones were identified by their absence of GFP, presence of ZFH1 or Tj, and position within 2 cell diameters from the hub. Statistical analysis on percentage testes with clones (normalized to basal clone induction rates obtained from non heat-shocked controls) was performed using the Fisher Exact or Chi-Squared tests.

In situ hybridization

To generate probes for in situ hybridization, cDNAs for *ken* (GH12495, Berkley *Drosophila* Genome Project [BDGP]) and *Ptp61F* (LP02164, BDGP) were PCR amplified with primers that contained restriction enzyme sites XbaI and EcoRI at the 5′ ends to allow for subsequent cloning. PCR amplified products were digested with XbaI and EcoRI, and then ligated into the pBluescript II KS(+) vector (Stratagene). Digoxigenin-labeled anti-sense RNA probes were transcribed in vitro using T3 RNA polymerase according to the manufacturer's instructions (Roche) from plasmid templates linearized with XbaI. Control sense probes were transcribed with T7 RNA polymerase from plasmids linearized with EcoRI. In situ hybridizations were performed as described (Terry et al., 2006) and visualized with an Olympus BX51 microscope.

Immunostaining

Testes were dissected from newly eclosed flies (less than 3 days old unless otherwise stated) and were fixed and immunostained as previously described (Matunis et al., 1997). To visualize *ken* expression in the *ken* enhancer trap lines, tyramide signal amplification was used to increase sensitivity of the anti- -galactosidase (-GAL) staining according to the manufacturer's instructions (Molecular Probes/Invitrogen). Antibodies used were rabbit anti-Vasa (d-260) (Santa Cruz Biotechnology; 1:400), rabbit anti-GFP (Torrey Pines Biolabs; 1:10,000), mouse anti- -GAL (Promega; 1:1000), affinity-purified rabbit anti-

Stat92E (E. Bach; 1:400) (Flaherty et al., 2010), guinea pig anti-ZFH1 (J. Skeath; 1:1000), mouse monoclonal antibody 1B1 (Developmental Studies Hybridoma Bank; 1:50), rabbit anti-phospho-Histone H3 (Upstate/Millipore; 1:200). Alexa 488- and Alexa 568-conjugated secondary antibodies were used (Molecular Probes/Invitrogen; 1:400 and 1:200, respectively). DNA was counterstained with 4,6-diamidino-2-phenylindole (DAPI) (Sigma; 1 g/ml). Confocal images were acquired with a Zeiss LSM 5 Pascal microscope and figures were assembled with Adobe Photoshop CS3 and Adobe Illustrator CS3.

Antibody generation and Western blotting

Rabbit polyclonal antiserum was raised to the following Ken peptide: DRKHLLEAQRNRAQSPE (Open Biosystems). Western blots were performed using standard methods (Harlow and Lane, 1988). Protein extracts were made from 20 pairs of testes or 10 adult males for each genotype. Testis protein extracts were prepared as previously described (Chen et al., 2005). Adult flies were homogenized in 50 l 2X SDS loading buffer, boiled for 5 minutes, and then centrifuged at 13,200 rpm for 1 minute. 5 l of the protein lysate (equivalent to one fly) was then loaded onto a 4-12% Bis-Tris gel (NuPage/Invitrogen). Following SDS-PAGE. proteins were transferred to nitrocellulose membranes and immunoblotting was performed using Anti-Ken antisera diluted 1:1000 in 5% dry milk in 1X PBS-Tween (0.1% Tween-20), peroxidase-conjugated anti-rabbit IgG secondary antisera diluted 1:10,000 (Jackson Immunoresearch Laboratories), and ECL Plus Western blotting detection reagents according to the manufacturer's instructions (Amersham/GE Healthcare).

In silico identification of potential Stat92E- and Ken-binding sites

We searched the promoter proximal regions of *Socs36E* and *Ptp61F* for Stat92E-binding sites TTC[N]$_3$GAA or TTC[N]$_4$GAA that were +/− 5 kb from the transcription start site in sequences obtained from the UCSC *Drosophila melanogaster* Genome Browser (April 2006 assembly). Stat92E$^+$ sites that were immediately followed by an extra A (TTC[N]$_3$GAAA or TTC[N]$_4$GAAA) represented potential Stat92E$^+$/Ken$^+$-binding sites.

Quantitative Real-Time PCR analysis

Fifty pairs of testes were dissected from 0-3 day old males of the appropriate genotype and separated from other reproductive tissues such as the seminal vesicles and accessory glands. RNA was extracted with TRIzol® Reagent (Invitrogen) and RNA cleanup was performed using QIAgen's RNeasy kit followed by treatment with DNase I, Amplification Grade (Invitrogen). cDNAs were synthesized from total RNA primed with oligo(dT) using SuperScript® III First-Strand Synthesis (Invitrogen). qPCR was performed with SYBR Green Supermix (Bio-Rad) and a CFX96 Real-Time PCR detection thermal cycler (Bio-Rad) using primers specific for *Socs36E*, *ken*, *Ptp61F*, and *Gapdh2*.

Socs36E (forward): GCCAACTAGCCAAAAGTAACG

Socs36E (reverse): TGCTGAGAACTTGCTAAGGTG

ken (forward): GCGAGAACAAAGTAAAGCTGC

ken (reverse): AAGTAGGTGGCATTCACGTC

Ptp61F (forward): ATCGATCCAATTCCAGGCC

Ptp61F (reverse): CTGTTTGTCCTCGTTCTCCC

Gapdh2 (forward): GAGTTTTCGCCCATAGAAAGC

Gapdh2 (reverse): CGATGCGACCAAATCCATTG

All reactions were performed in triplicate and the relative expression levels of each target gene were normalized to that of *Gapdh2*. qPCR analysis was performed with Excel and graphing was performed using Prism software (Graphpad). One representative experiment is shown (n = 3 or 4). *P* values were obtained using two-tailed Student's *t* test.

Results

ken is expressed in the *Drosophila* testis apex

The expression pattern of *ken* mRNA during *Drosophila* development is very dynamic and is present in many of the tissues where JAK-STAT signaling occurs (Arbouzova et al., 2006). To determine if this is also the case in the adult *Drosophila* testis niche, we generated a polyclonal antiserum to Ken to visualize the *ken* expression pattern in the testis. However, we could not detect endogenous Ken protein above background levels by immunofluorescence on whole testes (data not shown) or by Western analysis on extracts from testes or whole adult males (Supplemental Figure 1). Nevertheless, by Western analysis, this antiserum recognizes a specific band at approximately 67 kDa within 30 minutes of global ectopic Ken induction in transgenic adult males carrying *ken* wild-type cDNA driven by the *hsp70* promoter (*hs-ken*) (Supplemental Figure 1). Similarly, *ken* mRNA is undetectable by in situ hybridization in wild-type testes but is readily detected in testes with ectopic *ken* expression (data not shown). Taken together, these results indicate that *ken* is not expressed at high levels in adults or in testes. Although endogenous *ken* mRNA is undetectable by in situ hybridization, recent RNA-Sequencing (RNASeq) studies (Gan et al., 2010) have shown that the *ken* gene is expressed in *Drosophila* testes, which we have verified by performing our own real-time quantitative PCR (qPCR) of wild-type testes (data not shown). Therefore, *ken* is expressed in the *Drosophila* testis, albeit at low levels.

Since *ken* expression is not readily detectable by in situ hybridization or immunofluorescence, we used three independent enhancer detector lines inserted in the *ken* locus as tools to obtain further clues about the spatial distribution of *ken* expression in the testis (Figure 1). All three enhancer traps are expressed in this tissue with expression patterns restricted to the testis apex. In *ken[1]* heterozygous flies, -GAL staining is detected in both the germline and somatic lineages (Figure 1A). The highest levels are detected in the hub, in GSCs, and in all spermatogonial stages (Figure 1A) with an abrupt decrease in expression at the spermatogonialto-spermatocyte transition (Figure 1B, dotted line). Expression is detectable in CySCs and cyst cells as well (Figure 1A). *ken[02970]* and *ken[k11035]* heterozygous flies also express LacZ in hub cells, GSCs and early spermatogonia, as well as CySCs and cyst cells, albeit at lower levels than *ken[1]* flies (data not shown). Taken together, these results indicate that *ken* is expressed at low levels in the testis apex, in the hub as well as in both stem cell populations and their early progeny. Even though the enhancer trap lines might not reflect the full expression pattern of *ken*, their expression patterns are restricted to the testis apex, which suggests that *ken* could be functioning in the testis niche.

ken is required cell-autonomously for CySC but not GSC self-renewal

Since *ken* is expressed in both stem cell populations in the testis, we used mosaic analysis to determine whether *ken* is required in the GSCs and/or CySCs. The Flipase (FLP)-mediated mitotic recombination technique (Xu and Rubin, 1993) was used to generate *ken* mutant clones of three loss-of-function alleles in the testis. By counting the proportion of testes with mutant GSCs or CySCs at 2, 6, 10, and 14 days after clone induction (ACI), we found that *ken* mutant GSC clones are recovered as efficiently as wild-type clones (Table 1, Figure 2A) and are maintained at a rate comparable with wild-type clones over time (Table 1, Figure

2B). In contrast, even though a similar number of *ken* mutant and wild-type CySCs were initially induced (2 days ACI, Table 1), *ken* mutant CySCs are lost at a faster rate (Table 1, Compare Figure 2C to 2D). As the number of *ken* mutant CySCs diminishes over time, *ken* mutant cyst cells are still detected for up to two weeks (Table 1). These cyst cells are not likely to arise during the initial clonal induction event, since the entire process of spermatogenesis is complete in 10 days (Fuller, 1993). Instead, it is likely that *ken* mutant CySCs are able to generate cyst cell daughters. This suggests that *ken* mutant CySCs are lost from the tissue via differentiation, though we have not ruled out the possibility that apoptosis may play a role as well. Taken together, these data indicate that *ken* does not play a cell-autonomous role in GSCs for their maintenance or differentiation, but is required cell-autonomously in CySCs for their maintenance.

Since *ken* mutant CySCs are likely lost to differentiation, we analyzed the expression of ZFH1, a known JAK-STAT target required for CySC self-renewal (Leatherman and Dinardo, 2008), in *ken* CySC clones. ZFH1 is highly expressed in CySCs and is quickly downregulated in their daughters (cyst cells) (Leatherman and Dinardo, 2008). When we examined testes with *ken^1^, ken^02970^, or ken^k11035^* mutant CySC clones, we found that there is no discernible decrease in ZFH1 expression in *ken* mutant CySCs compared to neighboring wild-type CySCs (example in Figure 2E). Taken together, these data indicate that *ken* is required in CySCs for their self-renewal and *ken* mutant CySCs appropriately express ZFH1 prior to differentiating into cyst cells.

Ectopic *ken* expression in the CySC lineage causes an accumulation of somatic and germ cells that retain stem cell-like properties

Since we observed that CySCs autonomously require Ken for their maintenance, we speculated whether *ken* is sufficient to maintain CySC fate. To address this, we used the binary GAL4/UAS system (Brand and Perrimon, 1993) combined with a temperature-sensitive GAL80 (McGuire et al., 2004) to overexpress Ken in the CySCs and their daughters in newly eclosed males. This is sufficient to cause a dramatic accumulation of ZFH1-positive early somatic cells as well as early germ cells throughout the testis (Figure 3A). This is reminiscent of the phenotype seen when the JAK-STAT targets ZFH1 or Chinmo are overexpressed in the CySC lineage (Flaherty et al., 2010; Leatherman and Dinardo, 2008). Furthermore, overexpression of Ken in the germline (*nanos>ken*) does not result in any phenotypes (data not shown). Therefore, *ken* overexpression in CySCs, but not GSCs, results in the accumulation of GSC- and CySC-like cells. Taken together, these data are consistent with the emerging model that CySCs behave as a niche for GSCs, and under certain conditions, the somatic lineage can cause GSC-like cells to accumulate throughout the testis (Flaherty et al., 2010; Leatherman and Dinardo, 2008).

To further characterize the effects of ectopic Ken expression on the testis stem cells, we examined these testes for additional evidence of CySC identity. In wild-type testes CySCs undergo mitosis, but their daughters (cyst cells) exit the cell cycle. Sustained Ken expression in the cyst cell lineage causes somatic cells displaced far from the hub to undergo mitosis as single cells (Figure 3C). These data, as well as the expression of the CySC self-renewal factor ZFH1 throughout the testis, indicate that ectopic Ken is sufficient to promote CySC identity.

In testes ectopically expressing Ken, the germ cells intermingled with ZFH1-positive cells typically appear to be single cells or two interconnected cells, suggesting that they are GSCs or GSC-GB pairs. Thus, we assayed for different features of GSCs or GBs, which distinguish them from differentiating spermatogonia. First, we looked for the presence of spherical or dumbbell-shaped fusomes by 1B1 staining, a hallmark of GSCs or GSC-GB pairs. We found that most germ cells are found in pairs containing a dumbbell-shaped

fusome (Figure 3B). Furthermore, despite being far removed from the hub, these germ cells undergo mitosis as single cells or in pairs, much like GSCs or GSC-GB pairs, as shown by phospho-Histone H3 staining (Figure 3D). In wild-type testes, only GSCs and GBs cycle as single cells while differentiating spermatogonia divide synchronously. Finally, GSCs self-renewing far from the niche in testes ectopically expressing Ken display elevated levels of the BMP pathway activation indicator pMad (Compare Figure 4A to 4B). Together, these data indicate that expression of Ken in the somatic lineage causes an expansion of both germline and somatic stem cell populations in a manner very similar to that seen with ectopic expression of the Stat92E or its target ZFH1. This led us to speculate that *ken* could be acting either together with the Upd/JAK-STAT signaling pathway and its target ZFH1, or in a parallel pathway.

ken-induced CySC and GSC self-renewal is not due to ectopic JAK-STAT pathway activation

To determine whether the phenotype that we observed with Ken overexpression in the CySC lineage is due to the ectopic activation of the JAK-STAT pathway ligand Upd, we examined the expression of *upd* in testes with ectopic Ken expression by in situ hybridization. We found that levels of *upd* are not altered in Ken overexpressing testes (Compare Supplemental Figure 2A to 2B). We next asked whether ectopic Ken expression promotes the stabilization of Stat92E in the CySC- and GSC-like cells accumulating outside of the niche in these testes. However, unlike testes overexpressing Hop^{TumL} (Figure 5A), which are known to contain high levels of Stat92E in early germline and somatic cells far from the niche (Leatherman and Dinardo, 2008), Ken overexpressing testes do not express Stat92E in CySC-like cells far removed from the hub (Figure 5B). These data indicate that Ken overexpression is not sufficient to induce ectopic Upd or Stat92E activation outside of their normal domain. However, Ken overexpression is sufficient to induce high levels of ZFH1 expression, raising the possibility that Ken may induce ZFH1 in a Stat92E-independent manner.

To further explore the epistatic relationship between *ken*, *stat92E*, and *zfh1*, we asked whether overexpression of Ken could rescue the loss of CySCs caused by RNA interference (RNAi) of *stat92E* or *zfh1*. Expression of *stat92E*-RNAi in the CySC lineage causes a significant loss of CySCs, which in turrn leads to a loss of germ cells as well (Compare Supplemental Figure 3A to 3C and 3C'). Co-expression of Ken and *stat92E* (or *zfh1*) RNAi partially rescued the CySC loss phenotype (Supplemental Figure 3D' and 3E). Furthermore, CySCs in testes concomitantly overexpressing Ken and *stat92E*-RNAi in the CySC lineage continued to express ZFH1 (Supplemental Figure 3D'). While we cannot rule out that the presence of ZFH1 staining in these testes is partly due to incomplete knockdown of *stat92E*, this finding, along with our data above, suggest that ZFH1 expression in Ken overexpressing testes may not be Stat92E-dependent (Figure 5). This is consistent with data indicating that there may be additional inputs to ZFH1 expression other than Stat92E (Leatherman and Dinardo, 2008). Ken becomes a reasonable candidate for such an input.

ken is not a Stat92E target in the *Drosophila* testis

If Ken constitutes part of a JAK-STAT independent input promoting ZFH1 expression, *stat92E* should not be required for *ken* expression in the testis. To determine if *ken* expression is influenced by JAK-STAT signaling, we crossed the *ken* enhancer trap lines into transgenic flies carrying *upd* cDNA driven by the *hsp70* promoter (*hs-upd*) and then examined the expression pattern of *ken* before and after heat-shock induced activation of the JAK-STAT pathway. However, we did not observe any appreciable differences in the expression pattern of *ken* with and without ectopic JAK-STAT signaling (data not shown). Consistent with these results, we also did not detect any changes in *ken* mRNA expression

levels by qPCR in wild-type versus heat-shocked *hs-upd* testes (Figure 6A, left). However, these conditions are sufficient to significantly up-regulate the expression of a known Stat92E target, *Socs36E* (Issigonis et al., 2009) (Figure 6A, middle). Therefore, *ken* is not a Stat92E target in the testis. This distinguishes *ken* from the other known CySC maintenance factors, *zfh1* and *chinmo*, which are Stat92E targets in the testis (Flaherty et al., 2010; Leatherman and Dinardo, 2008).

Both Stat92E and Ken affect the expression of *Ptp61F*

All our data indicate that *ken* positively regulates JAK-STAT signaling in the testis niche. Similar to *Stat92E*, *ken* is autonomously required in CySCs to prevent CySC differentiation, and ectopic Ken expression in the CySC lineage leads to ectopic CySCs and GSCs. Our results are surprising, since previous studies have shown that Ken behaves as a selective inhibitor of JAKSTAT signaling by negatively regulating the expression of a subset of JAK-STAT targets in the embryo (Arbouzova et al., 2006). Therefore, *ken* may maintain CySCs either by activating genes required for CySC maintenance (e.g. JAK-STAT signaling) or by repressing an inhibitor of the pathway. Since Ken is known to behave as a transcriptional repressor, we hypothesized that it could be acting on *Socs36E* or *Protein tyrosine phosphatase 61* (*Ptp61F*), two known JAKSTAT inhibitors. *Socs36E* is expressed in the testis niche and is an induced antagonist of the JAK-STAT pathway (Callus and Mathey-Prevot, 2002; Issigonis et al., 2009; Karsten et al., 2002; Singh et al., 2010). However, previous results have demonstrated that *Socs36E* does not respond to Ken in the embryo (Arbouzova et al., 2006), and quantitative real-time PCR (qPCR) analysis of *Socs36E* in wild-type testes versus testes with ectopic JAK-STAT signaling revealed this to be the case in the testis as well (Figure 6B, middle). Therefore, we focused on the effects of Ken on the candidate JAK-STAT target and inhibitor *Ptp61F*. According to RNA-Seq data, *Ptp61F* is expressed in the testis (Gan et al., 2010) and has also been shown to be a JAK-STAT target in *Drosophila* (Baeg et al., 2005; Muller et al., 2005). Furthermore, an in silico search for Stat92E-binding sites (TTC[N]$_3$GAA and TTC[N]$_4$GAA) in the promoter proximal region of *Ptp61F* revealed a high number of Stat92E-binding sites, many of which are also potential Ken-binding sites (42/76 Stat92E^{+}/Ken[+] sites).

To examine the expression pattern of *Ptp61F* in the *Drosophila* testis, we performed in situ hybridization to *Ptp61F* mRNA and found that it is expressed at low levels in the testis apex (including the hub, CySCs, GSCs, and their daughters) and is slightly upregulated in late spermatocytes and in cyst cells (Figure 7). Since previous data have shown that, similar to *Socs36E*, *Ptp61F* is an induced antagonist of the JAK-STAT signaling pathway (Baeg et al., 2005; Muller et al., 2005), we asked whether *Ptp61F* expression is also controlled by JAK-STAT signaling in the testis. To do this, we performed quantitative real-time PCR (qPCR) analysis of *Ptp61F* in wild-type testes versus testes with ectopic JAK-STAT signaling. Surprisingly, *Ptp61F* expression is significantly downregulated in response to JAK-STAT pathway activation (Figure 6A, right). Taken together, these data suggest that *Ptp61F* is a target of JAK-STAT signaling and that Stat92E differentially regulates distinct targets, either by upregulating (i.e. *Socs36E*) or downregulating (i.e. *Ptp61F*) gene expression.

To test whether Ken can also modulate the expression of *Ptp61F*, we performed qPCR analysis of *Ptp61F* in wild-type versus Ken overexpressing testes. Since misexpression of both Upd and Ken lead to the same phenotype (ectopic CySCs and GSCs), we hypothesized that *Ptp61F* expression would decrease in testes with ectopic Ken (as it does in testes with ectopic Upd). We found that *Ptp61F* expression is significantly downregulated in Ken overexpressing testes (Figure 6B, right). However, not all Stat92E targets are similarly affected; *Socs36E* expression is unaffected by ectopic Ken expression (Figure 6B, middle). We conclude that *Ptp61F*, but not *Socs36E*, is a target of the transcriptional repressor Ken in

the testis, and that global ectopic expression of either Upd or Ken is sufficient to downregulate the expression of *Ptp61F*.

Although global induction of either JAK-STAT signaling or Ken throughout the testis is sufficient to reduce the levels of *Ptp61F* expression (Figure 6C and 6D), Ken is required specifically in the CySC lineage (Figure 2). Therefore, we sought to determine whether ectopic expression of Ken or Hop TumL specifically in the CySC lineage is sufficient to reduce PTP61F expression as detected via RT-PCR. Testes from *c587>hop^{TumL}* and *c587>ken* flies that have been shifted for 1 week at 31°C are wild-type in appearance (data not shown). However, induction of ectopic HopTumL in the CySC lineage is sufficient to yield a significant increase in JAK-STAT pathway activity as evidenced by an increase in *Socs36E* expression (Figure 6C, middle). Testes misexpressing Ken in the CySC lineage alone also exhibit a significant decrease in *Ptp61F* expression (Figure 6D, right). These data indicate that ectopic expression of either the JAK-STAT pathway or Ken specifically in the CySCs lineage is sufficient to downregulate the expression of *Ptp61F* in these cells.

Discussion

Here, we demonstrate that *ken*, the orthologue of the human oncogene *BCL6*, plays a novel and crucial role in adult stem cell maintenance. Furthermore, our data show that *ken* is sufficient to promote the self-renewal of CySCs outside of their normal niche, which in turn drives the nonautonomous self-renewal of GSCs. This is consistent with previous studies, which have shown that hyperactivation of JAK-STAT signaling or misexpression of the Stat92E targets ZFH1 or Chinmo are sufficient to induce ectopic CySCs and GSCs (Flaherty et al., 2010; Leatherman and Dinardo, 2008, 2010). This work also reveals a previously unappreciated role for Stat92E in the *Drosophila* testis-transcriptional repression of target genes.

Transcriptional repressors are essential for CySC self-renewal

This study demonstrates the importance of *ken* in maintaining CySC fate. The only three genes other than *Stat92E* currently known to be necessary and sufficient for CySC self-renewal are *ken*, *zfh1*, and *chinmo*. Remarkably, all three genes are known to behave as transcriptional repressors. Furthermore, both *ken* and *chinmo* encode proteins that share the same overall domain structure: an N-terminal BTB domain and C-terminal DNA-binding zinc fingers. The *Drosophila* genome encodes 32 BTB-ZF proteins, so it would be interesting to see whether other BTB-ZF proteins are also sufficient to induce ectopic CySCs and GSCs when expressed in the CySC lineage. BTB-ZF proteins regulate many important biological processes such as cell survival and differentiation and generally behave as transcriptional repressors (Beaulieu and Sant'Angelo, 2011; Perez-Torrado et al., 2006). Therefore, it is clear that transcriptional repression plays a critical role in regulating CySC fate (Figure 8).

It will be interesting to learn whether Ken, ZFH1, and Chinmo each control a distinct set of genes, or whether some of their targets are co-regulated. Both ZFH1 and BCL6, the mammalian homolog of Ken, are known to interact with the corepressor CtBP (C-terminal binding protein) (Mendez et al., 2008; Postigo and Dean). Furthermore, heterodimerization between different BTB-ZF family members has been shown to occur (Davies et al., 1999; Dhordain et al., 2000; Korutla et al., 2009; Phan et al., 2005). Since the transcriptional repressors Ken, ZFH1, and Chinmo have similar loss-of-function phenotypes (CySC loss) and gain-of-function phenotypes (ectopic CySCs), it seems likely that identifying their common targets will lead to identification of key effectors required to promote CySC self-renewal. An important parallel can be drawn to studies on embryonic stem (ES) cells which demonstrate that the main ES cell self-renewal factors OCT4, SOX2, and NANOG promote

stem cell fate by transcriptionally repressing genes required for differentiation. Interestingly, OCT4, SOX2, and NANOG have been shown to co-occupy a number of target genes (Boyer et al., 2005). Mapping Ken as well as ZFH1 and Chinmo to their binding sites within CySCs will reveal how these transcriptional regulators behave to promote self-renewal and block differentiation.

Previous studies have uncovered the dependence of the germ cells on CySCs for their self-renewal (Flaherty et al., 2010; Leatherman and Dinardo, 2008, 2010) and on cyst cells for their proper differentiation (Kiger et al., 2001; Matunis et al., 1997; Sarkar et al., 2007; Tran et al., 2000). However, further investigation is required to elucidate the mechanisms by which ectopic CySCs are induced, and how this consequently leads to GSC self-renewal. It is unknown whether blocking differentiation in CySCs is sufficient to stall GSCs in an undifferentiated state or whether CySCs send a signal to neighboring germ cells causing them to self-renew. This work and previous studies have begun to uncover the regulatory network comprised of transcription factors (Flaherty et al., 2010; Leatherman and Dinardo, 2008, 2010) and chromatin remodelers (Cherry and Matunis, 2010) in CySCs. In order to understand how these transcriptional regulatory networks control the decision between stem cell fate versus differentiation in CySCs, and how CySC self-renewal promotes GSC identity, one must identify the downstream target genes of these critical transcriptional regulators.

Global and specific JAK-STAT pathway inhibition is critical for stem cell maintenance

Previous work from several labs has shown the importance of JAK-STAT activity for the maintenance of both CySCs and GSCs (Kiger et al., 2001; Leatherman and Dinardo, 2008; Tulina and Matunis, 2001). In CySCs, JAK-STAT signaling promotes stem cell identity by activating the transcription of self-renewal factors, and in GSCs, pathway activation primarily regulates their adhesion to the hub (de Cuevas and Matunis, 2011; Leatherman and Dinardo, 2010). However, attenuation of JAK-STAT signaling is critical as well; expression of the Stat92E target *Socs36E* in CySCs is necessary to create a negative feedback loop that prevents CySCs from activating Stat92E at aberrantly high levels and consequently outcompeting neighboring GSCs (Issigonis et al., 2009). Therefore, differentially fine-tuning the overall global levels of JAK-STAT pathway activation in the two stem cell types is essential. But how do the stem cells precisely regulate which JAK-STAT targets are activated in the appropriate cell lineage? For example, even though the JAK-STAT pathway is activated in both CySCs and GSCs (albeit at different levels), the target genes *zfh1* and *Socs36E* are expressed in the CySCs but not the GSCs (Figure 8). It is possible that distinct STAT targets respond to different thresholds of STAT activation. Furthermore, certain co-activators or co-repressors may be uniquely expressed or may function exclusively in one cell lineage and not the other. For example, ZFH1 is only expressed in CySCs and is required for their maintenance. On the other hand, Chinmo is expressed in both GSCs and CySCs, but functions solely in the latter stem cell population for their maintenance. Ken is enriched in the testis apex, and similar to the transcriptional repressors ZFH1 and Chinmo, is required in CySCs, but not GSCs. However, in the testis, *ken* is not a target of the JAK-STAT pathway, unlike *zfh1* and *chinmo*. It is worth noting that although their loss-of-function phenotypes are similar, *ken* mutant CySC clones are lost more slowly than *stat92E*, *zfh1*, or *chinmo* mutant CySCs (which are depleted within ~2 days of clonal induction) (Flaherty et al., 2010; Issigonis et al., 2009; Leatherman and Dinardo, 2008). One reason for this difference may be attributed to the fact that the available *ken* alleles are not null. However, it is also possible that genes such as *zfh1* and *chinmo* may have stronger loss-of-function phenotypes because they play a primary role in CySC maintenance whereas Ken may perform secondary functions such as fine-tuning the transcriptional output of the JAKSTAT pathway. The *Drosophila* testis niche presents a unique opportunity to study how

a single signaling pathway regulates two different stem cell populations within a niche via (1) differential regulation of global antagonists (i.e. *Socs36E*), (2) activation of a distinct set of target genes exclusively in one stem cell type (i.e. *zfh1*), and (3) differential regulation by transcriptional repressors (i.e. *ken* and *chinmo*) (Figure 8).

Stat92E as a transcriptional repressor

An interesting discovery from this study is that Stat92E represses the expression of *Ptp61F* (Figure 8). STATs were originally discovered as activators of gene transcription in response to interferons (Schindler and Darnell, 1995; Shuai et al., 1993). Recently, however, increasing evidence indicates that in addition to their more familiar and well-documented role as transcriptional activators, STATs can also behave as functional repressors in an indirect manner (via STAT-induced activation of a repressor) or directly (through interactions with DNA methyltransferases, histone deacetylases, or heterochromatin proteins) (Shi et al., 2008; Tran et al., 2010; Walker et al., 2007; Zhang et al., 2005).

In *Drosophila*, JAK-STAT pathway activation is known to upregulate the transcription of some targets, while repressing others (Flaherty et al., 2009). However, how a transcription factor such as Stat92E can stimulate the expression of individual genes while inhibiting others that have potentially conflicting roles is not well understood. The *Drosophila* testis provides a good model system to study this problem; Stat92E is required for the self-renewal of CySCs, presumably by positively regulating genes required for stem-cell identity while repressing those which would lead to opposite fates (i.e. differentiation). Our results indicate that *Ptp61F* is negatively regulated by JAK-STAT signaling in the testis since the activation of JAK-STAT leads to a dramatic decrease in *Ptp61F* expression (Figure 8). Since *Ptp61F* expression was quickly downregulated in *hs-upd* testes after a single heat-shock pulse, we think that Stat92E may be directly repressing *Ptp61F* transcription instead of activating the expression of a *Ptp61F* repressor. Support for this comes from work performed in an ex vivo system using *Drosophila* haemocyte-like cells to identify JAK-STAT targets (Bina et al., 2010). Upd or Hop^{TumL} stimulation of these haemocyte-like cells leads to a significant increase in the transcript levels of the "immediate-early" JAK-STAT target *Socs36E*, which responds within two hours of pathway activation (Bina et al., 2010). We were able to recapitulate these observations in vivo as we observe a robust increase in *Socs36E* expression levels in response to our heat-shocking protocol in *hs-upd* testes. Similarly, the rapid response seen in *Ptp61F* expression levels upon JAKSTAT pathway activation may reflect a direct repression of this target as opposed to a secondary effect. Future studies will address the mechanism by which Stat92E represses the JAK-STAT inhibitor *Ptp61F* to promote CySC self-renewal.

Ken and its mammalian orthologue BCL6

While the mechanism by which Ken represses JAK-STAT targets is currently unknown, clues to how Ken may be behaving can be drawn from its orthologue BCL6, which interacts with chromatin modifiers such as SMRT, mSIN3A, N-CoR, BcoR, and histone deacetylases (HDACs) (Dhordain et al., 1997; Dhordain et al., 1998; Huynh and Bardwell, 1998; Huynh et al., 2000; Lemercier et al., 2002; Wong and Privalsky, 1998). This suggests that Ken may be acting through these partners to block transcriptional activation through chromatin modification. Another possibility is that Ken directly blocks Stat92E from binding to and transcriptionally activating expression of target genes. Furthermore, since Stat92E can either activate or repress expression of targets, it is also possible that Ken behaves as a Stat92E co-repressor. Any of these non-exclusive possibilities will further our understanding of how a signaling pathway is able to transcriptionally activate different target genes in different cell types and stages of development as opposed to eliciting the indiscriminate activation of all possible target genes at once.

Chromosomal rearrangements and point mutations that lead to the misregulation of BCL6 occur frequently in human lymphomas (Baron et al., 1993; Kerckaert et al., 1993; Ye et al., 1993). Furthermore, constitutive overexpression of BCL6 in mice promotes the development of lymphomas (Baron et al., 2004; Cattoretti et al., 2005). BCL6 has been shown to repress differentiation of B-cells and mammary cells (Dent et al., 1997; Fukuda et al., 1997; Logarajah et al., 2003; Ye et al., 1997). In this study, we find that Ken plays an analogous role in repressing differentiation of CySCs in the *Drosophila* testis. Future studies on *Drosophila* Ken and its targets will further our understanding of the mammalian oncogene BCL6.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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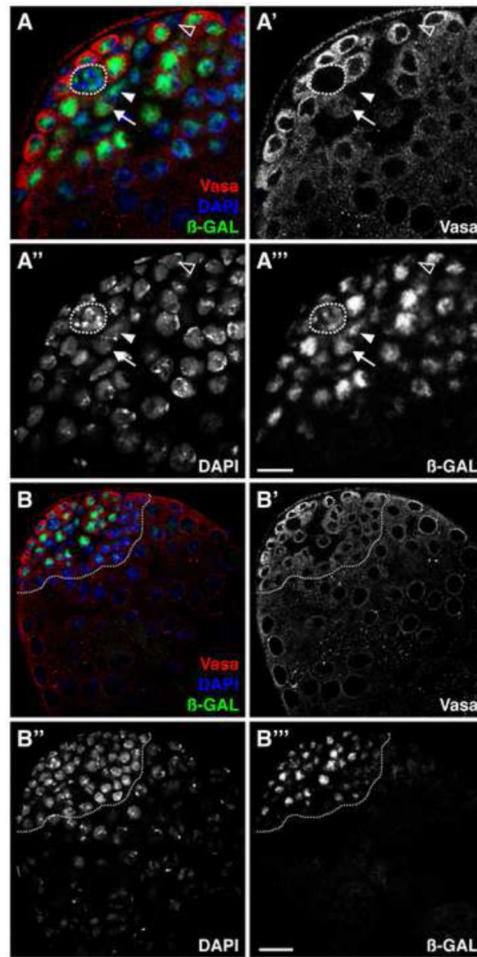
Figure 1. *ken* is expressed in the *Drosophila* testis apex

(A, B) *ken¹* heterozygous testes immunostained with anti-Vasa (germline, red), anti- -galactosidase (-GAL)(Ken, green), and DAPI (DNA, blue). (A) Ken is expressed in the hub (outlined), the CySCs (one indicated, arrowhead), the GSCs (one indicated, arrow), and their differentiating cyst cell (one indicated, open arrowhead) and germline daughters. Scale bar, 10 m. (B) Ken is expressed in the testis apex (outlined), but not in highly differentiated germ cells or somatic cells. Scale bar, 20 m.
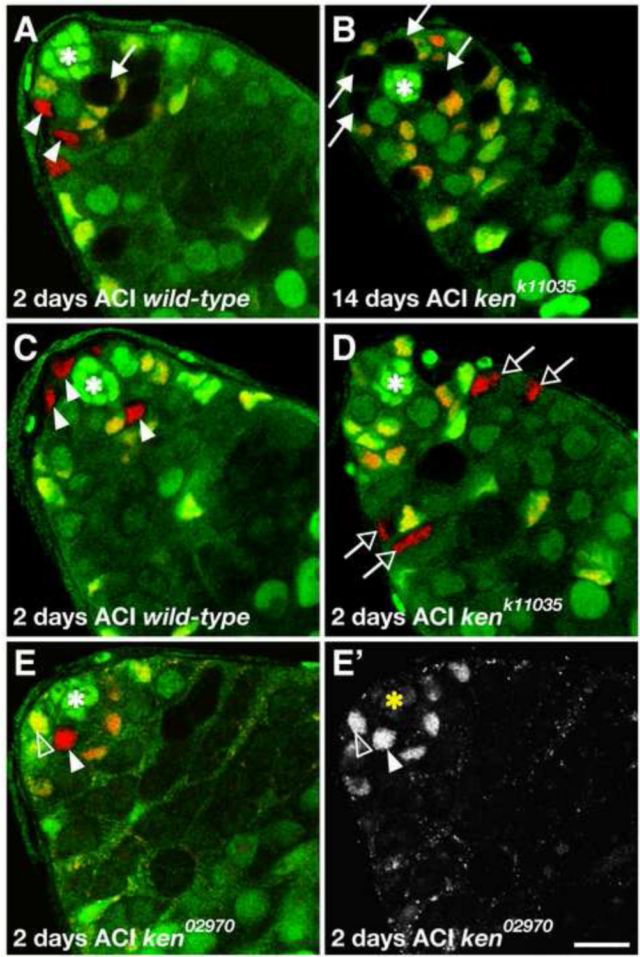
Figure 2. *ken* is required cell-autonomously for CySC but not GSC maintenance
(A-E) Confocal sections through the testis apex. Hubs denoted by asterisks. (A) Wild-type GSC clone (arrow) adjacent to the hub identified by absence of GFP (green) and Tj (red) at 2 days after clone induction (ACI). Wild-type CySC clones (arrowheads) identified by absence of GFP (green) and presence of Tj (red) are visible in this testis as well. (B) *ken^k11035* mutant GSC clones (arrows) are still present 14 days ACI and producing normal differentiating spermatogonia whereas there are virtually no remaining *ken^k11035* CySC clones by this time point. (C) Wild-type CySC clones (arrowheads) surrounding the hub at 2 days after clone induction (ACI). (D) Differentiating *ken^k11035* mutant cyst cell clones (open arrows) identified by absence of GFP (green), presence of Tj (red), and distance from the hub. There are no *ken^k11035* CySCs surrounding the hub indicating that these cyst cells originated from CySC clones generated 2 days prior. (E) *ken^02970* mutant CySC clone (arrowhead) 2 days ACI identified by the absence of GFP (green) and presence of ZFH1 (red) has similar ZFH1 levels relative to neighboring wild-type CySCs (one indicated, open arrowhead). (E') ZFH1 channel alone. Scale bar, 10 m.
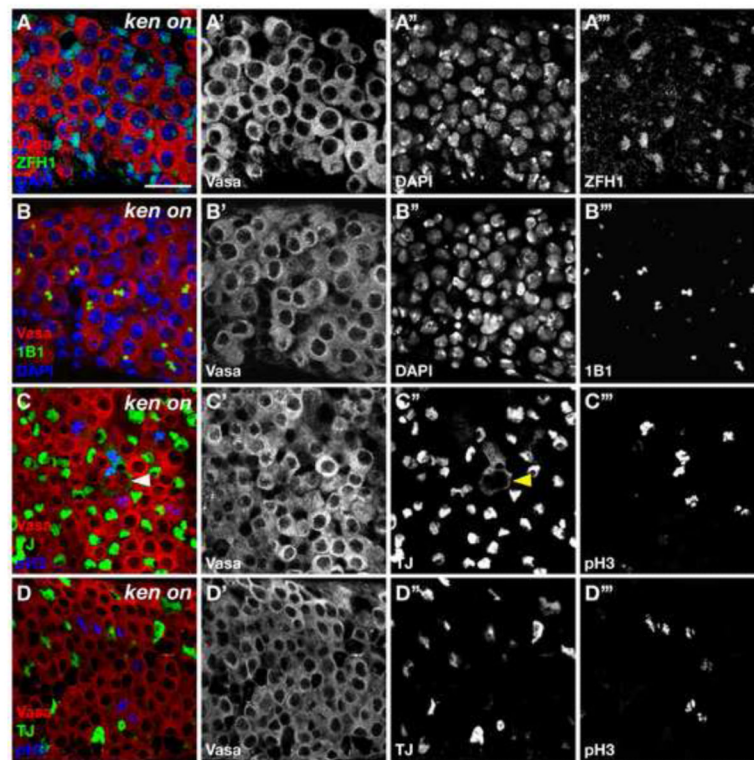
Figure 3. Misexpression of Ken in the CySC lineage causes an accumulation of CySC- and GSC-like cells

(A-D) Confocal section through Ken misexpressing testis. (A) Testis immunostained with anti-Vasa (red), anti-ZFH1 (green), and DAPI (blue). Ken misexpression in the CySC lineage leads to excess ZFH1-positive somatic cells, which are displaced from the hub. (B) Testis immunostained with anti-Vasa (red), anti-1B1 (green), and DAPI (blue). Ken misexpression in the CySC lineage causes early germ cells to accumulate throughout the testis. These germ cells are characterized by a dot or dumbbell-shaped fusome usually found only in GSCs and GBs in wild-type testes. (C, D) Testes immunostained with anti-Vasa (red), anti-Tj (green), and pH3 (blue). (C) Ectopic somatic cells cycle as single cells (Tj^{+}/pH3^{+}) displaced far from the hub. Note the cytoplasmic projections that a cycling CySC (arrowhead) forms around a GSC-GB pair. Breakdown of the nuclear membrane during mitosis in this cell causes the nuclear localization of Tj to accumulate in the cytoplasm. (D) Ectopic early germ cells only cycle as single cells (reminiscent of GSCs or GBs) or as GSC-GB-like pairs. Scale bar, 20 m.
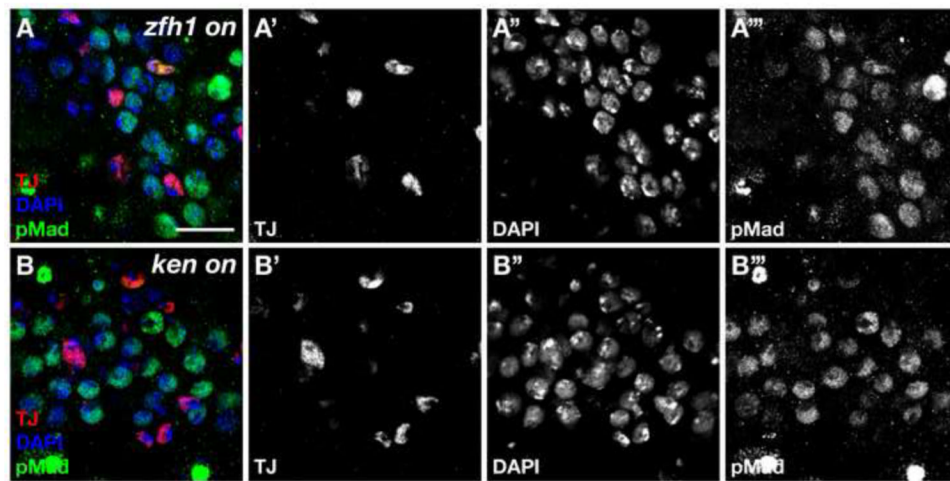
Figure 4. The BMP pathway is highly activated in ectopic GSCs caused by misexpression of Ken in the CySC lineage

(A, B) Confocal section through testis immunostained with anti-Tj (red), anti-pMad (green), and DAPI (blue). High levels of pMad indicative of BMP pathway activation accumulate in ectopic GSCs in ZFH1 misexpressing (A) and Ken misexpressing (B) testes. Scale bar, 20 m.
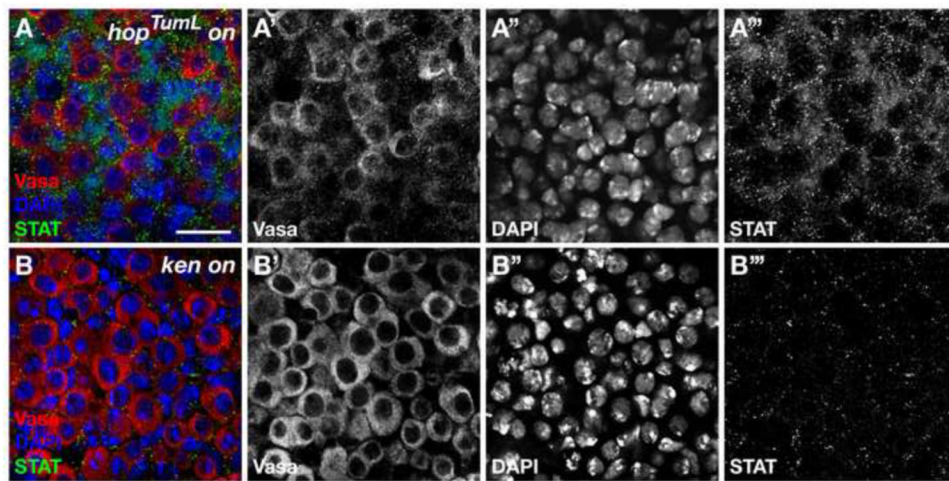
Figure 5. *ken*-mediated CySC and GSC self-renewal is not due to ectopic JAK-STAT pathway activation

(A, B) Confocal sections through testes immunostained with anti-Stat92E (STAT, green), anti-Vasa (red), and DAPI (blue). (A) In positive control testes with sustained *hop*^{TumL} expression in the CySC lineage, Vasa-negative somatic cells far away from the hub (not shown) express elevated levels of Stat92E due to ectopic JAK-STAT pathway activation. (B) In testes misexpressing *ken* in the CySC lineage, Stat92E expression is not detected above background levels in ectopic CySCs or GSCs. Scale bar, 20 m.
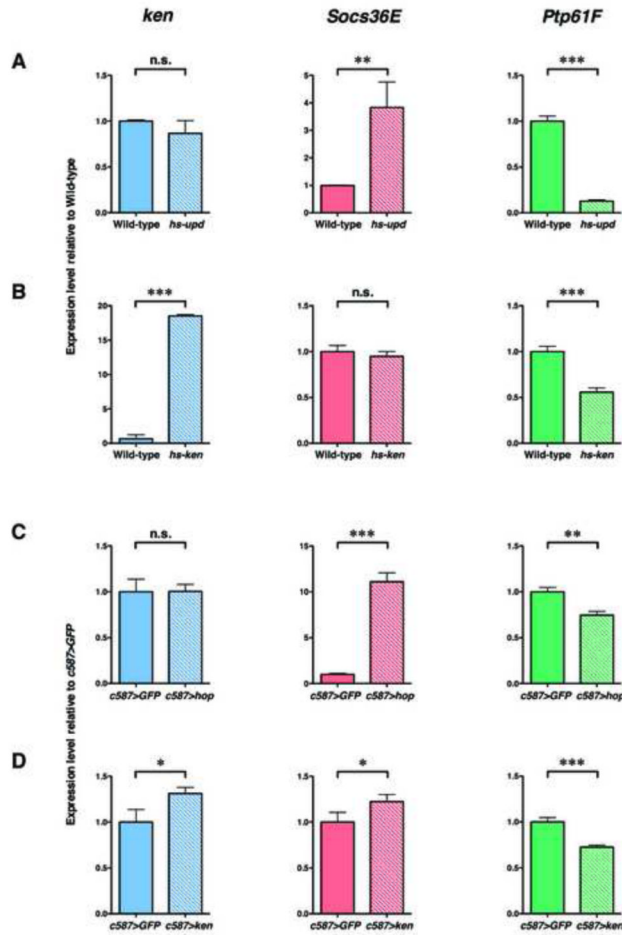
Figure 6. Stat92E and Ken both negatively regulate *Ptp61F* expression
(A) Quantitative RT-PCR analysis of *ken*, *Socs36E*, and *Ptp61F* transcript levels in wild-type and heat-shocked *hs-upd* testes. (A, left) *ken* expression does not appreciably change in response to ectopic JAK-STAT pathway activation. (A, middle) *Socs36E* expression levels increase 5-fold in response to ectopic JAK-STAT signaling. (A, right) Ptp61F levels significantly decrease due to JAK-STAT pathway activation. (B) qPCR analysis of *ken*, *Socs36E*, and *Ptp61F* transcript levels in wild-type and heat-shocked *hs-ken* (*ken* misexpressing) testes. (B, left) A single heat-shock pulse significantly upregulates *ken* in *hs-ken* testes. (B, middle) *Socs36E* expression is not influenced by ectopic *ken* expression. (B, right) Ken negatively regulates *Ptp61F* expression levels. For comparison, expression levels of each gene were normalized to wild-type (set to 1). (A) qPCR analysis of *ken*, *Socs36E*, and *Ptp61F* transcript levels in *c587>GFP* and *c587>hop^{TumL}* testes from flies that have been shifted to 31°C for one week. (C, left) *ken* expression does not appreciably change in response to ectopic JAK-STAT pathway activation. (C, middle) *Socs36E* expression levels increase more than 10-fold in response to ectopic JAK-STAT signaling. (C, right) Ptp61F expression significantly decreases in response to JAK-STAT pathway activation. (D) qPCR analysis of *ken*, *Socs36E*, and *Ptp61F* transcript levels in *c587>GFP* and *c587>ken* testes. (D, left) Shifting *c587>ken* flies to 31°C for one week causes a significant increase in *ken* expression. (D, middle) *Socs36E* expression is not influenced by ectopic *ken* expression. (D, right) Ken negatively regulates *Ptp61F* expression levels specifically in the CySC lineage. For comparison, expression levels of each gene were normalized to *c587>GFP* controls (set

to 1). Error bars represent standard deviations of the mean. n.s., not significant; **, $P < 0.01$; ***, $P < 0.001$ (Student's t test).
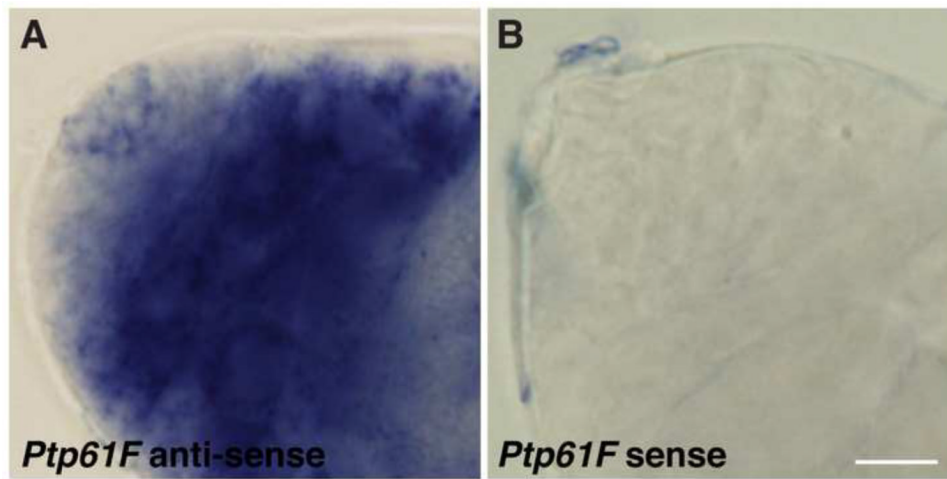
Figure 7. *Ptp61F* expression in the *Drosophila* testis apex
(A, B) In situ hybridization on wild-type testes. (A) *Ptp61F* mRNA is expressed at low levels around the niche (in the hub, GSCs, and CySCs), and is greatly upregulated in differentiating germ cells and somatic cells. (B) *Ptp61F* sense probe is shown as a negative control. Scale bar, 10 m.
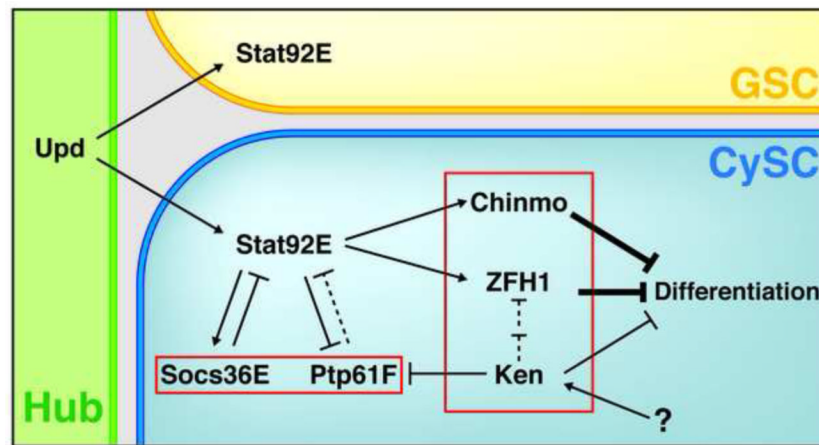
Figure 8. A model for the cell-autonomous requirement of ZFH1, Chinmo, and Ken in CySCs to prevent differentiation
Upd from the hub activates the JAK-STAT signaling pathway in adjacent GSCs and CySCs. In CySCs, activation of Stat92E leads to expression of its targets ZFH1 and Chinmo, which are both required for CySCs self-renewal. Similarly, Ken is cell-autonomously required for CySC self-renewal in a Stat92E-independent manner. Stat92E activates *Socs36E* and represses *Ptp61F* expression. Whether Ptp61F behaves as an inhibitor of Stat92E in the testis remains unknown (dashed line). See text for details.

Table 1

***ken* is necessary for the maintenance of CySCs, but not GSCs**

The percentage of testes with at least one clone is indicated. The total number of testes scored is shown in parentheses.

Days ACI	ken allele	Number of testes with one or more clones/total testes			
		GSC	CySC	SG[a]	CC[b]
no hs control	Wild-type	6% (36)	0% (36)	8% (36)	0% (36)
	1	0% (17)	0% (17)	0% (17)	0% (17)
	02970	0% (20)	0% (20)	0% (20)	0% (20)
	k11035	0% (17)	0% (17)	0% (17)	0% (17)
		n.s	n.s		
2	Wild-type	59% (51)	33% (51)	86% (51)	57% (51)
	1	49% (45)	49% (45)	71% (45)	76% (45)
	02970	57% (49)	53% (49)	80% (49)	71% (49)
	k11035	34% (47)	55% (47)	60% (47)	60% (47)
		n.s	n.s		
6	Wild-type	54% (57)	30% (57)	56% (57)	33% (57)
	1	39% (44)	5% (44)	41% (44)	11% (44)
	02970	48% (31)	16% (31)	55% (31)	19% (31)
	k11035	56% (25)	8% (25)	60% (25)	16% (25)
		n.s	P<0.01		
10	Wild-type	53% (43)	26% (43)	53% (43)	30% (43)
	1	39% (31)	0% (31)	39% (31)	0% (31)
	02970	54% (26)	15% (26)	54% (26)	15% (26)
	k11035	25% (44)	2% (44)	25% (44)	2% (44)
		n.s	P<0.001		
14	Wild-type	39% (79)	14% (79)	39% (79)	19% (79)
	1	33% (73)	4% (73)	34% (73)	10% (73)
	02970	37% (63)	5% (63)	38% (63)	5% (63)
	k11035	26% (38)	3% (38)	26% (38)	3% (38)

Days ACI	*ken* allele	Number of testes with one or more clones/total testes			
		GSC	CySC	SG*a*	CC*b*
		n.s	$P < 0.05$		

*a*SG, spermatogonial clone;

*b*CC, cyst cell clone; n.s., not significant (Chi square and Fisher's exact test).