

Non-autonomous cell redox-pairs dictate niche homeostasis in multi-lineage stem populations

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Abstract

Reactive oxygen species (ROS), predominantly derived from mitochondrial respiratory complexes, have evolved as key molecules influencing cell fate decisions like maintenance and differentiation. These redox-dependent events are mainly considered to be cell intrinsic in nature, on contrary our observations indicate involvement of these oxygen-derived entities as intercellular communicating agents. In multi-lineage *Drosophila* germline, neighbouring Germline Stem Cells (GSCs) and Cystic Stem Cells (CySCs) maintain differential redox thresholds where CySCs by virtue of their higher redox-state regulate physiological ROS levels of germline. Disruption of the intercellular redox equilibrium between the two adjoining stem cell populations results in deregulated niche architecture and loss of GSCs, which was mainly attributed to loss of contact-based receptions and uncontrolled CySC proliferation due to ROS-mediated activation of self-renewing signals. Our observations hint towards the crucial role of intercellular redox gradients originating from somatic progenitors, CySCs in niche stability where they function not only as a source of their own maintenance cues but also serve as non-autonomous redox moderators of germline immortality. Our findings underscore the complexity of niche homeostasis and predicate the importance of intercellular redox communication in understanding stem cell microenvironments.

eLife assessment

This work focuses on the role of Reactive Oxygen Species (ROS) signaling in cyst stem cells of the *Drosophila* testis. In particular, the authors suggest that ROS can act as signaling molecules between somatic and germ stem cells of the testis. The work is potentially **useful**, although the evidence that supports the authors' claims is **incomplete**.

Introduction

Studies from the past few decades have shown the apparent role of ROS in influencing various biological processes^{1,2,3}. Generally, the sources of ROS are compartmentalized in co-occurrence with their targets mediating important signalling events⁴. Mitochondria, a major source of oxidant species^{5,6}, localize dynamically towards nucleus, effecting oxidation induced reshaping of gene expression profiles⁷. Localized ROS production are contributed by NADH oxidases distributed across different cellular regions⁸. Intracellular hydrogen peroxide gradients maintained by thioredoxin system allow intercompartmental exchanges among endoplasmic reticulum (ER), mitochondria and peroxisomes^{9–11}.

The process of redox relays is conserved and plays a fundamental role in self-renewal and differentiation of stem cell populations¹². Stem cells whether embryonic or adult maintain a low redox profile, characterized by subdued mitochondrial respiration^{13–17}. Levels of ROS are tightly regulated and elevated amounts promote early differentiation and atypical stem cell behaviour¹⁸. However, leading evidences suggest that many transcription factors require oxidative environment for maintenance of pluripotent states. For instance, physiological ROS levels play a crucial role in genome maintenance of embryonic stem cells¹³. Multipotent hematopoietic progenitors and neural stem cells require relatively high baseline redox for their maintenance^{14,16,19}. Suppression of Nox system or mitochondrial ROS compromises the maintenance of these self-renewing populations and promote their differentiation or death¹⁸. These evidences point towards essentiality of a perfectly tuned redox state for balancing the pluripotent and differentiated states. But how the stem cells harmonize their redox potential by possessing a restrained oxidant system is not very clear.

We addressed this fundamental question in multi-stem cell lineage-based niche architecture in *Drosophila* testis. The testicular stem cell niche is composed of a central cluster of somatic cells called the hub which contacts eight to eleven GSCs arranged in a round array^{20,21}. A pair of cystic stem cells (CySCs) originating the somatic lineage cyst cells, enclose each GSC and make their independent connections with the hub and GSCs via adherens junctions^{22–26}. The hub cells secrete Unpaired family of cytokines that defines niche occupancy and self-renewal of both GSCs and CySCs through Jak-Stat signalling^{27,28}. CySCs also repress GSC differentiation by suppressing transcription of bag-of-marbles (Bam)^{29,30}. Concerted asymmetric division of both GSC and CySC is essential for proper cyst formation³¹. A developing cyst contains a dividing and differentiating gonialblast encircled by cyst cells that provide nourishment to developing spermatids^{32,33}. However, the maintenance of GSCs in the spatially controlled microenvironment is still not very clear.

We found the existence of a perfectly balanced redox differential between GSC and CySC essential for niche homeostasis. CySCs by virtue of their higher redox threshold and clustering of mitochondria at GSC-CySC interface, generate an intercellular redox gradient that maintain physiological ROS levels in GSCs. Alterations in CySC redox state affected self-renewal and differentiation propensities of the GSCs. Intercellular redox-imbalance induced niche disequilibrium through precocious differentiation of GSCs and deregulated CySC proliferation, due to activation of pro-proliferative transductions and loss of cell-cell contact signalling. Our results in multi-stem cell lineage-based germline system indicate a nuanced division of labour where a higher redox state in CySCs not only maintains their self-renewing processes but also non-autonomously foster GSCs maintenance.

Results

Redox state of GSC is maintained by asymmetric distribution of CySC mitochondria

The apical region of the *Drosophila* testis incorporates a cluster of differentiated cells the hub surrounded by GSCs in a rosette arrangement with each GSC being enclosed by two CySCs (Fig. 1A). The mitochondria morphology and distribution in this cluster was checked by immunostaining ATP5A subunit of mitochondrial ATPase. We observed fragmented mitochondria in Vasa⁺ GSCs as compared to a fused organization in Traffic jam (Tj)⁺ CySCs (Fig. 1B #, C, D). Distribution profiles of mitochondria showed its higher propensity to overlap with Tj, indicating CySCs to be a mitochondria dense region compared to GSC (Fig. 1F); and its mitochondria to be predominantly clustered near the GSC-CySC boundary, illustrated by intensity profile (Fig. S1A'') and X-Z and Y-Z sectioning (Fig. S1B-B'). The ATP5A labelling overlapped with *TFAM-GFP*, a mitochondrial transcription factor³⁴, further confirming the patterning observed between these two stem cell populations (Fig. S1C').

Mitochondria localization near the GSC-CySC boundary, together with its dispersion pattern was in accordance with the intensity variance of ROS reporter line *gstD1-GFP*³⁵ at the niche (Fig. 1H and S1D-D'). The redox gradient intensifies at the Tj-Vasa boundary and then gradually diffuses into Vasa⁺ GSCs, suggesting an establishment of GSC redox state through CySCs (Fig. 1Q; left panel). A GSC intrinsic oxidative environment should have resulted in a constant continuum of *gstD1-GFP* intensity across the Vasa-stained structure. We confirmed the greater ROS gradient at the GSC-CySC interface by 2D-Plot profile of Vasa, Tj and *gstD1-GFP* across the different cell types (Fig. S1E).

To affirm the role of CySC in influencing GSC redox state, we asked if disrupting dismutases in CySCs would influence the redox state of GSCs. We depleted the expression of superoxide dismutase 1 (Sod1) in CySCs and early differentiating cyst cells using *Tj-Gal4* driver (*Tj>Sod1i*). Higher CySC redox state resulted in corresponding increase in GSC ROS profile, as indicated by a greater degree *gstD1-GFP* intensity and DHE fluorescence at the Vasa-*gstD1GFP* (VNG) intersection (Fig. 1K, P, R and S1F), suggesting non-autonomous maintenance of GSC redox gradient by CySCs (Fig. 1Q; right panel). The surge in GSC ROS levels upon lowering of antioxidant defences in CySCs, was additionally confirmed through enhanced Vasa:*gstD1-GFP* colocalization intensities in *Sod1i* populations ($MCC^{\text{mean}} = 0.482$) with respect of controls ($MCC^{\text{mean}} = 0.276$) (Fig. 1S). These observations hinted towards a potential role of CySCs and its GSC polarized mitochondria in elevating the cellular ROS gradient at the interface between two stem cell populations.

Balanced CySC redox profile is crucial for its proliferation and GSC maintenance

Along with the shift in the redox gradient, we observed that high ROS in *Tj>Sod1i* had a striking effect on the increase in DAPI⁺-nuclei at the tip of testis (Fig. 1G and L). This overcrowding was attributed to an increase in number and positional shift in Tj⁺ CySCs and early differentiating cyst cells (Fig. 2A #, B' and E). The enhancement in the number of CySCs was also reflected by ~2-fold change in Zfh1⁺ cells (Fig. 2F). Vasa⁺ cells flanking the hub however, substantially reduced in number (Fig. 2C #'-D'' and G), which was validated through lower detectable levels of Vasa even when its expression per cell remained unchanged (Fig. S2O). The observations were further verified using *Sod1i* localized in different chromosome to avoid any chromosome or balancer-based biasness (Fig. S1G-L). However, the phenotypic effect of higher ROS was limited to its origin in CySCs only because ablation of GSC redox status did not cause any marked change in niche composition. *Nos-Gal4* driven knock-down of Sod1 in GSCs did not result in significant change in

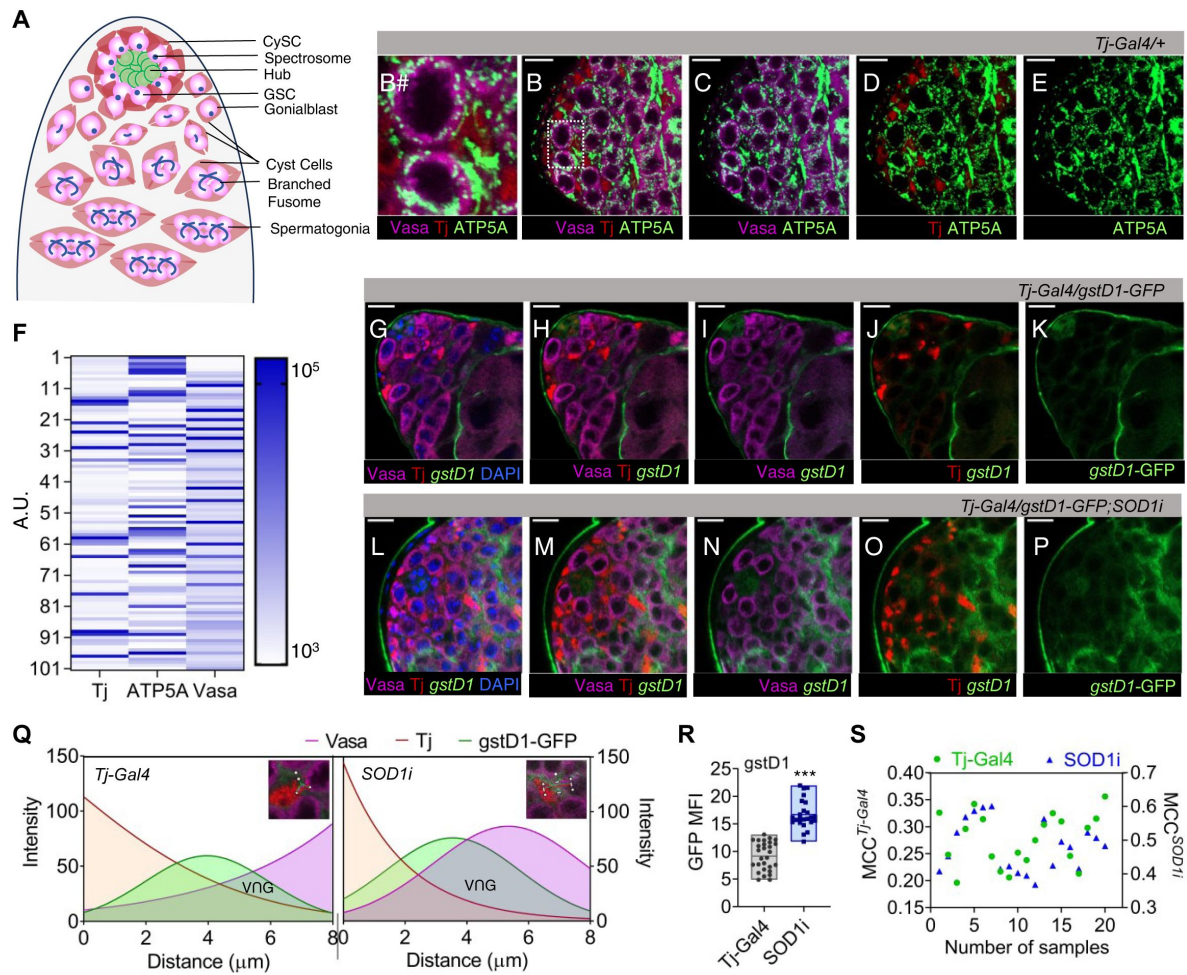


Figure 1.

Mitochondrial Distribution and Redox Profile of GSCs and CySCs in Adult *Drosophila* Testes

(A) Schematic representation of adult *Drosophila* testicular niche showing arrangement of different stem cell populations (GSC – Germinal Stem Cell, CySC – Cystic Stem Cell). (B-E) Differential distribution of mitochondria labelled with ATP5A (green) in Vasa⁺ GSCs and Tj⁺ CySCs of wild-type fly testis; B# shows digitally zoomed image of the dotted area. (F) Relative mitochondria density profile of GSCs and CySCs denoted through cooccurrence of ATP5A with Tj or Vasa. A.U. – arbitrary units. (G-P) Redox profiling of testicular stem cell niche using *gstD1*-GFP as intrinsic ROS reporter in control (G-K) and *Tj-Gal4* driven *Sod1RNAi* (*Sod1i*) testis (L-P). (Q) Gaussian distribution curve for the variations in *gstD1*-GFP fluorescence at CySC-GSC intersection region (inset) in *Tj-Gal4* control and *Sod1i* testis. (R) Quantification of mean *gstD1*-GFP fluorescence intensity (MFI) upon Sod1 knockdown represented as fold change over control. Data denotes mean $\pm$ s.e.m, n = 25 (S). Colocalization intensity between Vasa and *gstD1*-GFP expressed through Manders' co-localization coefficient (MCC) among control and *Sod1i* cells (G). Controls are the indicated driver line crossed with *Oregon R*⁺. Scale bar – 10 μ m. See also Figure S1.

CySC number (Fig. S2A', B' and E) but effected a considerable reduction on Vasa⁺ cells (Fig. S2C', D' and F), aligning with previous observations¹⁷. Although, Sod1 is the predominant enzyme which also localizes in the intermembrane space of mitochondria, we observed a similar result upon depleting the matrix localized Sod2 in both GSCs and CySCs (Fig. S2G-M), indicating involvement of mitochondrial ROS for the observed cellular phenotypes.

To confirm active proliferation of cells and rule out the possibility of arrested growth, we utilized fly-Fluorescent Ubiquitination-based Cell Cycle Indicator (FUCCI) system³⁶ which comprises of two reporter constructs marking G1/S transition (green), S (red) and G2/early mitosis (yellow) (Fig. 2H). *Tj>Sod1i* testes presented a ~2-fold increase in cell number across all stages of cell-cycle (Fig. 2I). The enhanced number of dividing cells was contributed mainly by progressive division of CySCs, showing >2-fold difference in accumulation of Zfh1⁺ nuclei in S and G2/M phases (Fig. 2J, S2PV-VII and QV-VII). The number of Zfh1 labelled cells in G1/S transition was also substantially more (Fig. S2PIII and QIII), indicating dysregulated redox ensued increased mitotic index of CySCs. Downregulation of Sod1 in GSCs through *Nos-Gal4* demonstrated no significant change in G1/S, S and G2/M phase numbers, confirming our previous observation that altering ROS levels in GSCs does not have any non-autonomous effect on CySCs (Fig. S2N).

CySC induced ROS couples precocious differentiation of stem cell lineages

Since, in *Sod1i* testis the number of Tj⁺ CySCs and early differentiating cells³⁷ were elevated and more than CySC-specific Zfh1⁺ cells (Fig. 2I and J), we sought to label these cells with another differentiation marker Eya (Eyes absent). We found that Eya⁺ cells clustered towards the hub along with a significant increase in their number in Sod1 depleted CySCs (Fig. 3A, B and D). The supposed premature expression of Eya resulted in a population of differentiating cyst precursor cells co-expressing Eya-Zfh1 (Fig. 3A", B" and D). This population was found to be deviating from the normal partitioning of wild type representative populations (Fig. 3C). On contrary, the same cells would not show a similar phenotypical arrangement when Sod1i depletion was driven in the germline lineage by *Nos-Gal4* (Fig. S3A-D). These observations hint towards an obvious change in redox state of GSCs affecting their number, even when generated non-autonomously from CySCs.

Together with reduction in Vasa⁺ GSCs shown earlier (Fig. 2G), we also detected gonialblast expressing differentiation-promoting factor Bam to be much closer to the hub in *Tj>Sod1i* testes (Fig. 3E" and F"). The mean distance between the hub and Bam⁺ cells was found to be shortened by ~7 microns (Fig. 3G). The relative advancement of spermatogonial differentiation towards the hub was also ascertained by tracking the transformation of GSC-specific spectroosome into branched fusomes connecting the multi-cell gonialblast (Fig. 1A). The branching fusomes were found more proximal to hub than controls (Fig. 3H, I" and J"), along with parallel reduction in number of spectroosomes, implying a decline in early-stage germ cells (Fig. S3F). This loss could possibly be attributed to a higher oxidizing environment in GSCs and loss of intercellular contacts, particularly with hub cells (Fig. 2C# and D#). The observation corroborated with decreased expression pattern of E-cadherin in *Tj>Sod1i* particularly near the hub (Fig. 3K-M and S3G) and could be one of the reasons for GSCs dissociation and differentiation (Fig. 2D#), due to absence of self-renewing signals. Indeed, disengagement of GSCs from the hub caused reduction in Socs36E and Ptp61F transcripts that act as drivers of E-cadherin expression and are induced by maintenance factor STAT originating from the hub (Fig. 3N and O). These results suggest that disruption of intercellular redox gradient effected premature differentiation of stem cells in the niche.

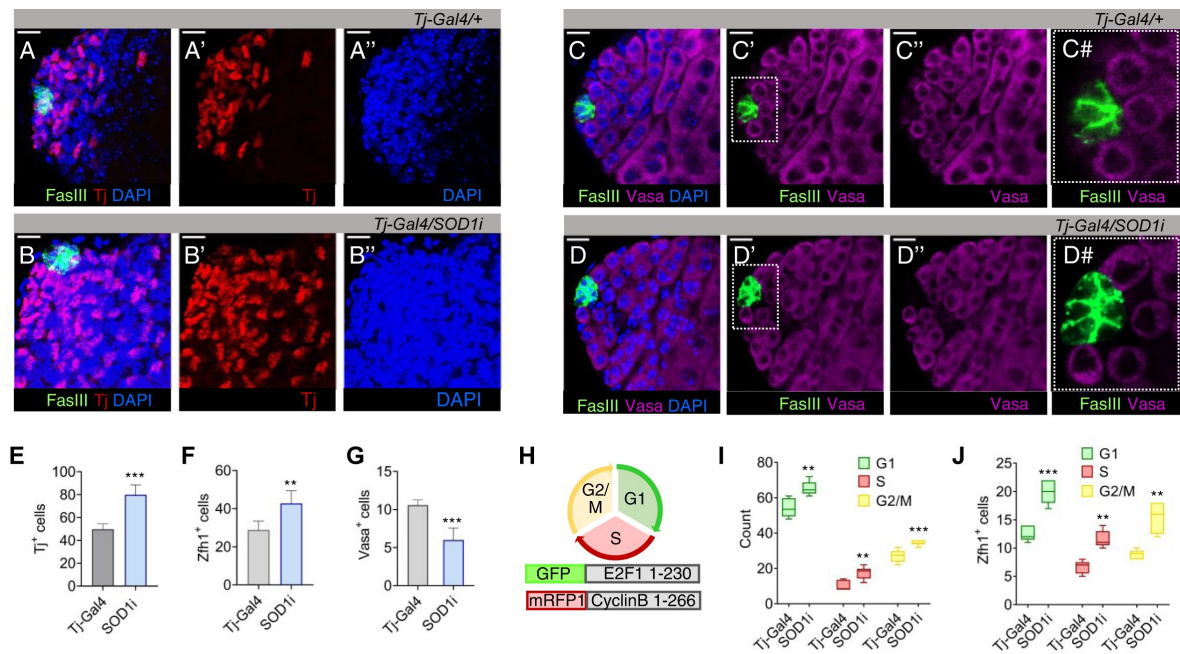


Figure 2.

Redox disequilibrium in Cyst stem cell lineage deregulates early CySCs and decreases the GSC number

(A-B) Distribution of *Tj*⁺ CySC/early cyst cell around the hub (FasIII) in control and driven *Sod1RNAi* testis. (C-D) Represents the rosette arrangement of *Vasa*⁺ GSCs flanking the hub. The digitally magnified region around the hub (dotted line) as seen in control (C#) and *Sod1RNAi* (D#). (E-G) Mean number of *Tj*⁺ cells, *Zfh1*⁺ early CySCs and GSCs in control and *Sod1RNAi* lines shown as mean $\pm$ s.e.m, n = 10. (H) Construct design of the FUCCI reporter line for tracking the cell cycle stages in *Drosophila* tissues, containing degons of Cyclin B and E2F1 proteins fused with RFP or GFP. G1, S and G2/M is represented by GFP⁺, RFP⁺ and dual labelled cells respectively. (I-J) Comparative changes in the total cell population (I) or *Zfh1*⁺ cells (J) present in G1, S and G2/M phase at the niche zone between control and *Tj>Sod1i*. Data points denotes mean $\pm$ s.e.m, n = 10. Scale bar – 10 μ m. See also Figure S2.

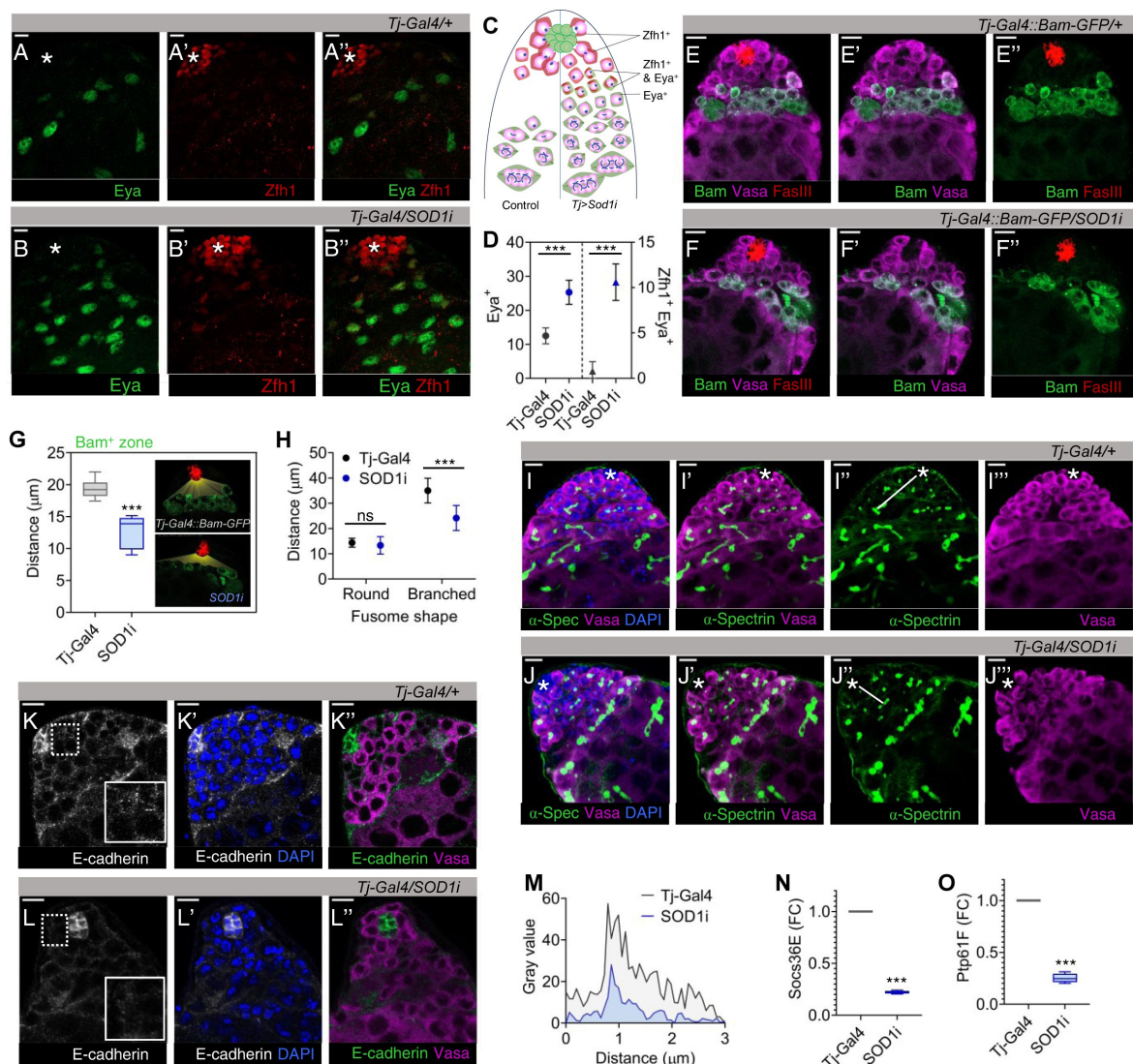


Figure 3.

ROS imbalance in CySCs promotes differentiation of both GSCs and CySCs

(A-D) Number of early CySCs ($Zfh1^+$) (A'-B'), late differentiating cyst cells (Eya^+) (A-B) and $Zfh1$, Eya co-expressing population were imaged (A''-B''), represented schematically (C) and quantified with respect to control and *Sod1RNAi* lines (D). Data points represent mean $\pm$ s.e.m, $n = 10$. (E-F) The effect of *Sod1* depletion in CySCs on the differentiation status of GSCs as observed using *Bam-GFP* reporter line. (G) The relative distance of the differentiation initiation zone (Bam^+) from the hub (red) in control and *Sod1i* testis shown as mean $\pm$ s.e.m, $n = 10$. (H-J) The shape and size of the spectrosomes marked with α -spectrin (green) were imaged (I''-J'') and quantified for their distance from the hub (marked with asterisk) (H), $n = 10$. Branched fusome marks differentiating populations (I'-J'). (K-L) Comparative staining of CySC-GSC contacts through adherens junction using E-cadherin (monochrome/green). Dotted area near the hub has been expanded as inset to show loss of E-cadherin network. (M) 3D surface intensity histogram for E-cadherin expression near the hub. (N-O) Fold change in expression of Stat dependent transcripts *Socs36E* (N) and *Ptp61F* (O) among control and *Sod1i* niche. Data is shown as mean $\pm$ s.e.m, $n = 3$. Scale bar - 10 μ m. See also Figure S3.

Disrupted niche architecture compromises GSC-CySC communication

GSCs and CySCs intercommunicate through redox dependent factors such as EGFR, PI3K/Tor to support CySC maintenance³⁸. EGF ligands secreted by GSCs maintain differences in cell polarity and segregates self-renewing from differentiating populations³⁹. *Tj>Sod1i* cells showed lower levels of cell-polarity marker Dlg (Fig. 4A", B" and S4A), that expanded more into the differentiated zone (Fig. S4B). Since, Dlg expression is dependent on EGFR⁴⁰, impairment in its reception was reflected in net reduction in pErk levels across sections in *Sod1i* testis (Fig. S4C). Due to loss of contact, this reduction was mainly limited to regions surrounding the hub, in CySC zone (Fig. 4C"-D" and E). Differentiated cyst cells presented relatively higher levels of pErk expression (Fig. 4E", S4D'), particularly in *Tj>Sod1i* tissues where the pErk area was significantly extended, mapping with that of Dlg (Fig. 4F") and located comparatively closure to the hub (Fig. S4D), supporting its redox-dependent enhancement to promote differentiation. The deregulated proliferation of cystic cells reconciled with higher levels of its self-renewing inducers PI3K effector, p4E-BP (Fig. 4G", H' and I) and Hedgehog (Hh) (Fig. 4M" and N')⁴¹ in *Tj>Sod1i* testis. The noticeably high p4E-BP was associated with Cyclin-D accumulation (Fig. 4J"). Similarly, depletion of Sod1 in CySC resulted in elevated expression of Hh receptor Patched (Ptc) (Fig. 4O") which extended to regions farther from the hub, overlapping with expanded *Tj*⁺ cells (Fig. 4M-N"). Since, Ptc itself is a transcriptional Hh target, its expression in CySCs suggest active Hh signalling⁴², further supported by higher levels and similar pattern of Hh transcriptional effector Cubitus interruptus (Ci) in *Sod1i* testis (Fig. 4P" and S4E"-F"). Since, Ptc, Ci and a GPCR-like signal transducer Smoothened (Smo) are all transcriptionally driven by Hh, and Hh itself is regulated by higher levels of Ci^{43,44}, we expectedly found higher levels of transcripts for these pathway genes (Fig. S4G-J), indicating ROS-mediated upregulation of Hh activity. The overt proliferation of CySCs under high ROS conditions was suppressed by reducing gene dosage either by single copy of *PI3K^{DN}* (dominant-negative allele) or Hh-RNAi (Fig. 4L" and Fig. S4K-N) which was also associated with an equivalent maintenance of GSC population (Fig. 4K" and S4O-R). Together these observations suggest that intercellular redox gradients balance the stem-cell populations in the niche through CySC maintenance factors.

Elevating CySC antioxidant defence promotes GSC self-renewal

To further substantiate the role of ROS in coordinating the multi-lineage stem populations, we strengthened the cellular defences against oxidants by overexpressing Sod1 in CySCs and monitored the relative GSC/CySC populations. Indeed, we observed an increase in the number of Vasa⁺ cells (Fig. 5A" and S5A"-B"), together with a slight reduction in *Tj*⁺ cells (Fig. 5B" and S5E"-F"). Any morphological anomalies associated with cell-cell adhesion or abnormal cellular dispersion, was not observed (Fig. S5A-B and E-F). Enhancing the levels of Sod1 in GSCs promoted the growth of GSC-like cells (Fig. S5C", D" and I) but did not have any prominent effect on CySCs (Fig. S5G", H" and J). The increment in Vasa⁺ GSCs under CySC-induced low redox conditions was also represented by parallel increase in spectroscopy number (Fig. 5C-E"). The uptick in GSC number due to scavenging of ROS in CySC, might be a result of delayed differentiation as indicated by displacement of Bam⁺ zone away from hub; thus, confirming non-cell-autonomous role of CySC ROS in maintaining GSC fate (Fig. 5F-H"). The data suggest that reducing redox profile of CySCs differentially affected their own self-renewing propensities along with GSC maintenance and optimum redox state of both the stem-cell populations is controlled by CySCs.

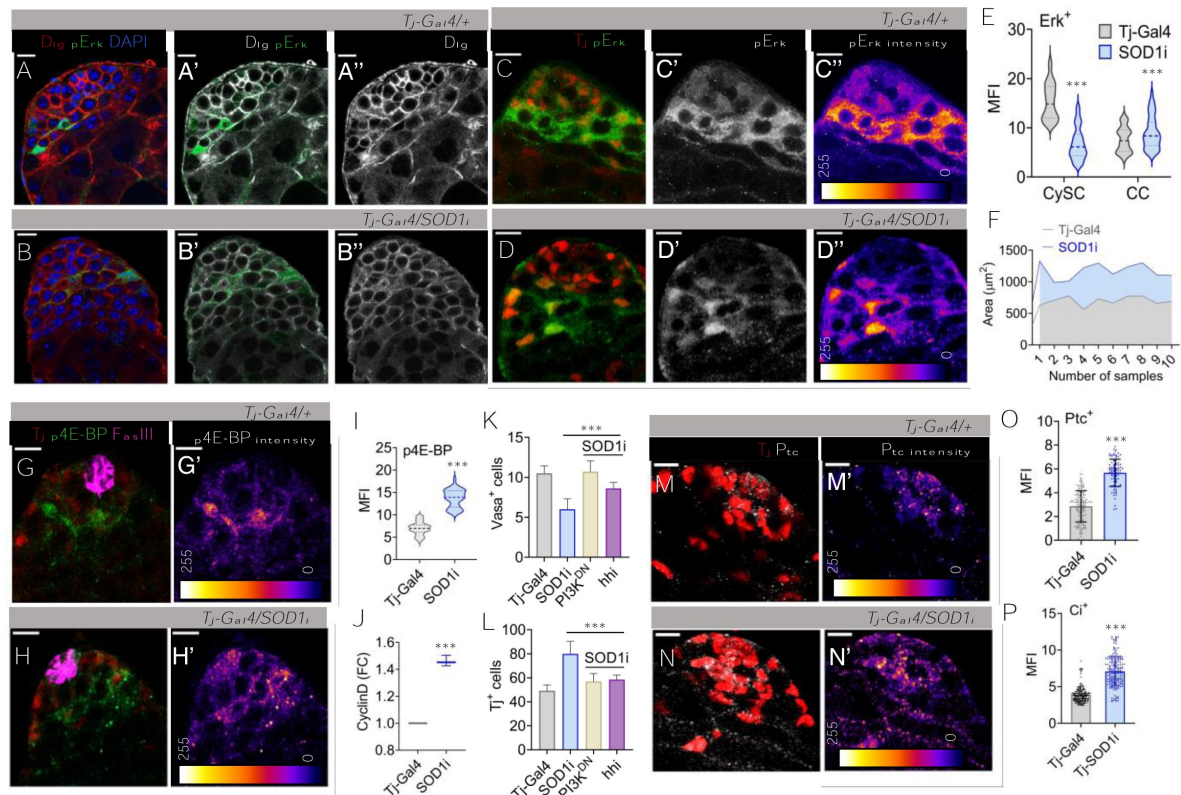


Figure 4.

Altered CySC redox state affects niche maintenance signals.

(A-B) The expression of cell polarity marker Dlg (red) in control and *Sod1i* stem-cell niche (A'-B''), (C-D) Representative image showing pErk distribution parallelly with Tj⁺ cells and its expression pattern through Fire LUT (C''-D''). Scale bar – 10 μm. (E) Segregated pErk expression patterns in CySCs near the hub and differentiating cyst cells (CC), mean ± s.e.m, n = 200. (F) Estimation of the differentiation zone through quantification of high pErk expressing regions. (G-I) Activation of PI3K/Tor in somatic lineage was adjudged through reporter p4E-BP in *Tj>Sod1i* (G'-H') and levels of the active form was quantified (I), MFI – mean fluorescence intensity shown as mean ± s.e.m, n = 40. (J) Induction of Cyclin D in *Sod1i* represented as fold change over controls by mean ± s.e.m, n = 3. (K-L) The number of Vasa⁺ (K) and Tj⁺ (L) cells across control, *Sod1i*, *Sod1i/PI3K^{DN}* (dominant negative allele) and *Sod1i/Hhi* rescue samples depicted as mean ± s.e.m, n = 10. (M'-N') Representative image showing distribution of Patched (Ptc) in Tj⁺ cells in control and *Tj>Sod1i*. (O-P) Quantification of Ptc (O) and Hh effector Ci (P) expression through fluorescence intensity as mean ± s.e.m, n (Ptc) = 90, n (Ci) = 200. See also Figure S4.

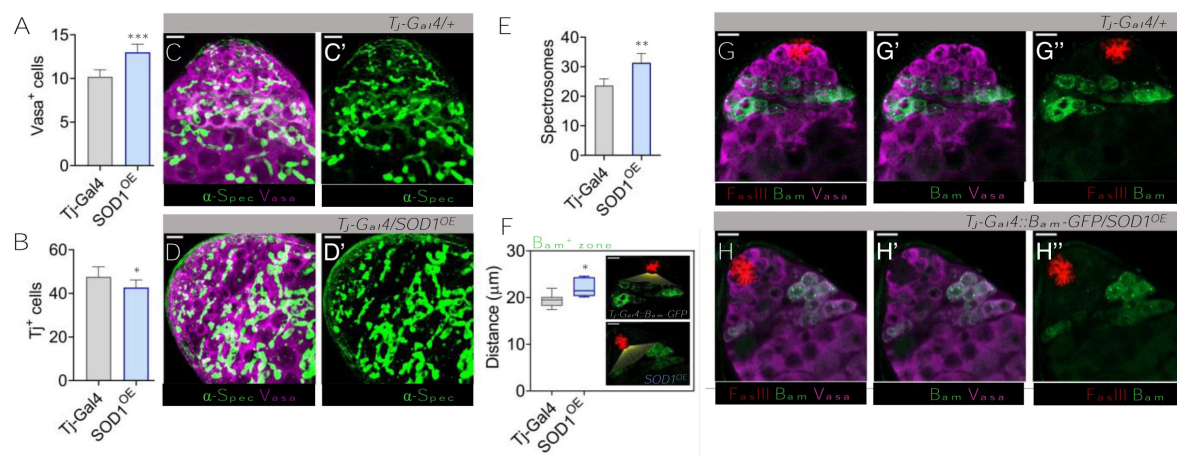


Figure 5.

Low CySC ROS sustains GSC maintenance

(A-B) Bar graphs illustrating variations in Vasa⁺ GSC (A) and Tj⁺ CySCs (B) numbers upon overexpressing Sod1 (*Tj>Sod^{OE}*), represented as mean $\pm$ s.e.m, n = 10. (C-E) The spectrosomes labelled with α -spectrin (α -spec) were imaged (C'-D') and their number was quantified (E). (F-H) The initiation of gonialblast differentiation was determined by measuring the distance of Bam⁺ zone from hub (red), n = 10 (F), (G'-H'') shows distribution of Bam⁺ reporter expressing cells. Scale bar – 10 μ m. See also Figure S5.

Discussion

ROS is a crucial mediator of stem cell maintenance where these species act as second messengers to induce different post-translational protein modifications thereby, affecting cell fate^{45,46}. Effectuation of ROS is mainly considered to be autocrine in nature where generation of these oxidative species is compartmentalized with their site of action. Recent studies have reported non-autonomous generation of ROS by NOX enzymes or upon stimulation by growth factors, to play a critical role in regenerative growth^{47–49}. However, in either of the cases, the target cell serves as the source and consequent response. Our observations in multi-lineage *Drosophila* stem cell niche suggested a previously unaccounted transmission of redox signals inter-cellularly that act as an organizer of niche homeostasis. The higher ROS threshold of CySCs and resultant CySC-GSC oxidative gradient maintained the physiological redox state of GSC. Reduced ROS in GSC was accompanied with its proliferation⁵⁰, phenocopied when Sod1 was overexpressed in its neighbours (Fig. 6).

The GSCs are attached to the hub via adherens junction to receive the maintenance signals like, Upd that trigger the JAK-STAT pathway^{27,28}, suppression of which activates Bam, acting as a switch from transit amplifying cells to spermatocyte differentiation^{51,52}. E-cadherin levels ensure only undifferentiated stem cells to persist within the niche, while differentiating GSCs lose the competition for niche occupancy²⁵. Elevated ROS in CySC possibly affected STAT phosphorylation through S-glutathionylation^{53,54}, causing reduced presence of dependent transcripts, Socs36E and E-cadherin thereby, resulting in detachment of GSCs from the neighbours as under E-cadherin depleted conditions^{55–58}. This resulted in compromised GSC-CySC paracrine receptions such as EGFR which play a crucial role in maintenance of cell-polarity that segregates self-renewing CySC populations from the ones receiving differentiation cues^{39,59,60}, leading to accumulation of cells co-expressing both stemness and differentiation markers. In contrast to GSCs, ROS imbalance in CySCs induced accelerated differentiation due to deregulation of PI3K/Tor and Hedgehog pathways. Although, redox modulation of PI3K pathway components has been previously demonstrated⁶¹, in this study we found Hedgehog to be probably susceptible to redox regulation.

However, we do not rule out the possibilities of hub cells playing an equivalent role in GSC maintenance, given their parallel effect in suppressing GSC differentiation²⁸. We had tried using E-132 driver to express *Sod1i* lines in hub cells but were challenged by substantial adverse effect on fly viability. We also avoided usage of Gal80 dependent clonal populations to maintain a homogenous genetic background and prevent false readouts due to diffusion of ROS signals in unaltered neighbourhood.

The proposed concept of intercellular ROS communication can be of importance in deciphering the biochemical adaptability and plasticity of different niches influencing stem cell fate, ensuring niche size and architecture to prevent stem cell loss and aging. This has been observed in neural stem cells where inflammation-induced quiescence recovers the regenerating capacity of aging brain⁶². In addition to metabolic variations, dependence of the germline on somatic neighbours for its redox state might be one the reasons behind its presumed immortal nature and renitence to aging⁶³. The same can also be applied to cancer stem cells which maintain niche occupancy and resist oxidative stress induced apoptosis probably, by receiving redox cues from the environment. However, further work is required to elucidate the myriad of driver mechanisms intersecting at the realm of redox regulation that extend beyond the present system into broader translational areas.

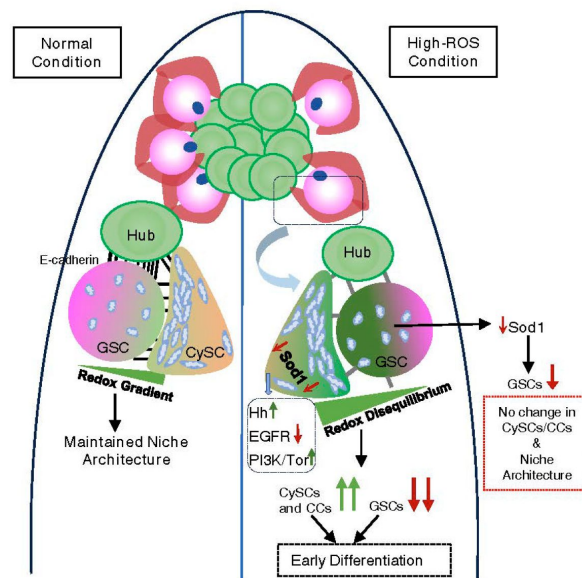


Figure 6.

Proposed role of intercellular redox gradients in GSC maintenance and differentiation

Mitochondrial abundance in GSC-CySC boundary maintains redox differential between two stem cell population. Disequilibrated intercellular redox gradient enhances precocious GSC differentiation and enhances CySC proliferation, thereby disrupting the homeostatic balance between the two stem populations.

Materials and Methods Fly strains

Fly stocks were maintained and crosses were set at 25 °C on normal corn meal and yeast medium unless otherwise indicated. All fly stocks (BL-24493) *UAS SOD1 RNAi*, (BL-32909) *UAS SOD1 RNAi*, (BL-29389) *UAS SOD1 RNAi*, (BL-32983) *UAS SOD2 RNAi*, (BL-24754) *UAS SOD1*, (BL-32489) *UAS hhRNAi*, (BL-8288) *UAS PI3K^{DN}*, (BL-25751) *UAS Dcr-nos*, (BL-55122) *UAS-FUCCI*, were obtained from Bloomington Drosophila Stock Centre. *Nos-Gal4*, *Tj-Gal4*, *Tj-Gal4-BamGFP*, *gstD1-GFP*, *TFAM-GFP* were kind gift from U. Nongthomba, P. Majumder, K. Ray, B. C. Mandal, Hong Xu labs respectively. For optimal Gal4 activity parent crosses were shifted to 29 °C till the eclosion of adults. 3-5 days old males were taken for all the experiments. The control lines for all experiments are the corresponding Gal4-line crossed with wild type *OregonR⁺*.

Dissection and immunostaining

Anesthetized flies were dissected in 1X Phosphate Buffer Saline (PBS). All incubations were carried out at room temperature (25 °C) unless otherwise mentioned. Testes were fixed in 4% paraformaldehyde for 30 minutes, followed by multiple washes with 0.1% PBTX (PBS + TritonX 100). Post-blocking in 0.5% Bovine Serum Albumin, testis was incubated overnight at 4°C for primary antisera, followed by washing in 0.1% PBTX 3 times 15 minutes each before incubating with secondary antibody. The tissues were counterstained with DAPI (4,6-diamidino-2-phenylindole) for 20 minutes, followed by three washes in 0.1% PBTX 10 minutes each. For PTC staining, a modified protocol utilizing PIPES-EGTA buffer was used as described⁶⁴. The dpErk was labelled by dissecting fly testes in Schneider's media, followed by fixation in 4% PFA for 30 min, 3x wash in 0.1% PBTX, blocked and incubated in primary antibody overnight; each step supplemented with phosphatase inhibitor cocktail 2 (1:100, Sigma, cat#P5726). Samples were mounted in DABCO anti-fade medium prior imaging.

The following primary antibodies were used in the experiments – anti-FasIII (7G10) (1:120, DSHB (Developmental Studies Hybridoma Bank)), anti-Eya (1:50, DSHB), anti-DEcad (DCAD2) (1:50, DSHB), anti- α -Spectrin (3A9) (1:20, DSHB), anti-Ptc (1:100, DSHB), anti-Ci (1:50, DSHB), anti- β tubulin (1:300, DSHB), anti-pERK (1:100, Cell Signaling (4370)), ATP5A (1:700, Abcam (ab14748)), anti-Tj (1:5000), anti-Vasa (1:4000), anti-Zfh1 (1:2000). The primary labelling was detected using appropriate Alexa-Flour tagged secondary antibody (ThermoFisher Scientific).

To assess superoxide levels, testes were dissected in Schneider's medium (SM) and immediately incubated in 30 μ M Dihydroxyethidium (DHE) at 25 °C in dark. Testes were washed thrice with SM for 7 minutes each before quantifying the emitted fluorescence using SpectraMax iD5 (Molecular Devices).

Quantitative Reverse transcription-PCR

For semi-quantitative RT-PCR and qRT-PCR analyses, total RNA was isolated from testes of 3-5 days old male flies using Qiagen RNA extraction kit. RNA pellets were resuspended in nuclease-free water and quantity of RNA was spectrophotometrically estimated. First strand cDNA was synthesized from 1-2 μ g of total RNA as described earlier. The prepared cDNAs were subjected to real time PCR using forward and reverse primer pairs as listed below, using 5 μ l qPCR Master Mix (SYBR Green, ThermoFisher Scientific), 2 pmol/ μ l of each primer per reaction for 10 μ l final volume in ABI 7500 Real time PCR machine. The fold change in expression was calculated through $2^{-\Delta\Delta C_t}$ method. The primers used are listed in Supplementary Table S1.

Imaging and image analysis

Confocal imaging was carried out using Zeiss LSM 900 confocal microscope, using appropriate dichroics and filters. Images were further analyzed and processed for brightness and contrast adjustments using ImageJ (Fiji). Mean intensity measurements were carried out using standard

Fiji plug-ins, 2D surface plot quantification or Zeiss Zen software as indicated. Quantification of cell number/nuclei in single or co-stained populations (Tj, Vasa, Zfh1, Eya, Fucci) were performed from individual slices of z-stacks using semi-automated Cell Counter plugin in Fiji for all the mentioned genotypes. The relative distance from the two reference points in the images was estimated through Inter-edge Distance ImageJ Macro v2.0 from Github. All images were assembled using Adobe Photoshop version 21.2.1.

Immunoblot Analysis

Protein was extracted from the dissected testes using RIPA buffer as described previously and quantified using Bradford reagent (Biorad). Equivalent concentration of lysate was denatured using 1X Laemmli Buffer with 1 M DTT at 95°C, separated in 10% SDS-PAGE and transferred onto PVDF membranes. Membranes were blocked with commercial blocking solution in TBST base before sequential primary antibody incubation with anti-Vasa and anti- β -Tubulin (DSHB, E7). Secondary detection was performed using HRP-tag anti-mouse antibody (GE Amersham).

Statistical analyses

All data points represent at least three biological replicates and the sample sizes carrying adequate statistical power are mentioned in the figure legends. Statistical significance for each experiment were calculated using two tailed Student's t-test unless otherwise mentioned, using GraphPad Prism 8 software. The P values were calculated through pairwise comparison of the data with wild type or driver alone and driven RNAi lines. Significance values for sample sizes mentioned in figure legends were represented as *P < 0.01, **P < 0.001, or ***P < 0.0001.

Author contribution

D.S. and O.M conceived the study and designed the experiments. O.M, A.C and T.C performed the experiments. D.S and O.M analyzed the data. D.S, O.M. and A.C. wrote the manuscript.

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Declaration of interest

The authors declare no competing interests

Data availability

The data that support the findings of this study are available within the main text and its Supplementary Information file. The lead contact will share all raw data associated with this paper upon request. This study did not utilize or generate any unique datasets or codes.

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Reviewer #1 (Public Review):

The manuscript by Majhi and colleagues describes the effects of manipulating ROS levels in somatic stem cells of the testis on overall testis architecture, signaling, and function. The conclusions made by the authors are somewhat difficult to judge as the changes to the testis cell types are mostly not apparent in the representative images shown. This is true in examining *gstD1*-GFP expression and in the analysis of cell types and behaviours (e.g. cell cycle) and cell signaling pathway activity. Thus, the reader is left to try and interpret the quantification of the data to justify the authors' conclusions, but it is often not clear how the quantification was accomplished. For example, it is not clear how CySC vs. GSC quantification is done when the molecular markers used do not define the surface of these cells (plasma membrane) and mark different cellular compartments (Tj is nuclear while Vasa is perinuclear or cytoplasmic). Why the changes reported in quantification are not apparent in the specific example images chosen for the figures is worrisome. I'm much more used to being able to clearly see what the authors are reporting in the images, and then using the quantification to illustrate the range of data observed and demonstrate statistical significance. For this reason, I'm very concerned about the strength and validity of the conclusions. In addition, while many different characteristics of the testis somatic and germline cells are analyzed, a general and consistent view of how ROS affects these cells is not presented. In particular, one of the principle conclusions, that ROS signaling in the CySCs affects ROS signaling in the GSCs, is not well-supported by the data presented.

Specific Comments:

In Figure 1, it is very difficult to identify where CySCs end and GSCs begin without using a cell surface marker for these different cell types. In addition, the methods for quantifying the mitochondrial distribution in GSCs vs. CySCs are very much unclear, and appear to rely on colocalization with molecular markers that are not in the same cellular compartment (Tj- nuclear vs Vasa-perinuclear and cytoplasmic), the reader has no way to determine the validity of the mitochondrial distribution. Similarly, the labeling with *gstD1*-GFP is also very much unclear - I see little to no GFP signal in either GSCs or CySCs in panels 1G-K. Lastly, while the expression of SOD in CySCs does increase the *gstD1*-GFP signal in CySCs, the effects on GSCs claimed by the authors are not apparent.

In Figure 2, while the cell composition of the niche region does appear to be different from controls when SOD1 is knocked down in the CySCs, at least in the example images shown in Figures 2A and B, how cell type is quantified in Figures 2E-G is very much unclear in the figure and methods. Are these counts of cells contacting the niche? If so, how was that defined? Or were additional regions away from the niche also counted and, if so, how were these regions defined?

In Figure 3, it is quite interesting that there is an increase in Eya⁺, differentiating cyst cells in SOD1 knockdown animals, and that these Eya⁺ cells appear closer to the niche than in controls. However, this seems at odds with the proliferation data presented in Figure 2, since Eya⁺ somatic cells do not normally divide at all. Are they suggesting that now differentiating cyst cells are proliferative? In addition, it is important for them to show example images of the changes in Socs36E and ptp61F expression.

Overall, the various changes in signaling are quite puzzling-while Jak/Stat signaling from the niche is reduced, hh signaling appears to be increased. Similarly, while the authors conclude that premature differentiation occurs close to the niche, EGF signaling, which occurs from germ cells to cyst cells during differentiation, is decreased. Many times these changes are contradictory, and the authors do not provide a suitable explanation to resolve these contradictions.

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Reviewer #2 (Public Review):

Summary:

In this work, the authors investigate the role of the Superoxide dismutase 1 (Sod1) enzyme, which acts to reduce the reactive oxygen species load, in the *Drosophila* testis. They show that the knockdown of Sod1 in somatic cells impacts stem cell numbers both autonomously in the soma and non-autonomously in the germline. Somatic stem cell numbers are increased, while germline stem cells are decreased and differentiate prematurely. The authors then show that in somatic Sod1 knockdowns, several signalling pathways are disrupted and that these may be responsible at least in part for the phenotypes observed. Finally, over-expression of Sod1 in the soma results in opposite phenotypes, suggesting that ROS levels do play a role in maintaining the balance between both stem cell populations in the testis.

Strengths:

The main strength of this work is to show a previously unappreciated role for Sod1 (and presumably by extension of ROS) in the *Drosophila* testis and in the regulation of stem cell self-renewal and differentiation. The authors use multiple readouts to show that the knockdown of Sod1 in the soma increases the number of somatic cells and also drives a non-autonomous, premature differentiation of germ cells. They also quantify the early differentiation of the germline using two different methods. Importantly, overexpression of Sod1 produces opposite phenotypes to knockdown, strengthening the conclusions.

Weaknesses:

Although the data presented are interesting, an important weakness of the manuscript as it currently stands is that many statements are not fully supported by the data. In particular, the authors do not provide any evidence of "cell redox-pairs" as indicated in the manuscript title, nor of intercellular redox gradients, as stated in several places throughout. While the data are consistent with non-autonomous regulation of ROS levels, this would not constitute a gradient. However, and crucially, there is no evidence provided to show that Sod1

manipulation in the soma is affecting ROS levels in the germline and that any of the phenotypes observed are a consequence of elevated ROS in the germline, rather than indirect effects caused by dysregulation of somatic self-renewal and differentiation, which is known to impact the germline. Indeed, there are many published reports of autonomous manipulations in the soma that influence either germline stem cell number (eg PMID: 19797664 among others) or differentiation (eg PMID: 17629483). The latter example is particularly relevant as the authors show altered somatic ERK levels, and the role of somatic ERK in promoting germ cell development is well established (PMID: 11048722, 11048723,...). Thus, whether Sod1 plays any non-autonomous role in controlling germ cell fate through ROS in the germline directly, or whether the phenotypes observed can all be explained by autonomous effects on somatic cell behaviour is debatable, but the experiments presented here do not distinguish between these two hypotheses. The only evidence presented by the authors for a non-autonomous role of Sod1 is the expression of a GFP reporter for *gstD1*. The quantifications and images are not clear and do not show unambiguously that this reporter is expressed in germ cells. Indeed, the quantifications show overlap between somatic and germline markers, suggesting that either the images themselves or the way they are quantified does not allow the authors to distinguish between the two cell types. Similarly, the claim that somatic mitochondria are enriched at the CySC-GSC interface and that this distribution maintains the redox balance in the niche is not supported by any experimental data. CySCs are extremely thin cells and much of the space is occupied by the nucleus (PMID: 114676), therefore it is likely that mitochondria would be enriched at the periphery. A careful analysis would be necessary to show that this enrichment is specific to the interface with GSCs. Moreover, no experiments are conducted to test whether mitochondrial distribution in CySCs has any impact on GSCs. Finally, no experiments are conducted to show definitively that the phenotypes observed upon Sod1 knockdown are indeed due to increased ROS, while this claim is made several times in the text. At present, the data presented here can support a role for Sod1 in somatic CySCs, but much more caution is required in attributing this to either ROS or intercellular ROS signaling. Therefore, several claims made in the title and throughout the text are not supported by evidence.

Besides this central point, there are other areas that should be improved. In particular, the data using the Fucci reporter to show accelerated proliferation do not appear convincing. It would seem that the proportions of cells in each phase are roughly similar, just that there are more cycling cells. A careful analysis of these results would distinguish between these two and determine whether Sod1 knockdown simply impairs differentiation (and therefore results in more somatic cells proliferating) or whether it speeds up the cell cycle (resulting in an increased mitotic index as suggested, but this requires a ratio to be shown). Similarly, several quantifications are not clearly explained, making it hard to understand what is being measured. As an example, while the decrease in pERK in CySCs is clear from the image and matched in the quantification, the increase in cyst cells is not apparent from the fire LUT used. The change in fluorescence intensity therefore may be that more cells have active ERK, rather than an increase per cell (similar arguments apply to the quantifications for p4E-BP or Ptc). Therefore, it is hard to know whether Sod1 knockdown results in increased or decreased signaling in individual cells.

Impact of study:

Demonstrating intercellular communication through ROS and its importance in maintaining the balance between two stem cell populations would be a finding of interest to a broad field. However, it remains to be demonstrated that this is the case, and given this, this study will have a limited impact.

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Reviewer #3 (Public Review):**Summary:**

The authors want to prove that there is a redox potential between germline stem cells (GSCs) and somatic cyst stem cells (CySCs) in the *Drosophila* testis, with ROS being higher in the former compared to the latter. They also want to prove that ROS travels from CySCs to GSCs. Finally, they begin to characterize the phenotypes caused by loss of SOD (which normally lowers ROS levels) in the *tj*-lineage and how this impacts the germline.

Strengths:

The role of SOD in somatic support cells is an under-explored area.

Weaknesses:

The authors fall short of accomplishing their goals. There are issues with the concept of the paper (ROS gradient between cells that causes a transfer of ROS across membranes for homeostasis), the data, the figures, and the scholarship of the testis. I have discussed each of the points in detail below. These weaknesses negatively impact the conclusions put forward by the authors. In short, their data is not compelling: there is no evidence provided by the authors that ROS diffuses from CySCs to GSCs as most of the claims about stem cells are founded on data about differentiating germ and somatic cells. The somatic SOD depletion phenotype is incompletely characterized and several pathways appear to change in these cells, including reduced *Egfr* signaling, increased *Tor* signaling, and increased *Hh* signaling. None of these results are sufficiently followed up on. And none of them are considered relative to their known roles in the testis. For example, high *Hh* signaling in CySCs increases their competitiveness with GSCs. Increased *Tor* signaling in all CySCs does not affect the CySC lineage. Reduced *Egfr* signaling in CySCs reduces the number of CySCs and reduces/inhibits abscission between GSCs-gonialblasts.

Major issues:**(1) Data**

- a. Problems proving which mitochondria are associated with which lineage.
- b. There is no evidence that ROS diffuses from CySCs into GSCs.
- c. The changes in *gst-GFP* (redox readout) are possibly seen in differentiating germ cells (i.e., spermatogonia) but not in GSCs. This weakens their model that ROS in CySC is transferred to GSCs.
- d. Most of the paper examines the effect of SOD depletion (which should increase ROS) on the CySC lineage and GSC lineage. One big caveat is that *tj-Gal4* is expressed in hub cells (Fairchild, 2016) so the loss of SOD from hub cells may also contribute to the phenotype. In fact, the niche in Figure 2D looks larger than the niche in the control in Figure 2C, arguing that the expression of *Tj* in niche cells may be contributing to the phenotype. The authors need to better characterize the niche in *tj>SOD-RNAi* testes.
- e. The *tj>SOD-RNAi* phenotype is an expansion of the *Zfh1+* CySC pool, expansion of the *Tj+ Zfh1-* cyst cells (both due to increased somatic proliferation) and a non-autonomous disruption of the germline.
- f. I am not convinced that MAPK signaling is decreased in *tj>SOD-i* testes. Not only is this antibody finicky, but the authors don't have any follow-up experiments to see if they can restore SOD-depleted CySCs by expressing an *Egfr* gain of function. Additionally, reduced *Egfr* activity causes fewer somatic cells (not more) (Amoyel, 2016) and also inhibits abscission between GSCs and gonialblasts (Lenhart 2015), which causes interconnected cysts of 8- to 16 germ cells with one GSC emanating from the hub.
- g. The increase in *Hh* signaling in SOD-depleted CySCs would increase their competitiveness against GSCs and GSCs would be lost (Amoyel 2014). The authors need to validate that *Hh*

protein expression is indeed increased in SOD-depleted CySCs/cyst cells and which cells are producing this Hh. Normally, only hub cells produce Hh (Michel, 2012; Amoyel 2013) to promote self-renewal in CySCs.

h. The increase in p4E-BP is an indication that Tor signaling is increased, but an increase in Tor in the CySC lineage does not significantly affect the number of CySCs or cyst cells (Chen, 2021). So again I am not sure how increased Tor factors into their phenotype.

i. The over-expression of SOD in CySCs part is incomplete. The authors would need to monitor ROS in these testes. They would also need to examine with *tj*>SOD affects the size of the hub.

(2) Concept

Why would it be important to have a redox gradient across adjacent cells? The authors mention that ROS can be passed between cells, but it would be helpful for them to provide more details about where this has been documented to occur and what biological functions ROS transfer regulates.

(3) Issues with scholarship of the testis

a. Line 82 - There is no mention of BMPs, which are the only GSC-self-renewal signal. Upd/Jak/STAT is required for adhesion of GSCs to the niche but not self-renewal (Leatherman and Dinardo, 2008, 2010). The author should read a review about the testis. I suggest Greenspan et al 2015. The scholarship of the testis should be improved.

b. Line 82-84 - BMPs are produced by both hub cells and CySCs. BMP signaling in GSCs represses bam. So it is not technically correct to say the CySCs repress bam expression in GSCs.

c. Throughout the figures the authors score Vasa+ cells for GSCs. This is technically not correct. What they are counting is single, Vasa+ cells in contact with the niche. All graphs should be updated with the label "GSCs" on the Y-axis.

(4) Issues with the text

a. Line 1: multi-lineage is not correct. Multi-lineage refers to stem cells that produce multiple types of daughter cells. GSCs produce only one type of offspring and CySCs produce only one type of offspring. So both are uni-lineage. Please change accordingly.

b. Lines 62-75 - Intestinal stem cells have constitutively high ROS (Jaspar lab paper) so low ROS in stem cell cells is not an absolute.

c. Line 79: The term cystic is not used in the *Drosophila* testis. There are cyst stem cells (CySCs) that produce cyst cells. Please revise.

d. Line 90 - perfectly balanced is an overstatement and should be toned down.

e. Line 98 - division of labour is not supported by the data and should be rephrased.

f. Line 200 - the authors provide no data on BMPs - the GSC self-renewal cue - so they should avoid discussing an absence of self-renewal cues.

(5) Issues with the figures

a. The images are too small to appreciate the location of mitochondria in GSCs and CySCs.

b. Figure 1

i. cell membranes are not marked, reducing the precision of assigning mitochondria to GSC or CySCs. It would be very helpful if the authors depleted ATP5A from GSCs and showed that the puncta are reduced in these cells and did a similar set of experiments for the *tj*-Gal4 lineage. It would also be very helpful if the authors expressed membrane markers (like myr-GFP) in the GSC and then in the CySC lineage and then stained with ATP5A. This would pinpoint in which cells ATP5A immunoreactivity is occurring.

ii. The presumed changes in *gst*-GFP (redox readout) are possibly seen in differentiating germ cells (i.e., spermatogonia) but not in GSC.

iii. Panels F, Q, and S are not explained and currently are irrelevant.

c. Figure 3K - The evidence to support less Ecad in GSCs in *tj*>SOD-i testes is not compelling as the figure is too small and the insets show changes in Ecad in somatic cells, not GSC.

d. Figure 4:

- i. Panel A, B The apparent decline (not quantified) may not contribute to the phenotype.
- ii. dpERK is a finicky antibody and the authors are showing a single example of each genotype. This is an important experiment because the authors are going to use it to conclude that MAPK is decreased in the *tj>SOD-i* samples. However, the authors don't have any positive (dominant-active *Egfr*) or negative (*tj>mapk-i*). As is standing the data are not compelling. The graph in F does not convey any useful information.
- e. Figure S1D - cannot discern green on black. It is critical for the authors to show monochromes (gray scale) for the readouts that they want to emphasize. I cannot see the green on black in Figure S1D.
- f. Figure S4 - there is no quantification of the number of Tj cells in K-N.

(6) Issues with Methods

- a. Materials and Methods are not described in sufficient depth - please revise.
- b. Note that *tj-Gal4* has real-time expression in hub cells and this is not considered by the authors. The ideal genotype for targeting CySCs is *tjGal4, Gal80TS, hh-Gal80*. Additionally, the authors do not mention whether they are depleting throughout development into adulthood or only in adults. If the latter, then they must have used a temperature shift like growing the flies at 18C and then upshifting to 25C or 29C during adult stages.
- c. The authors need to show data points in all of the graphs. Some graphs do this but others do not.
- d. The authors state that all data points are from three biological replicates. This is not sufficient for GSC and CySC counts. Most labs count GSCs and CySCs from at least 10 testes of the correct genotype.

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