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String (Cdc25) regulates stem cell maintenance, proliferation and aging in *Drosophila* testis

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SUMMARY

Tight regulation of stem cell proliferation is fundamental to tissue homeostasis, aging and tumor suppression. Although stem cells are characterized by their high potential to proliferate throughout the life of the organism, the mechanisms that regulate the cell cycle of stem cells remain poorly understood. Here, we show that the Cdc25 homolog String (Stg) is a crucial regulator of germline stem cells (GSCs) and cyst stem cells (CySCs) in *Drosophila* testis. Through knockdown and overexpression experiments, we show that Stg is required for stem cell maintenance and that a decline in its expression during aging is a critical determinant of age-associated decline in stem cell function. Furthermore, we show that restoration of Stg expression reverses the age-associated decline in stem cell function but leads to late-onset tumors. We propose that Stg/Cdc25 is a crucial regulator of stem cell function during tissue homeostasis and aging.

KEY WORDS: Cdc25, String, *Drosophila*, Stem cell

INTRODUCTION

Stem cells contribute to tissue homeostasis by producing differentiating daughter cells. To achieve this, stem cells maintain the ability to proliferate throughout the life of the organism and a decline in this ability is proposed to underlie tissue aging and degenerative disease. Despite the fact that stem cells are characterized by their high potential to proliferate, it remains poorly understood how the cell cycle of stem cells is regulated in what is possibly a stem cell-specific manner and how such regulation relates to stem cell function and aging. For example, stem cell division must be finely modulated according to how many differentiated cells are required by the tissue at a particular moment, a factor that constantly changes during tissue homeostasis, repair and aging. Furthermore, tissues that contain multiple stem cell populations would require an even more complicated regulation of stem cell proliferation, as differentiating cells of distinct lineages must be produced in the correct ratio.

Consistent with the importance of cell cycle regulation in stem cell/progenitor compartments, a tight correlation between cell cycle regulators, aging and tumorigenesis has been reported. For example, it is known that the Ink4a (Cdkn2a) tumor suppressor, which encodes a cyclin-dependent kinase (cdk) inhibitor, accumulates with age in many tissues and its removal can rescue the age-associated decline in stem cell/progenitor number and function (Janzen et al., 2006; Krishnamurthy et al., 2006; Molofsky et al., 2006). Conversely, the phosphatase Cdc25, a crucial cdk activator, is overexpressed in many human cancers (Kristjansdottir and Rudolph, 2004; Boutros et al., 2007). Although it has been reported that Cdc25 is required for stem cell maintenance in mouse small intestine (Lee et al., 2009; Lee et al., 2011), it remains unclear whether Cdc25 has specific functions in the stem cell compartment.

The *Drosophila melanogaster* testis contains two stem cell populations that cohere and regulate each other: germline stem cells (GSCs) and cyst stem cells (CySCs). At the testis apical tip, both GSCs and CySCs attach to the hub cells, a major component of the stem cell niche, via adherens junctions (Fig. 1A) (Yamashita et al., 2003; Voog et al., 2008). Unpaired (Upd; Outstretched – FlyBase) ligand secreted from the hub cells activates the Janus kinase-signal transduced and activator of transcription (JAK-STAT) pathway in both GSCs and CySCs, maintaining the stem cell identity of these cells (Kiger et al., 2001; Tulina and Matunis, 2001; Leatherman and Dinardo, 2008). CySCs perform dual roles as a part of the GSC niche (Leatherman and Dinardo, 2008; Leatherman and Dinardo, 2010) and as stem cells to generate cyst cells (CCs). A pair of CCs encapsulates differentiating germ cells [gonialblasts (GBs), spermatogonia and spermatocytes] and provides essential signals to promote differentiation (Fuller, 1993; Kiger et al., 2000; Tran et al., 2000). We have shown that both GSCs (Yamashita et al., 2003) and CySCs (Cheng et al., 2011) divide asymmetrically, which can explain, in part, how GSCs and CySCs coordinate the numbers of their progeny. However, it remains unclear whether, and if so how, these two stem cell populations coordinate their proliferation.

Here we show that a *Drosophila* homolog of Cdc25, String (Stg), is highly expressed in GSCs and CySCs and is quickly downregulated in differentiating cells. RNAi-mediated knockdown and overexpression analyses reveal that Stg is required for maintenance and proliferation of GSCs/CySCs. Furthermore, we show that expression of Stg declines with age specifically in GSCs and that this decline is a major determinant of age-associated decline in GSC and CySC function. Although restoring Stg expression in GSCs reverses age-associated phenotypes in GSCs and CySCs, it leads to late-onset tumors in aged testis, implying that the reduction in Stg expression in aging testis is a mechanism to prevent tumorigenesis. Collectively, we propose that Stg/Cdc25 is a crucial regulator of stem cell maintenance and proliferation during tissue homeostasis and aging.

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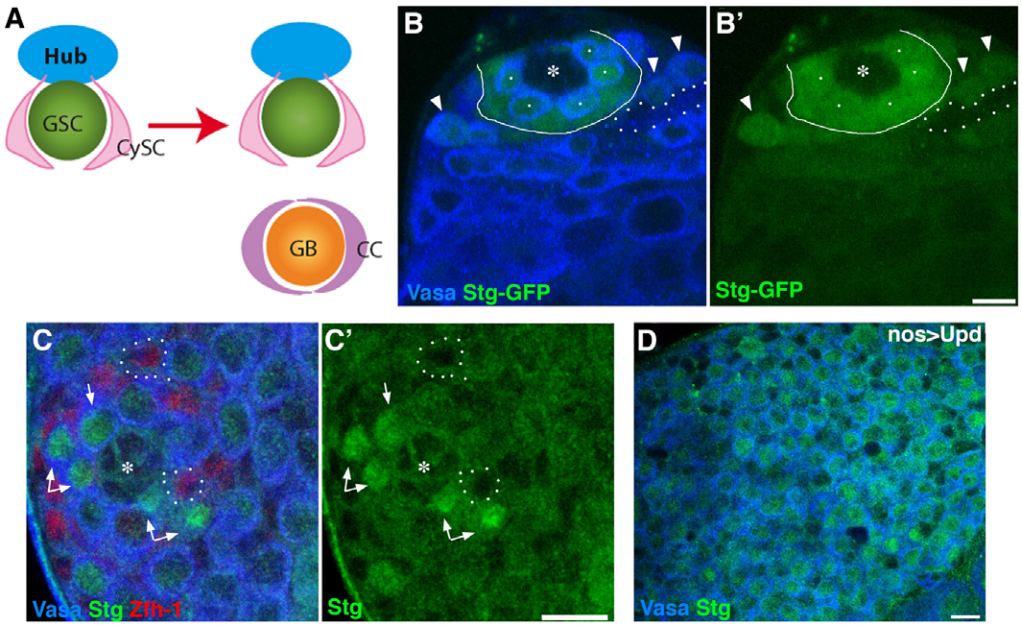

Fig. 1. Stg is highly expressed in *Drosophila* GSCs and CySCs and is required for their maintenance. (**A**) Germline stem cell (GSC) and cyst stem cell (CySC) divisions. GSCs and CySCs attach to the hub cells. Each GSC is encapsulated by a pair of CySCs. Each gonialblast (GB), a differentiating daughter of a GSC, is encapsulated by a pair of cyst cells (CCs), which are the progeny of CySCs. (**B**,**B′**) FlyTrap Stg-GFP is highly expressed in GSCs and CySCs. A rosette of GSCs (white dots) and CySCs surrounding the hub (asterisk) is indicated by the white line. Arrowheads indicate GBs and spermatogonia that express a moderate level of Stg-GFP. The dotted line indicates a CC with a minimal level of Stg-GFP expression. Green, Stg-GFP; blue, Vasa (germ cells). (**C**,**C′**) Stg expression detected by anti-Stg antibody. The arrow indicates a GSC expressing a high level of Stg. Paired arrows indicate GSC-GB pairs that express a high level of Stg in their nuclei. Dotted lines indicate CySCs with cytoplasmic Stg expression. Blue, Vasa; green, Stg; red, Zfh1 (Zfh-1; CySCs). (**D**) Stg is highly expressed in a GSC/CySC tumor induced by Upd overexpression. Scale bars: 10 μm.

MATERIALS AND METHODS

Fly husbandry and strains

Fly stocks were raised in standard Bloomington medium at 25°C. The following fly stocks were used: Stg-GFP (YD0246 and YD0685), Chd64-GFP (CB03690) [FlyTrap Project (Kelso et al., 2004; Buszczak et al., 2007)], c587-gal4 (Decotto and Spradling, 2005), nos-gal4 (Van Doren et al., 1998), UAS-Stg, UAS-Cdc2-myc (Bloomington Stock Center), UAS-Stg^{RNAi} (GD8177 from Vienna Drosophila Research Center, and TRiP.JF03235 from Bloomington Stock Center), UAS-Upd (Zeidler et al., 1999), hs-FLP; Act>stop>gal4 UAS-GFP (Yu Cai, Tamasek Lifesciences Laboratory, Singapore), UAS-Cdk4; UAS-Cyclin D [Laura Buttitta (Meyer et al., 2002)].

Immunofluorescence staining

Immunofluorescence staining was performed as described previously (Cheng et al., 2008). Briefly, testes were dissected in PBS, transferred to 4% formaldehyde in PBS and fixed for 30-60 minutes. The testes were then washed in PBST (PBS containing 0.1% Tween 20) for at least 30 minutes, followed by incubation with primary antibody in 3% bovine serum albumin (BSA) in PBST at 4°C overnight. Samples were washed for 60 minutes (three 20-minute washes) in PBST, incubated with secondary antibody in 3% BSA in PBST at 4°C overnight, washed as above, and mounted in VECTASHIELD with DAPI (Vector Labs). The primary antibodies used were: mouse anti-Adducin-like (Hu li tai shao – FlyBase) [1:20; developed by H. D. Lipshitz and obtained from Developmental Studies Hybridoma Bank (DSHB)]; rabbit anti-Thr3-phosphorylated Histone H3 (1:200; Upstate); rat anti-Vasa (1:40; DSHB); and rabbit anti-Zfh1 (1:4000; a gift from Ruth Lehmann, Skirball Institute of Biomolecular Medicine, NY, USA). Anti-Stg antibody was generated by injecting a peptide (DGLGGATGRLKKSRSRLML) into guinea pigs. Images were taken using a Leica TCS SP5 confocal microscope with a 63× oil-immersion objective (NA=1.4) and processed using Adobe Photoshop software.

RESULTS

Stg is highly expressed in GSCs and CySCs in *Drosophila* testis

Using FlyTrap strains of Stg [YD0246 and YD0685 (Kelso et al., 2004; Buszczak et al., 2007)], which express GFP-fused Stg protein driven by its native promoter, we found that Stg was strongly expressed in GSCs and CySCs and rapidly downregulated in their differentiating daughters (GBs/spermatogonia and CCs) (Fig. 1B). Some GBs and spermatogonia expressed moderate levels of Stg-GFP, presumably reflecting their status as transit-amplifying cells (Fig. 1B, arrowheads; see also Fig. 4A for additional images of the Stg-GFP expression pattern). By contrast, CCs, which do not divide further, had undetectable levels of Stg-GFP expression (Fig. 1B, dotted line).

A similar expression pattern of Stg was observed using anti-Stg antibody. However, unlike the Stg-GFP reporter, which was expressed in the nucleus and the cytoplasm of both GSCs and CySCs (Fig. 1B), Stg protein detected by anti-Stg antibody was localized in the GSC nucleus and CySC cytoplasm (Fig. 1C), and its level appeared to fluctuate during the GSC cell cycle, as only some GSCs showed high Stg expression (Fig. 1C). Nonetheless, the highest level of Stg expression was always observed in GSCs at the testis apical tip. This difference in localization might be because the Stg-GFP trap line is a loss-of-function allele, although it works as an enhancer trap; in fact, the Stg-GFP trap line was homozygous lethal, and lethal when combined with another loss-of-function allele, *stg*^{4}, in trans.

The correlation between a high level of Stg expression and stem cell identity was further indicated by the elevated expression of Stg in stem cell tumors induced by the expression of Upd (Fig. 1D), a

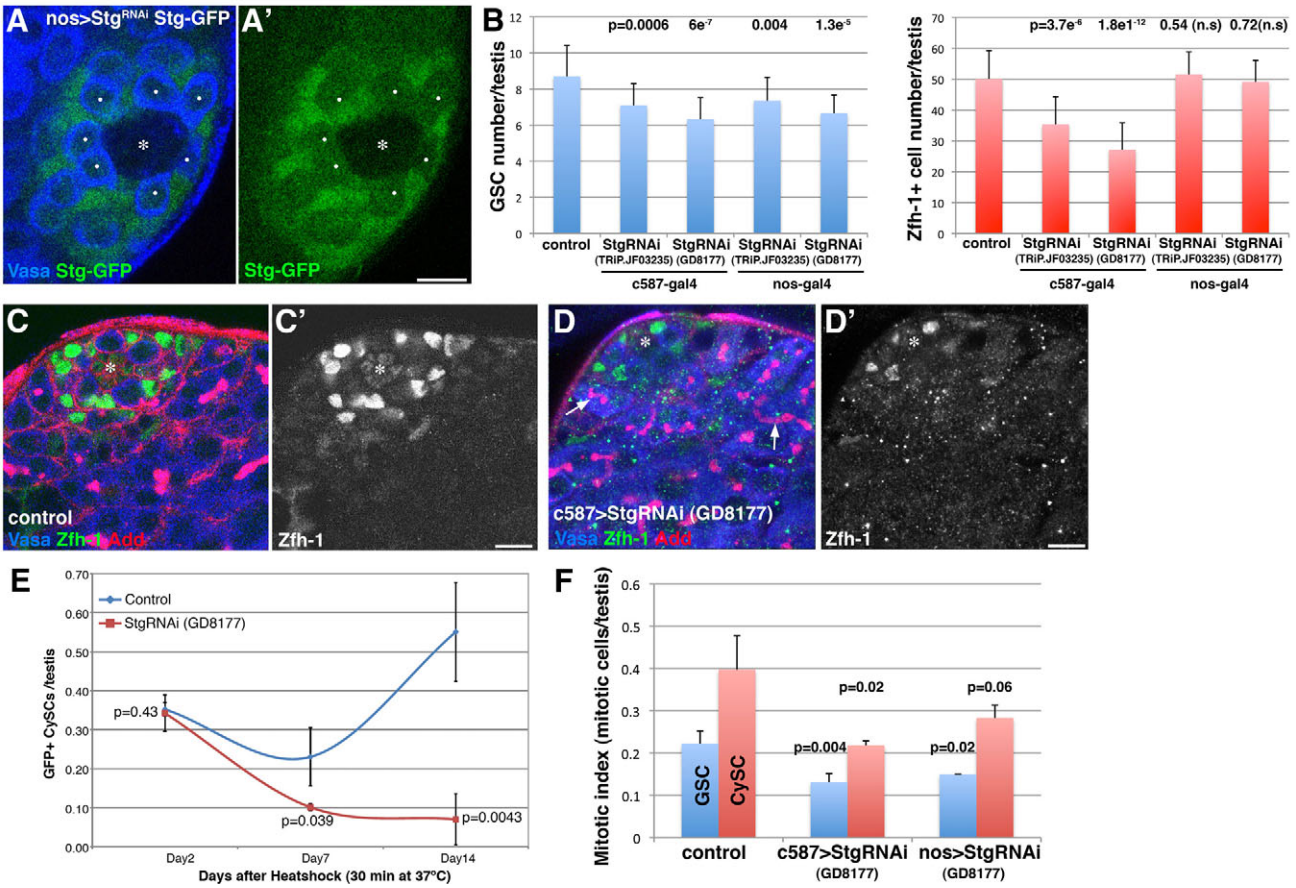

Fig. 2. Stg is required for stem cell maintenance in the *Drosophila* testis. (**A**,**A'**) Stg is specifically downregulated by Stg^{RNAi} expressed in the germline [nos>Stg^{RNAi} (GD8177)]; compare with Fig. 1B. Blue, Vasa; green, Stg-GFP. The hub is indicated by an asterisk, and GSCs are indicated by dots. (**B**) Number of GSCs and CySCs (Zfh1-positive cells) following RNAi-mediated knockdown of Stg in the CySC lineage (*c587-gal4* driver) or germline (*nos-gal4* driver). Mean ± s.d. The *P*-value (two-tailed Student's *t*-test) is provided for comparison with the control. *n*>25 testes per data point. n.s., not significant. (**C-D'**) Apical tip of the testis stained for Vasa (blue), Zfh1 (green) and Adducin-like (red) for control (C,C′) and c587>Stg^{RNAi} (D,D′) testes, demonstrating reduction in the number of CySCs upon knockdown of Stg in the CySC lineage. Arrows indicate branching fusomes, indicating normal differentiation of spermatogonia. Adducin-like stains the spectrosome and fusome. (**E**) CySC clones that express Stg^{RNAi} are lost over time. Flies (Hs-FLP; act>stop>gal4 UAS-GFP with or without UAS-Stg^{RNAi}) were subjected to heat shock (37°C, 30 minutes) and GFP-positive clones were scored 2, 7 and 14 days after heat shock. Error bars indicate mean ± s.d. (**F**) Mitotic indices (measured as mitotic cells per testis) of GSCs and CySCs upon knockdown of Stg. *n*>120 testes per data point. Error bars indicate mean ± s.d. Scale bars: 10 μm.

ligand that is normally secreted from the hub cells and which activates the JAK-STAT pathway within GSCs and CySCs to specify stem cell identity (Kiger et al., 2001; Tulina and Matunis, 2001; Leatherman and Dinardo, 2008; Leatherman and Dinardo, 2010).

In the following experiments, we addressed the function of Stg in GSCs/CySCs by specifically knocking down or overexpressing Stg using germline- or CySC lineage-specific drivers (*nos-gal4* or *c587-gal4*, respectively). Although *Drosophila* possess another Cdc25 homolog, Twine, it is expressed only in meiotic cells, where it regulates meiotic cell cycle progression (Alphey et al., 1992) and so we did not address its function in GSC/CySC behavior.

Stg is required for the maintenance and division of GSCs and CySCs

First, we used RNAi-mediated knockdown of Stg to address its function in GSCs/CySCs. Stg-GFP was specifically downregulated in GSCs when two independent UAS-Stg^{RNAi} (GD8177 and TRiP.JF03235) were expressed in the germline (Fig. 2A), thus validating these UAS-Stg^{RNAi} lines. When UAS-Stg^{RNAi} was expressed in the CySC lineage using the *c587-gal4* driver, the number of CySCs and GSCs was significantly reduced (Fig. 2B,C,D). GSCs were identified as Vasa^{+} cells that attach to the hub cells. CySCs were identified as Zfh1^{+} cells (Zfh1 is also known as Zfh-1), although they are likely to also include some differentiating CCs (Leatherman and Dinardo, 2008). The decrease in GSC number might be secondary to the decrease in CySCs, as CySCs play an instructive role in specifying GSC identity (Leatherman and Dinardo, 2008; Leatherman and Dinardo, 2010). When Stg^{RNAi} was expressed in the germline using the *nos-gal4* driver the number of GSCs, but not CySCs, was significantly decreased (Fig. 2B). The requirement for Stg in CySC maintenance was confirmed by generating Stg^{RNAi} CySC clones in a background of wild-type cells (by a heat shock of 37°C for 30 minutes to *hs-FLP; Act>stop>gal4, UAS-GFP, UAS-Stg^{RNAi}* flies). Control clones without UAS-Stg^{RNAi} were well maintained for the period of 14 days after clone induction and showed clonal expansion (which was likely to be due to occasional symmetric divisions of CySCs). By contrast, Stg^{RNAi} clones were not well maintained and did not show clonal expansion (Fig. 2E). It should be noted that UAS-

Stg^{RNAi} driven by either *nos-gal4* or *c587-gal4* yielded viable adult flies, although strong loss-of-function alleles of *stg* cause embryonic lethality, as mentioned above. This might be explained by the limited expression pattern of these *gal4* drivers and/or only partial reduction of Stg expression by RNAi. However, as judged by the Stg-GFP signal upon knockdown of Stg in CySCs (Fig. 2A), the RNAi-mediated knockdown appears to reduce Stg expression in GSCs to the level in GBs/spermatogonia, suggesting that RNAi-mediated knockdown is likely to address the importance of strong Stg expression in the stem cell compartments. Together, these data suggest that Stg is required for the maintenance of GSC and CySC numbers.

We next asked whether Stg is required for GSC/CySC division. First, we examined the mitotic index (defined as the number of mitotic cells per testis) of GSCs and CySCs. Mitotic cells were identified by immunofluorescent staining of whole-mount testes with anti-phosphorylated Histone H3 (PH3) antibody. During the course of this study, we realized that the mitotic index of cells in testes (and possibly also in other tissues that we have not examined) is very sensitive to CO_2 anesthetization, a routine method used to handle flies: within 5-7 minutes of CO_2 anesthetization, the mitotic index quickly and dramatically dropped (typically by more than 50%). Therefore, we carefully limited the duration of CO_2 anesthetization to 5-7 minutes to obtain a consistent mitotic index of ~0.2 mitotic GSCs/testis and ~0.4 mitotic CySCs/testis. For example, out of 1379 testes from young wild-type flies, the mitotic index of GSCs was 0.2±0.05 (mean ± s.d.) and that of CySCs was 0.41±0.1. However, Stg knockdown in the CySC lineage or GSC lineage resulted in a significant decrease in the proliferation rate (mitotic index) of CySCs and/or GSCs, whereas control flies (cross siblings that do not contain UAS-Stg^{RNAi}) showed mitotic indices similar to those of wild type (Fig. 2F).

When Stg was knocked down in CySCs (c587>UAS-Stg^{RNAi}), the mitotic index of both CySCs and GSCs was reduced, suggesting that GSC division requires CySC division in a non-cell-autonomous manner. When Stg was knocked down in GSCs (nos>UAS-Stg^{RNAi}), the mitotic index of GSCs was significantly reduced (two-tailed Student's *t*-test, P=0.02) and the mitotic index of CySCs was also slightly reduced (P=0.06) (Fig. 2F). Together, these results demonstrate the requirement for Stg in stem cell division in *Drosophila* testis and reveal a potential coordination between GSC and CySC division (see below). It should be noted that the reduction in mitotic index might be partly due to the reduction in stem cell number (Fig. 2B). However, the decrease in the proliferation rate (Fig. 2F) was always more dramatic than the decrease in stem cell number (Fig. 2B), suggesting that the division rate of each stem cell is also reduced upon knockdown of Stg. Based on these findings, we conclude that Stg is required for GSC/CySC maintenance and division in *Drosophila* testis.

Overexpression of Stg induces CySC/CC mitosis but does not confer CySC identity

To further define the function of Stg in GSCs/CySCs, we examined the effect of Stg overexpression in germline or CySC lineages. Upon overexpression of Stg in the CySC lineage (c587>Stg), the CySC mitotic index was elevated 2.5-fold (1.0±0.07/testis), whereas the mitotic index of GSCs was unaffected (Fig. 3A). Although overexpression of Stg in the germline (nos>Stg) did not change the mitotic index of GSCs or CySCs (Fig. 3A), simultaneous overexpression of Stg in the germline and CySC lineage (nos, c587>UAS-Stg) dramatically increased the mitotic indices of both GSCs and CySCs relative to controls (2-fold for GSC, 2.5-fold for CySC; Fig. 3A). These findings, together with the data obtained with Stg^{RNAi} described above, suggest that CySC division is mostly regulated cell-autonomously, whereas GSC division is under the influence of CySC divisions. However, the role of CySCs in GSC division is not instructive (as induction of CySC division does not increase GSC divisions) but instead permissive.

In addition to the elevation of CySC mitosis, mitosis was also induced in CCs upon overexpression of Stg (c587>Stg; supplementary material Fig. S1). Whereas the mitosis of somatic cells was limited to CySCs in wild-type/control flies (Cheng et al., 2011) (supplementary material Fig. S1A,C), we frequently observed mitosis of CCs far from the hub in testes expressing Stg (supplementary material Fig. S1B,C). It should be noted that these mitotic CCs were not scored as 'mitotic CySCs' when calculating the mitotic index for CySCs shown in Fig. 3A; 'mitotic CySC' was defined as being in mitosis (PH3 positive) and maintaining attachment to hub cells. In addition to 1.0 CySC mitoses/testis, 0.81 mitotic CCs/testis were observed upon expression of Stg in the somatic lineage (c587>Stg). Increased CySC/CC division was associated with an increase in the number of somatic cells that express Zfh1 or another marker for CySCs/early CCs, Chd64-GFP [FlyTrap CB03690 (Kelso et al., 2004; Buszczak et al., 2007; Sheng et al., 2009)] (Fig. 3B,C,D). Despite a significant increase in the number of Zfh1^{+} cells upon expression of Stg in the CySC lineage (c587>Stg), these cells do not appear to have adopted bona fide CySC identity, as the increase in Zfh1^{+} cells was not associated with an increase in GSC number or tumor formation, as observed with overexpression of Upd or Zfh1 (Kiger et al., 2001; Tulina and Matunis, 2001; Leatherman and Dinardo, 2008), and differentiation appeared to be unaffected, as judged by the presence of branching fusomes (Fig. 3E). Whereas there was no increase in GSC or CySC number upon expression of Stg in the germline (nos>Stg), combined expression of Stg in the CySC lineage and germline (c587, nos>Stg) led to a slight increase in GSC number (P=0.03, Fig. 3B) and to a dramatic increase in CySC number (compared with control as well as c587>Stg; Fig. 3B). Together, these data support the notion that Stg is not sufficient for GSC/CySC identity.

Stg expression in GSCs declines with age, correlating with a decline in division rate

Using a Stg-GFP protein trap (Fig. 4A,B) or anti-Stg antibody (supplementary material Fig. S2), we found that Stg expression decreased with age specifically in GSCs, whereas the expression level in CySCs as detected by the Stg-GFP trap was unchanged. This correlated with the mitotic indices of each stem cell type: the GSC mitotic index significantly decreased at days 10-20, when Stg expression in GSCs was reduced (Fig. 4B,C); by contrast, CySCs, in which Stg expression remained unchanged during aging, did not show a decrease in the mitotic index (Fig. 4B,C). Interestingly, centrosome misorientation in GSCs, which is reported to cause a decline in GSC proliferation (Cheng et al., 2008), increases gradually with age [~20% misorientation at day 10, ~30% misorientation at day 20 (Cheng et al., 2008)], whereas the decrease in GSC mitotic index did not progress between days 10 and 20 (Fig. 4C). This implies that centrosome misorientation is not the sole factor responsible for reduced GSC division during aging. Importantly, RNAi-mediated knockdown of Stg did not affect centrosome orientation [8.6±2.4% misorientation (n=163 GSCs) in nos>Stg^{RNAi} GSCs compared with 8.9±2.1% (n=158) in control]. Furthermore, nos>Stg expression, which suppressed the

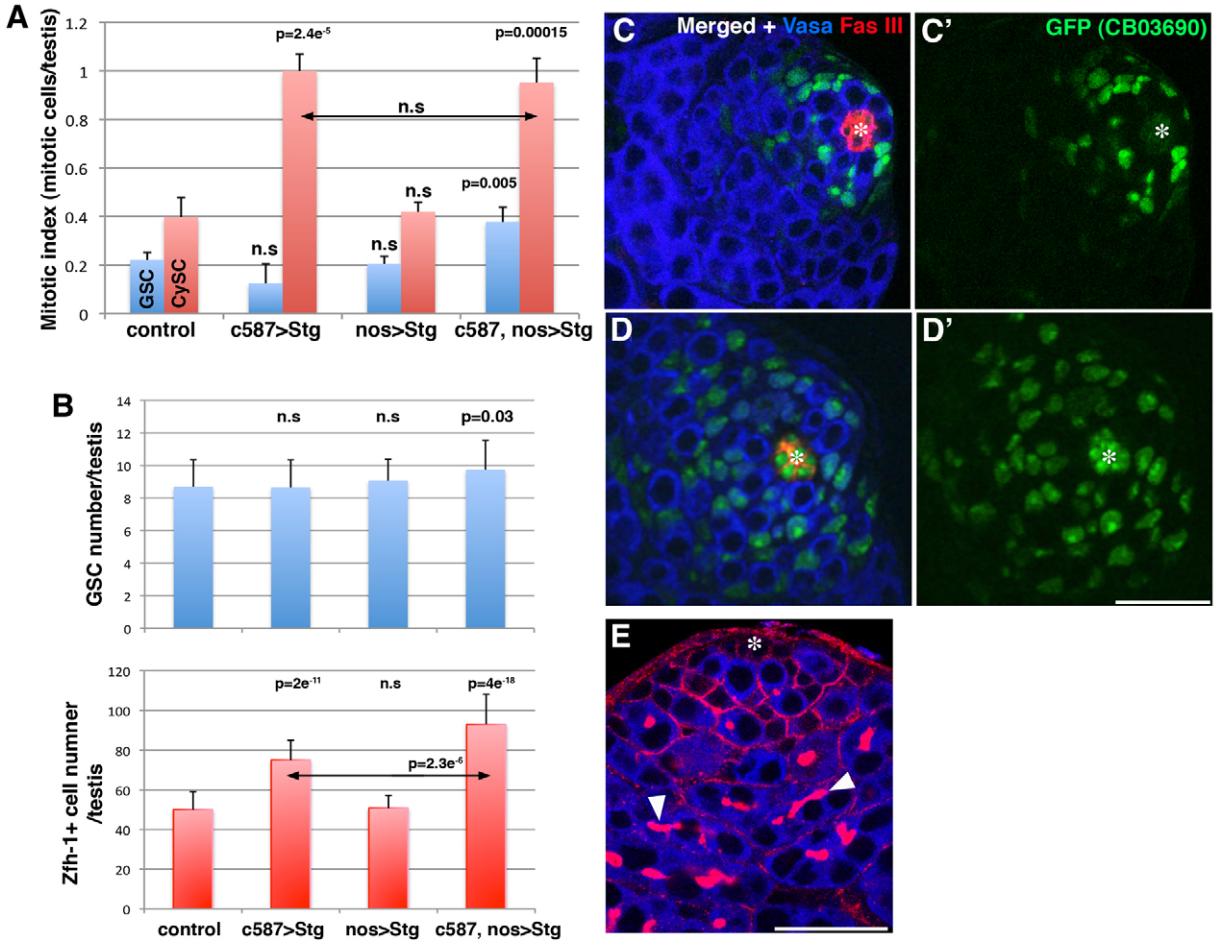

Fig. 3. Overexpression of Stg leads to an increase in the number of CySCs/CCs and mitoses. (**A**) Mitotic indices of GSCs and CySCs (mitotic cells/testis) upon expression of Stg in the germline and/or CySC lineage. Flies carrying the *c587-gal4* and *nos-gal4* drivers without UAS-Stg are shown as controls. The *P*-value (two-tailed Student's *t*-test) is provided for comparison with the control. *n*>120 testes for each data point. (**B**) Number of GSCs and CySCs (Zfh1-positive cells) upon overexpression of Stg in the germline and/or CySC lineage. *n*>30 testes for each data point. (A,B) Error bars indicate mean ± s.d. (**C-D'**) Examples of testis apical tip in control (C,C') or in testis expressing Stg in the CySC lineage (c587>Stg) (D,D'). Green, Chd64-GFP (CB03690, a CySC marker); red, Fas III (a marker of hub cells); blue, Vasa (germ cells). Asterisk indicates the hub. (**E**) Increased number of CySC-like cells upon expression of Stg does not lead to GSC tumor development. Red, Adducin-like (spectrosome/fusome); blue, Vasa (germ cells). Arrowheads indicate branched fusomes (stained with Adducin-like), a hallmark of differentiation. Scale bars: 25 μm.

decline of the mitotic index in aging GSCs (see below, Fig. 5), did not suppress centrosome misorientation associated with age (supplementary material Fig. S3). These results suggest that the decline in Stg expression in aging GSCs is neither a cause nor a result of centrosome misorientation, and that factors other than centrosome misorientation, including a decline in the Stg expression level, contribute to the age-associated decline in stem cell proliferation.

Interestingly, whereas GSC number did not significantly decrease by day 20 (Fig. 4D) (Boyle et al., 2007; Cheng et al., 2008), CySC number dramatically decreased with age (Fig. 4D,E,F). It should be noted that the decrease in CySC number combined with the sustained CySC mitotic index (CySC mitoses per testis; Fig. 5C) suggests that each CySC divides more frequently in aged than in young testis. These results reveal distinct kinetics of aging in GSCs and CySCs: GSCs exhibit decreased Stg expression and mitotic index but maintain their number, whereas CySCs decrease in number without reducing their division rate.

Expression of Stg in the germline prevents the age-associated decline in GSC proliferation and CySC number but leads to late-onset tumors

The correlation between the Stg expression level and mitotic index in GSCs and CySCs prompted us to examine whether restoration of Stg expression in GSCs might prevent or delay some age-associated phenomena. Importantly, expression of Stg in the germline (nos>Stg) did not lead to any obvious changes in stem cell number or division rate, as described above (Fig. 3); however, as the flies aged the nos>Stg testes showed considerable suppression of aging phenotypes. First, the GSC mitotic index was sustained in nos>Stg testes (Fig. 5A), whereas in wild-type flies the GSC mitotic index was halved by day 10 (see Fig. 4C). Second, nos>Stg testes maintained a high number of CySCs with age, in contrast to control flies (Fig. 5B). These findings suggest that restoration of Stg in the germline reverses multiple features of tissue aging. In contrast to the expression of Stg in the germline, expression of Stg in the CySC lineage did not suppress the aging

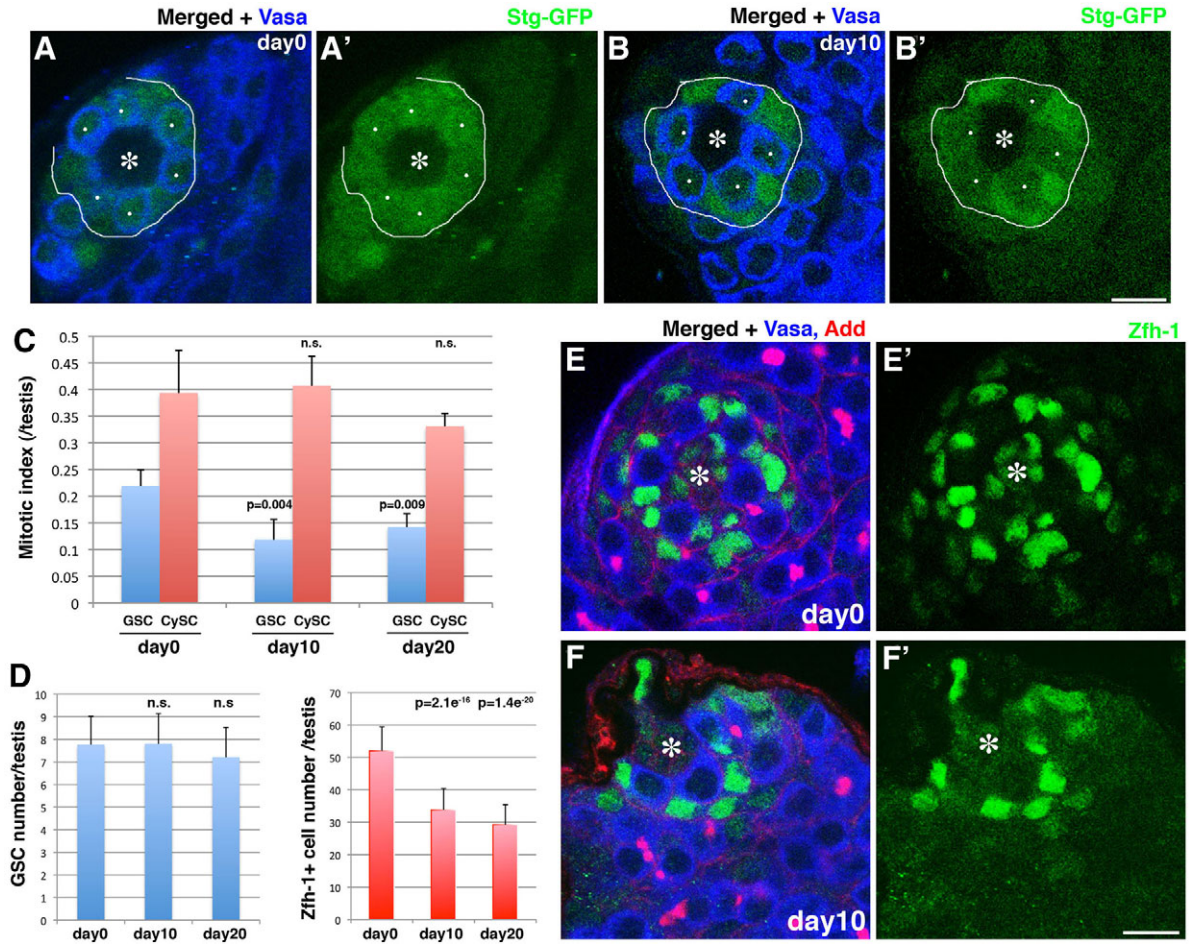

Fig. 4. GSCs and CySCs show distinct aging kinetics. (**A-B′**) Expression of Stg-GFP in flies at day 0 (A,A′) and day 10 (B,B′). The first tier of cells (GSCs and CySCs) is indicated by the white line. GSCs are indicated by white dots. Green, Stg-GFP; blue, Vasa (germ cells). Asterisk indicates the hub. (**C**) Mitotic indices of GSCs and CySCs with age. The *P*-value (two-tailed Student's *t*-test) is provided for comparison with day 0. *n*>120 testes for each data point. (**D**) Number of GSCs and CySCs with age. Whereas GSC number did not decrease significantly by day 20, CySC number significantly decreased by day 10. *n*>30 testes for each data point. (C,D) Error bars indicate mean ± s.d. (**E-F′**) Representative apical tip of the testis at day 0 (E,E′) and day 10 (F,F′) stained for Vasa (blue), Adducin-like (red) and Zfh1 (green), showing a dramatic decrease in Zfh1-positive CySCs with age. Scale bars: 10 μm.

phenotype (supplementary material Fig. S4). Although CySC number was increased dramatically by c587>Stg at day 0 (Fig. 3), it quickly fell to the level of the control by day 10 (supplementary material Fig. S4). Thus, expression of Stg in the CySC lineage, in which Stg expression does not decrease with age, does not suppress the aging phenotype.

Although expression of Stg in the germline suppressed age-associated phenotypes, nos>Stg testes developed tumors that were filled with undifferentiated germ cells and Zfh1^{+} CySC-like cells by day 30 (79% of testes examined, *n*=122 testes; Fig. 5C-H). Such tumorous testes were much less frequent in control siblings (30%, *n*=82 testes) and very rare in wild-type flies (6.4%, *n*=47 testes). The germ cell tumors in nos>Stg flies appear to be a mixture of GSCs and spermatogonia, as judged by the morphology of spectrosomes/fusomes (Fig. 5G,H). These tumors were infrequent (occurring in less than 3% of testes examined) at day 20 and non-existent at earlier time points; therefore, such testes with tumors were excluded from scoring of GSC and CySC numbers when obtaining the data on days 0-20 shown in Fig. 5A,B. Together, these data show that restoration of Stg expression in the germline reverses the aging phenotype in middle-aged (day 10-20) flies, but leads to late-onset tumorigenesis.

DISCUSSION

The present study reveals the function of Stg in stem cell proliferation in the *Drosophila* testis. The highly specific expression pattern of Stg in GSCs and CySCs indicates its function in stem cells. Specifically, Stg contributes to the maintenance of stem cells, as its knockdown leads to a decrease in GSC and CySC numbers, whereas its overexpression causes an increase in CySC number. It is currently unknown whether stem cells that are defective in Stg (or with reduced Stg expression) are lost due to cell death or differentiation. It is possible that cells that do not 'qualify' as stem cells undergo differentiation, as shown for melanocyte stem cells (Inomata et al., 2009). The reduced proliferation of GSCs and/or CySCs is unlikely to be a direct cause of decreased stem cell numbers: RNAi-mediated knockdown of Stg did not influence

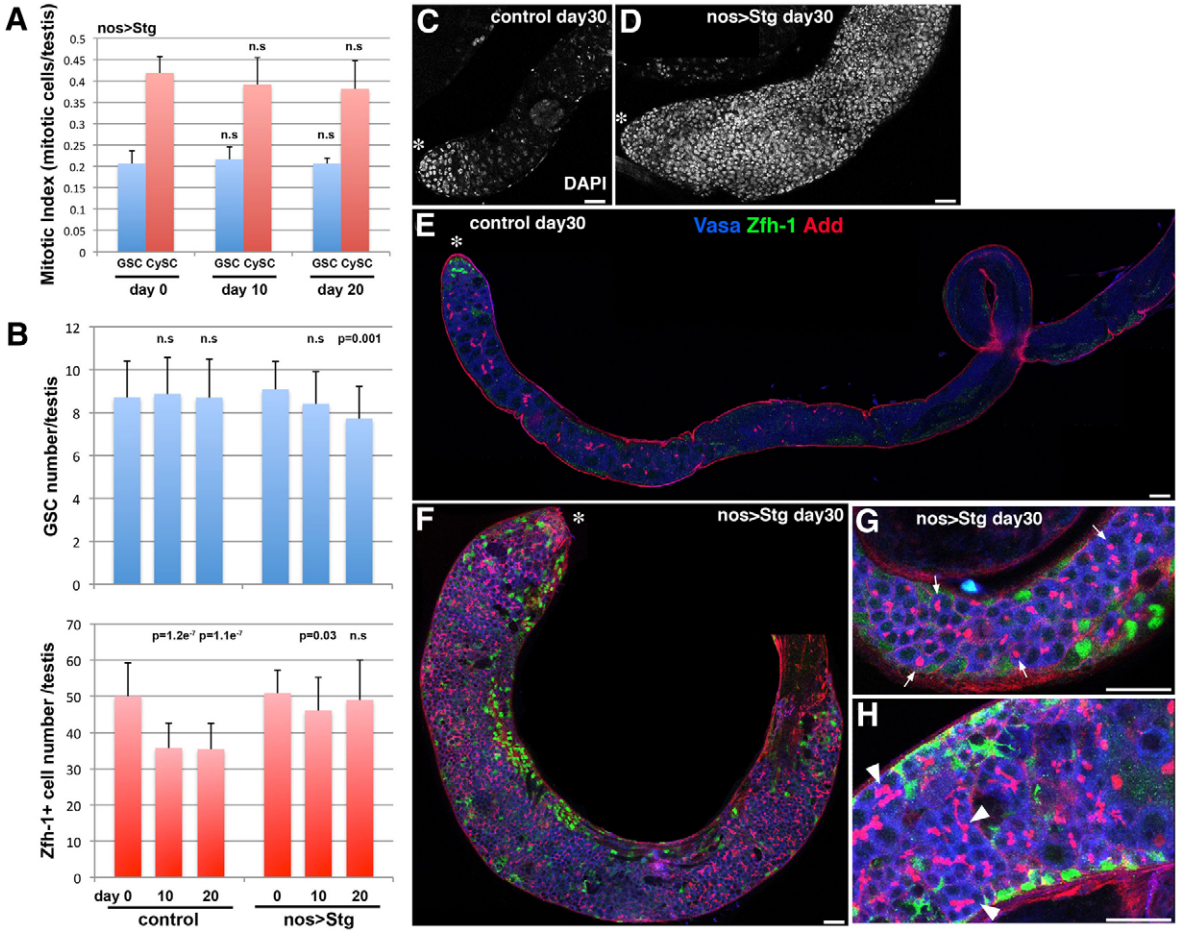

Fig. 5. Expression of Stg in the germline reverses aging phenotypes but leads to late-onset tumors. (**A**) Mitotic index of GSCs and CySCs with age upon expression of Stg in the germline (nos>Stg). The mitotic index of Stg-expressing GSCs did not decrease with age (for comparison, see Fig. 4C for mitotic indices in wild type). *n*>120 testes for each data point. (**B**) Change in stem cell number with age. CySC number was well maintained upon expression of Stg in the germline, whereas control flies lost a considerable number of CySCs with age. By contrast, whereas control flies maintained GSC number with age, Stg-expressing testes showed a slight but statistically significant decrease in GSC number. The *P*-value (two-tailed Student's *t*-test) is provided for comparison with day 0. *n*>30 testes for each data point. (A,B) Mean ± s.d. n.s., not statistically significant when compared with day 0 of each cell type. (**C**,**D**) DAPI staining of testes apical tip from control (C) and Stg-expressing (nos>Stg) (D) testis at day 30. Stg-expressing testes frequently developed tumors by day 30. Asterisks indicate the apical end of the testis. (**E**,**F**) Testis from control (E) and Stg-expressing (F) flies stained for Vasa (blue), Zfh1 (green) and Adducin-like (red). (**G**,**H**) High magnification of Stg-expressing testis. Some germline tumors contained spectrosomes (G, arrows), whereas others contained branched fusomes (H, arrowheads). Scale bars: 25 μm.

spindle orientation in GSCs or CySCs (data not shown) and thus GSCs are likely to be dividing asymmetrically, preserving GSC number. It is possible that GSCs with a reduced proliferation rate are somehow sensed by a quality control mechanism, leading to apoptosis or the differentiation of such GSCs and thus to a reduction in GSC number.

Are the effects of Stg manipulation described in this study specific to Stg function, or are they general consequences of manipulating the stem cell cycle? We favor the first possibility for the following reasons. First, other cell cycle regulators, such as Cyclins A, B and E, do not show stem cell-specific high-level expression (Lilly et al., 2000; Boyle et al., 2007; Monk et al., 2010), implying that these cell cycle regulators are equally important in stem cell compartments and transit-amplifying cells. Second, overexpression of Cdc2 (Cdk1) or Cdk4 together with Cyclin D (Meyer et al., 2002) did not lead to overproliferation of the CySCs (supplementary material Fig. S5), in contrast to the effect of Stg overexpression in CySCs. Given the specific, high-level expression of Stg in the stem cell compartments, Stg might function as a rate-limiting factor of cell cycle progression in stem cells.

Our data also indicate that CySCs are likely to play a permissive (but not instructive) role in GSC division, whereas GSCs play a minor role in CySC division. This leads to a higher CySC:GSC division ratio (the CySC mitotic index is more than twice the GSC mitotic index) under certain conditions: when CySC divisions are induced by overexpression of Stg in these cells, and when GSC divisions are reduced in aging testis leaving the CySC division rate unchanged. The fact that the GSC:CySC division ratio can diverge from a strict 1:2 ratio suggests that the nature of the coordination is not so tight that any modulation in the division rate in one population is reflected in the division rate of the other population. Instead, it seems that the system only provides a 'cap' on GSC division.

Stg expression specifically decreases in GSCs, but not in CySCs, with increasing age. Although re-expression of Stg in the germline suppressed the age-associated decline in GSC division and CySC number, it led to late-onset tumor development. This implies that, although the reduction in Stg expression contributes to tissue aging, it is an important tumor-suppressor mechanism; indeed, CDC25 has been shown to be overexpressed in many human cancers (Kristjansdottir and Rudolph, 2004; Boutros et al., 2007). Our work raises the possibility that the cells-of-origin of cancers that express CDC25 might be stem cell populations. It remains unclear whether the overexpression of Stg is sufficient to induce tumors or merely enhances a tumorigenic phenomenon inherent to *Drosophila* testes during aging. Indeed, wild-type and control flies also develop tumors at a lower frequency and Stg overexpression does not immediately lead to tumorigenesis, in contrast to the overexpression of 'stem cell factors' such as Upd and Zfh1 (Kiger et al., 2001; Tulina and Matunis, 2001; Leatherman and Dinardo, 2008). Therefore, we favor the possibility that the overexpression of Stg/Cdc25 enhances tumor formation when combined with other tumorigenic circumstances, such as the genetic background and age-associated imbalance of stem cell proliferation, as indicated in the present study.

Our study presents a potential hurdle in harnessing stem cells for therapeutics. Although it has been speculated that restoration of stem cell activity might help prevent aging or tissue-degenerative diseases, our data indicate that reversing the aging phenotype of one stem cell population does not necessarily lead to desirable consequences. To harness stem cell potential for therapeutic use, it will be crucial to understand how distinct stem cell populations decline with age and with unique kinetics, and how these distinct kinetics are coordinated among multiple stem cell populations over the course of aging to achieve tissue homeostasis and tumor suppression. In summary, the present study provides a cellular mechanism that links cell cycle regulation, stem cell identity, tissue aging and tumor-suppression mechanisms.

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Competing interests statement
The authors declare no competing financial interests.

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