

SOD2, the principal scavenger of mitochondrial superoxide, is dispensable for embryogenesis and imaginal tissue development but essential for adult survival

Subhas Mukherjee, Renee Forde, Amy Belton[†] and Atanu Duttaroy*

Biology Department; Howard University; Washington, DC USA

[†]Present address: Johns Hopkins University Medical School; Baltimore, MD USA

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Abbreviations: MnSOD, manganese superoxide dismutase; ROS, reactive oxygen species

Definitive evidence on the impact of MnSOD/SOD2-deficiency and the consequent effects of high flux of mitochondrial reactive oxygen species (ROS) on pre-natal/pre-adult development has yet to be reported for either *Drosophila* or mice. Here we report that oocytes lacking maternal SOD2 protein develop into adults just like normal SOD2-containing oocytes suggesting that maternal SOD2-mediated protection against mitochondrial ROS is not essential for oocyte viability. However, the capacity of SOD2-null larvae to undergo successful metamorphosis into adults is negatively influenced in the absence of SOD2. We therefore determined the impact of a high superoxide environment on cell size, progression through the cell cycle, cell differentiation and cell death and found no difference between SOD2-null and SOD2⁺ larva and pupa. Thus loss of SOD2 activity clearly has no effect on pre-adult imaginal tissues. Instead, we found that the high mitochondrial superoxide environment arising from the absence of SOD2 leads to the induction of autophagy. Such autophagic response may underpin the resistance of pre-adult tissues to unscavenged ROS. Finally, while our data establish that SOD2 activity is less essential for normal development, the mortality of *Sod2*^{-/-} neonates of both *Drosophila* and mice suggests that SOD2 activity is indeed essential for the viability of adults. We therefore asked if the early mortality of SOD2-null young adults could be rescued by activation of SOD2 expression. The results support the conclusion that the early mortality of SOD2-null adults is largely attributable to the absence of SOD2 activity in the adult per se. This finding somewhat contradicts the widely held notion that failure to scavenge the high volume of superoxide emanating from the oxidative demands of development would be highly detrimental to developing tissues.

Introduction

The growth and development of multicellular eukaryotes is fueled by oxygen (O₂)-dependent high-energy metabolism. Indeed, the extent to which aerobic organisms go to insure adequate oxygen supply to developing young attests to the host of injurious effects that can arise from oxygen deprivation during development. Ironically, the pathways of oxygen utilization in mitochondrial respiration and oxidative phosphorylation also generate copious amounts of the unstable superoxide radical¹ which is rapidly and spontaneously reduced to other reactive oxygen species (ROS) of which some are highly toxic and cause irreparable cellular damage. Accretion of such unrepaired ROS-mediated cellular damage is causally implicated in many human diseases and in processes linked to organismal aging.²⁻⁵ Moreover, accumulation of ROS-damaged mitochondria puts the organism at a much

higher risk for a variety of pathophysiological conditions and for reduced cognitive and physiological functions underpinning overall fitness.^{6,7}

Much of what we know about the role of ROS in multicellular eukaryotes has come from studies of adult-stage organisms. Some of the most informative of these studies have focused on the phenotypic consequences of genetic deficiency for enzymes that function to detoxify specific ROS. For example, genetic ablation of SOD2, a manganese-containing superoxide dismutase and the principal scavenger of mitochondrial superoxide, negatively affects the survival of organisms as diverse as yeast,^{8,9} *Drosophila*^{10,11} and mice.^{12,13} SOD2-deficient pups from SOD2 mutant strains of mice present a multitude of cell and tissue damage culminating in complete neonatal mortality.^{12,13} Importantly, present evidence suggests that the lack of SOD2 does not overtly affect the prenatal health or viability of *Sod2*^{-/-} mouse embryos

*Correspondence to: Atanu Duttaroy; Email: aduttaroy@howard.edu
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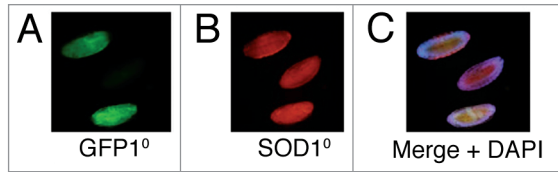


Figure 1. Maternal transmission of SOD2 protein. Shown here are three developing embryos obtained from a heterozygous *Sod2^{n283/+}* mother. (A) Two of the three embryos express GFP by virtue of a GFP-marked, *Sod2⁺* Balancer chromosome. The GFP negative embryo should be *SOD2-null* (*Sod2ⁿ²⁸³/Sod2ⁿ²⁸³*). (B) The same embryos stained with anti-SOD2 antibody (red). The presence of SOD2 protein in the *Sod2ⁿ²⁸³/Sod2ⁿ²⁸³* embryo, indicates maternal transmission from the *SOD2^{n283/+}* mother. (C) Merge with DAPI (blue). Genetic constructs are described in the Materials and Methods section.

when compared to their SOD2-positive (*Sod2^{+/+}*, *Sod2^{+/-}*) littermates.¹³

Likewise, SOD2-null mutants of *Drosophila* exhibit complete adult mortality within a day of pupal eclosion.¹⁰ However, definitive evidence on the impact of SOD2-deficiency on pre-natal/pre-adult development has yet to be reported for either *Drosophila* or mice. Here we report data, which lead to the conclusion that pre-adult development in *Drosophila* is less susceptible to high ambient mitochondrial superoxide arising from the complete absence of SOD2. These results, together with the mammalian data, above, allow us to hypothesize that while SOD2-mediated scavenging of mitochondrially-generated superoxide is in fact required for post-natal (post-eclosion) survival in animal taxa as widely divergent as mammals and holometabolous insects, its function is less required for normal prenatal (pre-eclosion) development. If confirmed, this hypothesis would serve to identify an important transition between pre-and post-natal (post-eclosion) development in animals generally in the generation and/or metabolism ROS.

Results

Maternally contributed SOD2 is not essential for embryogenesis. Earlier studies showed that *Drosophila* oocytes normally accumulate a large store of *Sod2* transcripts.¹⁴ To investigate whether embryos contain SOD2 protein and if so, whether it is of maternal vs. embryonic origin, we used a SOD2-specific antibody to identify SOD2 protein in *Sod2^{-/-}* and *Sod2^{+/-}* embryos produced from *Sod2^{+/-}* females. **Figure 1** shows the presence of copious amounts of SOD2 protein in both *Sod2^{-/-}* and *Sod2^{+/-}* embryos, indicating that some if not all of the SOD2 protein in embryos is of maternal origin. A plausible explanation for the stockpiling of SOD2 by oocytes is to provide protection from oxidative damage until the zygotic transcription of *Sod2* begins. To test this possibility, we made germ cell (oocyte) clones devoid of maternal SOD2 and followed the development of these eggs to adulthood (**Fig. 2**). To our surprise, oocytes lacking maternal SOD2 protein develop into adults and the recovery of a large number of *Sod2ⁿ²⁸³* adult animals (total of 67) proves this point beyond any doubt (**Fig. 2C**). We therefore conclude that maternal

SOD2-mediated protection against mitochondrial ROS is not essential during the process of oocyte development.

We then asked how long maternal SOD2 actually persists during development. By western analysis, we are unable to detect maternal SOD2 from the third larval instar onwards (**Fig. 3**). We then determined the capacity of SOD2-null larvae to undergo successful metamorphosis into adults. Interestingly, complete lack of SOD2 has some influence on metamorphosis as evident from the failure of significant number of *Sod2ⁿ²⁸³* pupae to turn into adult flies (**Table 1**). However, *Sod2^{n283/+}* heterozygotes show no significant influence on metamorphosis (**Table 1**).

Development and differentiation are not perturbed in high ROS environment. We then asked if we could find evidence of cell or tissue damage, which although perhaps insufficient to hinder development, would be evidence of ROS-mediated pathology. Dihydroxy ethidium (DHE) is an efficient and selective detector of superoxide in vivo, which acts by emitting a red fluorescence in the presence of superoxide.¹⁵ DHE staining of fat body, brain and eye discs tissues revealed that steady-state levels of tissue superoxide are significantly enhanced in SOD-null larva and pupa as compared to the controls (**Fig. 4A–D**).

To evaluate the pathological potential of this high superoxide environment in vivo, we compared tissues from SOD2-null and SOD2⁺ larvae and pupae using four different parameters of cell size, cell growth and differentiation, cell death and autophagy. To determine the impact of a high superoxide environment on cell size, we used phalloidin staining of fat body from SOD2-null and SOD2⁺ larva (**Fig. 4E and F**) and pupa: no apparent difference in cell size was detected. To determine the effect on cell growth and differentiation, we used *Drosophila* eye disc tissue, which develops from 6–23 epithelial cells that are set aside at the cellular blastoderm stage. These disc mother cells proliferate to form up to 9,700 cells by late third instar larval stage and give rise to all ommatidial precursors.¹⁶ The proliferative capacity of ommatidial cells from SOD2-null and SOD2⁺ larvae was compared following BrdU incorporation. Our data indicate that neither the progression of the cell cycle nor the cell differentiation events are affected by the absence of SOD2 (**Fig. 4G and H**). In addition to the eye disc we also compared cell proliferation in the brain and found no effect of the lack of SOD2 on brain cell proliferation (not shown).

We then looked for an association between the high superoxide environment in tissues of SOD2-null larvae and in situ cell death using two well defined programmed cell death reporters for *Drosophila*: breakdown of nuclear Lamin protein¹⁷ and activation of cellular caspase activity.¹⁸ Fat body cells obtained from SOD2-null larvae show no signs of nuclear lamin degradation; the lamin signal appeared as a well-defined halo around the nuclei similar to the control (**Fig. 4I and J**). With regard to caspase activation, the cell death effector gene *hid⁹* causes extensive cell death, therefore many cells appeared positive for active caspase (**Fig. 4K**). Some natural caspase activity appears in the developing eye disc of *Drosophila* in a temporally restricted pattern.¹⁸ SOD2-null eye discs exhibit a level of caspase activity equivalent to the controls, thereby indicating no additional cell death activity arising from the absence of SOD2 (**Fig. 4L and M**). In similar experiments, no additional caspase activity was seen in the *Sod2*-null brain

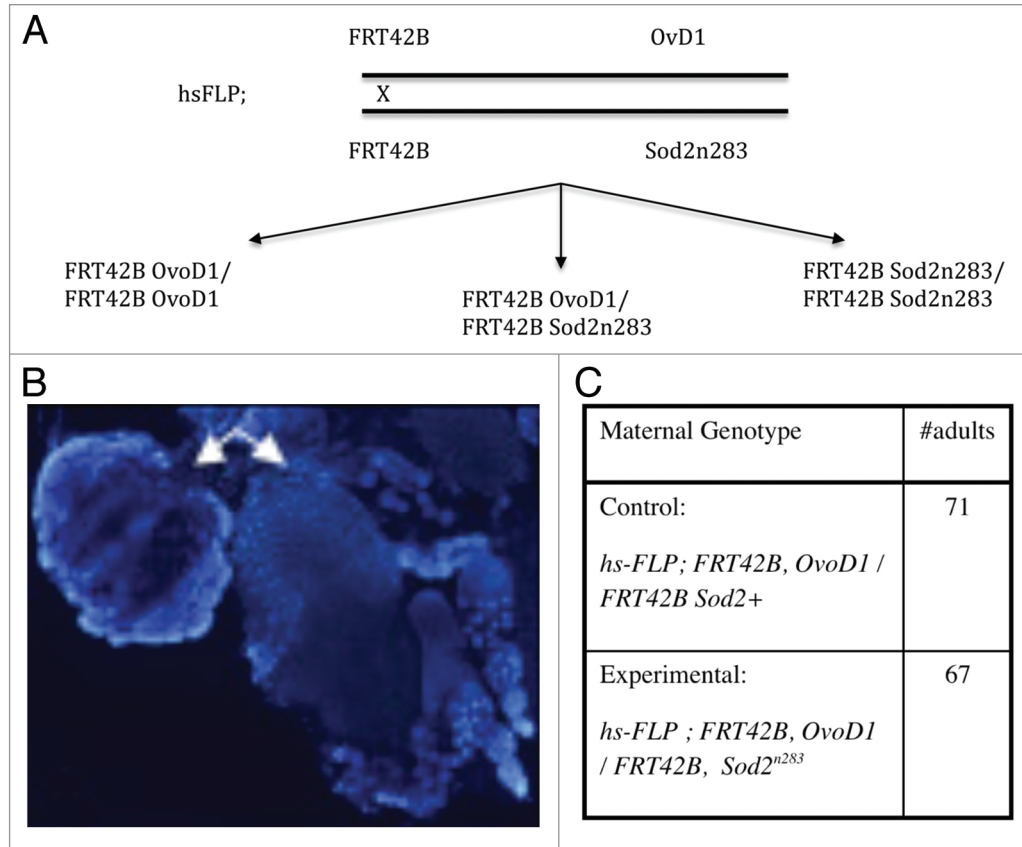


Figure 2. Making SOD2-null oocytes. (A) Mitotic recombination was induced in premeiotic germ cells using a standard FLP-based recombination system. (B) Success of the recombination system is demonstrated when ovarioles on the left appear dystrophic and carry no functional eggs due to the presence of the dominant *OvoD1* mutation. Ovarioles on the right are (*Sod2ⁿ²⁸³/Sod2ⁿ²⁸³*) and grow normally within a fully formed gerarium. Ovaries are stained with DAPI. (C) Maternal genotypes indicate that if adult animals appeared from the experimental mother then they must arise from the *Sod2ⁿ²⁸³* eggs, since the *OvoD1* carrying eggs will degenerate. That sufficient (67) adult progeny were recovered from the *Sod2ⁿ²⁸³* eggs indicates that absence of SOD2 in the oocytes does not impede fertilization and subsequent development to adulthood. Beyond this, the actual numbers of adult animals recovered in the control (71) and the experimental (67) crosses bear no quantitative significance.

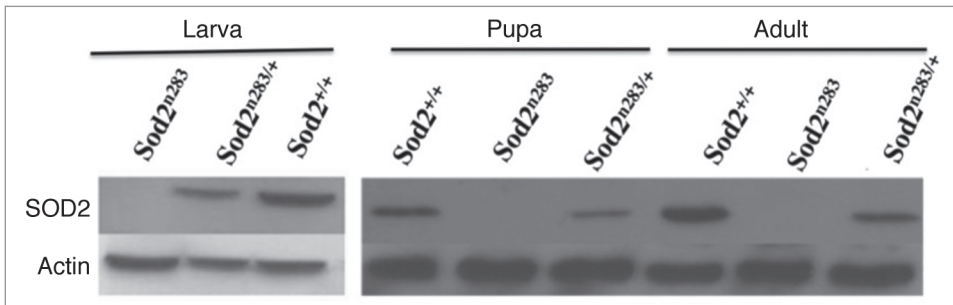


Figure 3. Developmental loss of maternal SOD2. Western analysis with anti-SOD2 antibody shows that maternal SOD2 does not persist into the third larval instar onwards in *Sod2ⁿ²⁸³* progeny derived from a *Sod2ⁿ²⁸³/CyO, GFP* stock.

Table 1. Pre-adult development in the absence of SOD2

	Larvae			Pupae			Adults		
	Dead	Alive	Total	Dead	Alive	Total	Dead	Alive	Total
KG0654R (Control)	0	250	250	0	250	250	23	227	250
<i>Sod2ⁿ²⁸³/CyO</i>	0	250	250	4	246	250	33	213	246
<i>Sod2ⁿ²⁸³</i>	0	250	250	16	234	250	37	197	234

At 95% confidence interval significantly less number of adults emerged out from *Sod2ⁿ²⁸³* pupae compared to KG08654R control ($\chi^2 = 4.87, p = 0.05$). However, no significant difference in adult metamorphosis was noted between the KG08654 and *Sod2ⁿ²⁸³/CyO* ($\chi^2 = 2.199, p = 0.05$) and between the *Sod2ⁿ²⁸³/CyO* and *Sod2ⁿ²⁸³* ($\chi^2 = 0.553, p = 0.05$).

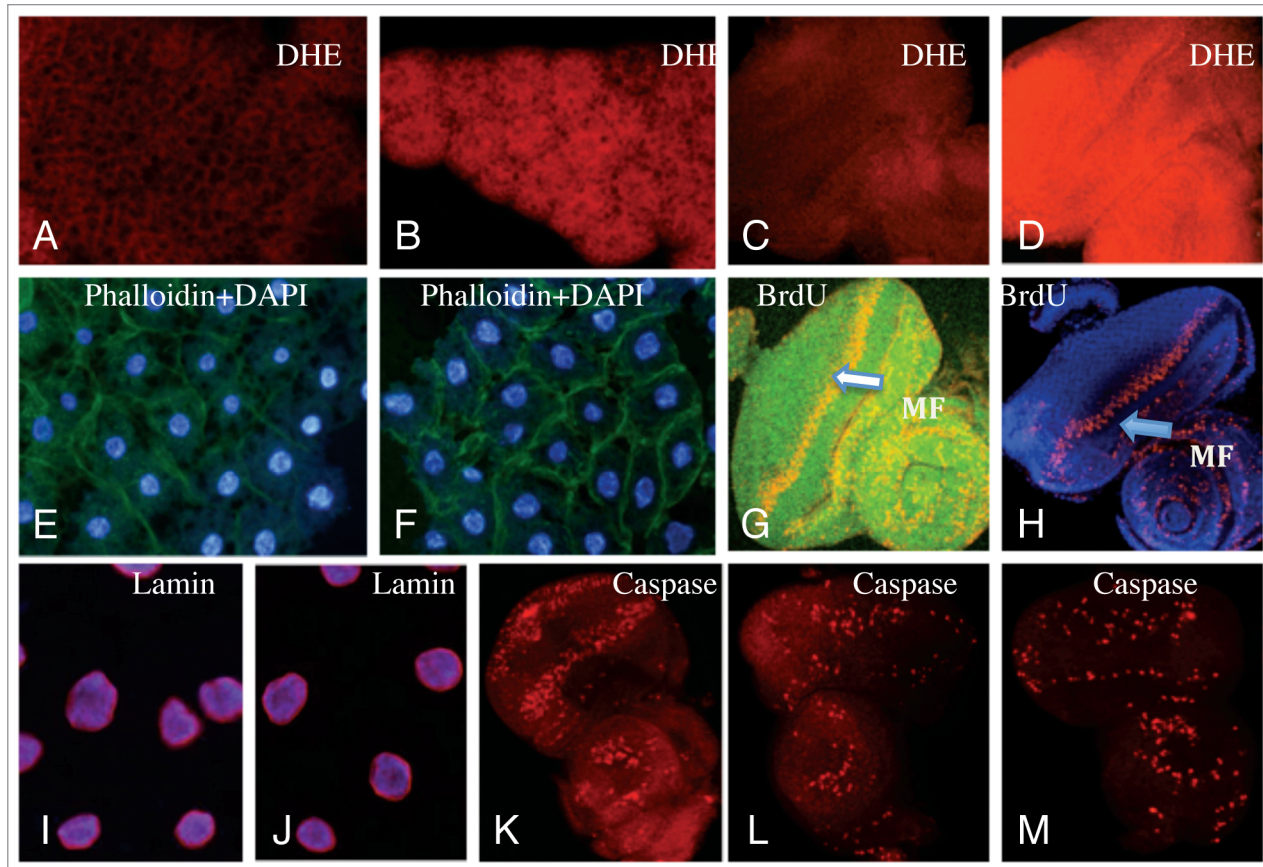


Figure 4. Assessment of superoxide content and tissue damage in SOD2-null larvae. (A–D) DHE staining of developing fat body (A and B) and eye disc (C and D) from *Sod2ⁿ²⁸³* larvae indicates high steady-state levels of superoxide compared to *Sod2⁺* controls. (A and C) KG06854R (*Sod2⁺*); (B and D) *Sod2ⁿ²⁸³*. Phalloidin staining, which demarcates cell boundaries, shows similar cell sizes in *Sod2ⁿ²⁸³* (E) and *Sod2⁺* control (F). BrdU incorporation, which identifies the S-phase of the cell cycle, demonstrates that cell cycle progression in the morphogenetic furrow (arrow heads) appears normal in *Sod2⁺* control (G) and *Sod2ⁿ²⁸³* eye discs (H). Induction of apoptosis. The nuclear lamin, which appears as a purple halo around the nucleus, remains intact in *Sod2ⁿ²⁸³* (J) compared to the *Sod2⁺* control (I). The induction of apoptosis by activation of the *hid* gene expression in the eye disc generates an abundance of caspase positive cells as shown by the use of an activated caspase antibody (K). The same activated caspase antibody picks up only a few caspase positive cells in *Sod2ⁿ²⁸³* (M), which is no more than in the *Sod2⁺* control (L).

(not shown). In summary, our data suggests that the high superoxide environment of SOD2-null tissues does not promote cell death activity in developing tissues.

Unscavenged mitochondrial superoxide promotes autophagy. Stress conditions such as starvation or oxidative stress are known to promote autophagy,²⁰⁻²⁴ a process that also happens naturally during metamorphosis.^{25,26} Autophagy, which is mediated through lysosomes, is fundamentally a catabolic process for eliminating or recycling damaged macromolecules within cells.²⁷⁻³⁰ We anticipated that the high flux of superoxide radicals might cause protein damage and consequently autophagic induction in *Sod2ⁿ²⁸³*. Upon induction of autophagy, lysosomal vesicles and autophagosomes appear as punctate structures in the cytoplasmic compartment when stained with lysotracker, a lysosome specific fluorescent dye.²³ We used lysotracker staining to monitor induction of autophagy in SOD-null tissue. Fat body cells from SOD2-null larvae show marked accumulation of lysosomal puncta compared to the SOD2⁺ control where fewer autophagic vesicles are evident (Fig. 5A and B). To further establish this point, *Sod2^{-/-}* cell clones were generated in a background of *Sod2^{+/-}* cells in the

fat body tissue by mitotic recombination. LysoTracker staining shows that the lysosomal puncta are restricted to the *Sod2^{-/-}* clone (Fig. 5C and D) confirming that high levels of unscavenged mitochondrial superoxide lead to autophagy.

SOD2 activity is essential for adult viability. While the above data establish that lack of SOD2 activity has only a limited effect on developmental processes, it is equally well established from the premature deaths of *Sod2^{-/-}* neonates that SOD2 activity is essential for adult viability. In principle, the inviability of SOD2-null adults could originate either from (a) latent damage to imaginal tissues arising from inadequate superoxide scavenging during larval and pupal life, or from (b) a vital requirement for SOD2 activity that arises within and is confined specifically to post-eclosion adults. Observations presented above already eliminated the first possibility. In order to investigate the second possibility we activated ubiquitous expression of SOD2 in newly-eclosed SOD2-null (*Sod2ⁿ²⁸³*) adults. Using a *Tubulin-P[Switch] GAL4* driver, GAL4-dependent expression of a UAS-linked *Sod2⁺* transgene was induced by feeding the *P[Switch]* activator, RU486 (Fig. 6A and B) and the expression of SOD2 protein in

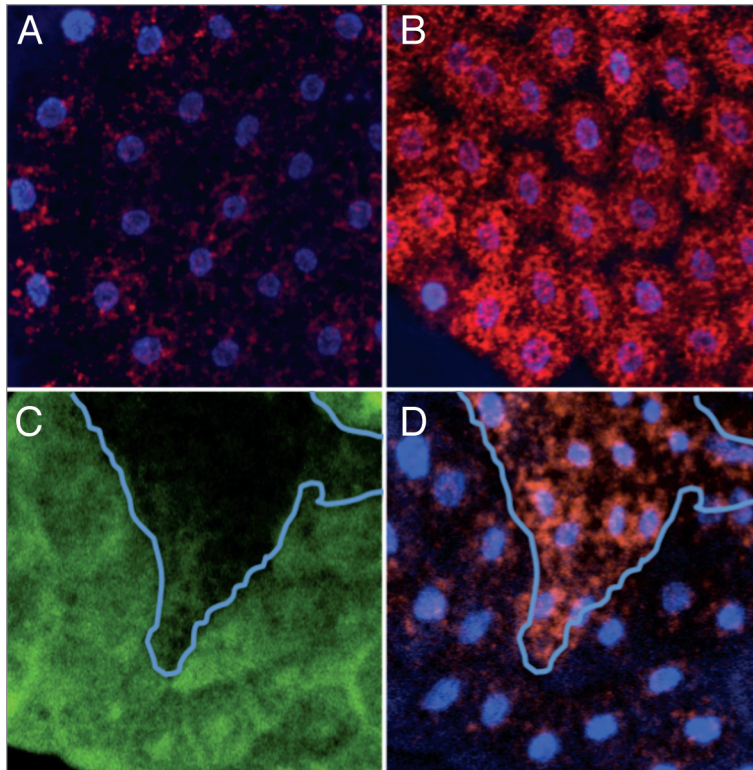


Figure 5. Absence of SOD2 induces autophagy. Lysotracker staining shows few lysosomal puncta in the control fat body cells from third instar larvae (A) where as numerous lysotracker positive cells appear in the *Sod2ⁿ²⁸³* (B). *Sod2ⁿ²⁸³* cell clones (GFP negative area) are induced by mitotic recombination (C). Lysotracker positive cells appear heavily in *Sod2ⁿ²⁸³* cell clones (D) suggesting that absence of SOD2 induces autophagy.

Sod2ⁿ²⁸³; Tubulin-P[Switch]/UAS-SOD2 adults was confirmed (Fig. 6D). Activation of SOD2 expression in *Sod2ⁿ²⁸³* adults improves their survival compared to the un-induced controls of the same genotype (Fig. 6C). On average, *Sod2ⁿ²⁸³; Tubulin-P[Switch]GAL4/UAS-SOD2* adults survive up to five days (mean life span), with a maximum survival up to eight days (Fig. 6C). This is a marked improvement over the survival of *Sod2ⁿ²⁸³* with mean and maximum life spans of 0.5 and one day, respectively.¹⁰ This data supports the conclusion that adult viability is dependent on SOD2 activity in the adult per se. It is worth noting that activation of SOD2 expression in *Sod2ⁿ²⁸³* adults does not fully restore normal lifespan. One possible reason could be that SOD2 is not completely non-essential during metamorphosis, which is now documented (Table 1). The other reason may be due to the inadequacy of the *P[Switch]* system to fully restore SOD2 activity during acute demand or due to the possible contribution of injury in pre-adult imaginal tissues is not discernible from this data. Clearly, SOD2 activity in the post-eclosion adult per se is required for normal adult lifespan.

To investigate this point further, we used the gene switch system in conjunction with *Sod2RNAi*¹¹ to inactivate the expression of SOD2 in *Sod2⁺* adults. Activation of an *UAS-SOD2IR* transgene with the *Tubulin-P[Switch]GAL4* driver in newly-eclosed adults produces a sharp reduction in SOD2 expression

(Fig. 6F) and a precipitous early adult mortality equivalent to that seen in SOD2-null mutant adults (Fig. 6E). Together, these manipulations of SOD2 expression support the conclusion that the early mortality of SOD2-null adults is largely if not fully attributable to the absence of SOD2 activity in the adult per se and definitely not to the latent effect of the lack of SOD2 activity in pre-adult imaginal tissues.

We believe that the feeding-dependent mechanism of GeneSwitch induction remains the main obstacle toward a complete rescue of *Sod2ⁿ²⁸³* life span because a critical but unavoidable window of non-protection will always exist between eclosion, feeding and activation of SOD2 expression. We therefore attempted to circumvent this issue by reducing the ambient oxygen level for *Sod2ⁿ²⁸³; Tubulin-P[Switch]GAL4/UAS-SOD2* flies which were simultaneously fed with RU486. The hypoxic condition by itself has little impact on the survival of *Sod2ⁿ²⁸³* adults (Fig. 6) same as pointed out earlier by Wicks et al.³¹ but following the activation of SOD2 with RU486, these flies can now live up to 25 days (average life span) under hypoxic environment, presumably due to reduced oxygen metabolism (Fig. 6). This observation further reinforces that the amount of ROS production can directly influence the adult life span.

Discussion

The presumed importance of the antioxidant protection system in the oocyte as well as during embryonic development has not been examined carefully with molecular genetic tools. Existing information suggests that mammalian embryos generate little mitochondrial ROS and therefore less oxidative stress, until mitochondrial oxidative phosphorylation begins at the blastula stage when embryos switch to high energy metabolism from a low energy state.^{32,33} Through the use of genetic manipulation, we demonstrate here that SOD2 depleted oocytes are viable, can be fertilized, undergo embryogenesis and complete development. If the same is true for mammalian oocytes, then our observations clarify how a transplanted SOD2^{-/-} ovary in a *Sod2^{+/+}* mouse host can give rise to viable progeny.³⁴ We are therefore left with an enigma: the high abundance SODs in *Drosophila* oocytes and by extension in murine oocytes, appear to be completely dispensable for normal development.^{14,35-37}

We then inquired how postembryonic development reacts to a high level of unscavenged mitochondrial ROS. Lack of SOD2 activity in *Sod2ⁿ²⁸³* leaves plenty of unscavenged ROS in post-embryonic tissues yet no overt cellular damage could be identified. Because tissue replenishment is a general characteristic of Dipteran insect metamorphosis,⁴⁸ it could be argued that in *Sod2ⁿ²⁸³* ROS-damaged tissues are replenished during the process of metamorphosis thereby enabling their survival. That we found no net difference in damage between tissues that undergo histolysis (like the fat body) and the non-replaceable tissues such as the eye and the brain argues against this mechanism. So, we looked

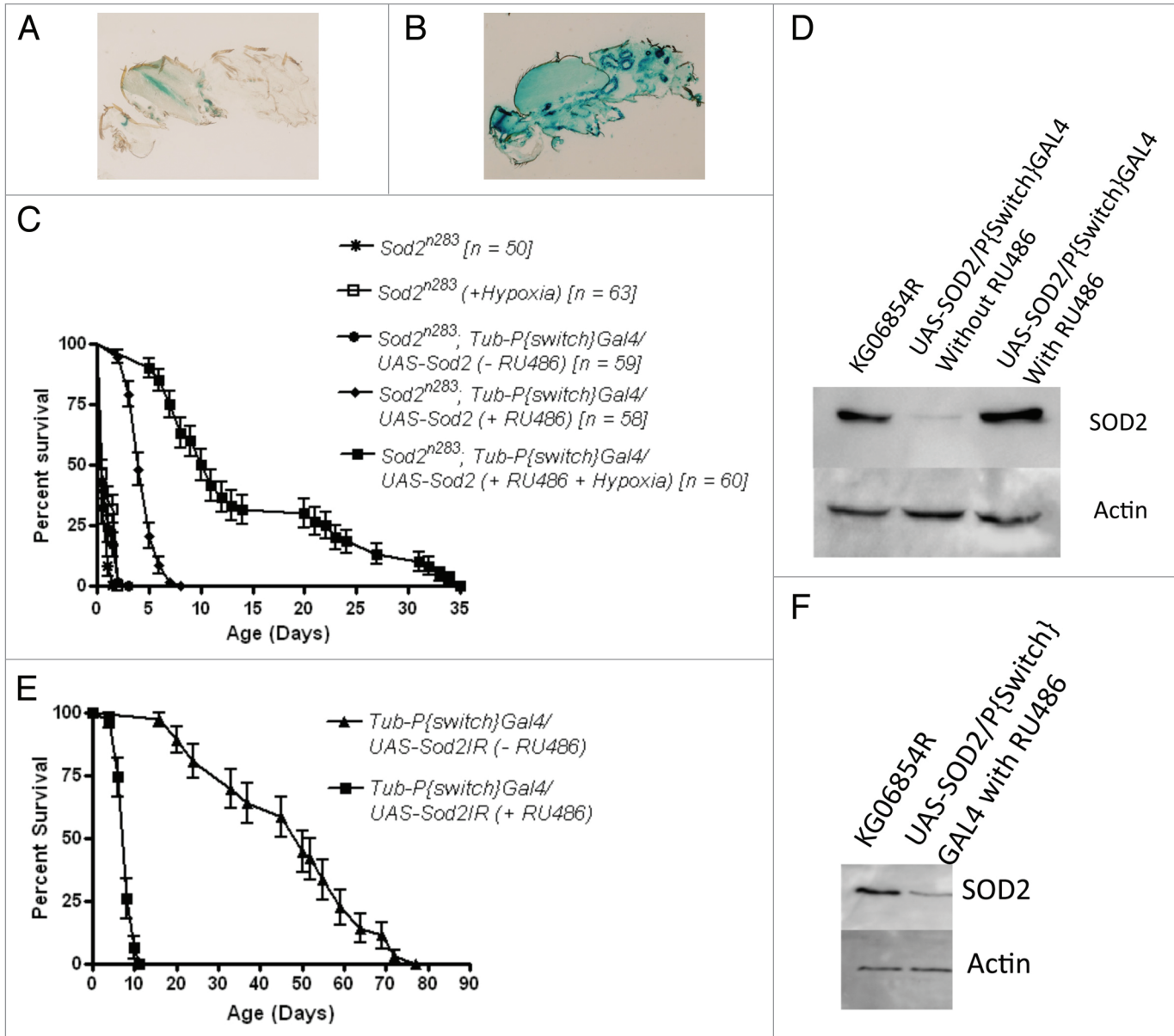


Figure 6. Activation of SOD2 expression in *Tub P[Switch]GAL4/UAS-Sod2* in *Sod2ⁿ²⁸³* adults improves survival. (A) Background levels of LacZ reporter without RU486 feeding of *Tub P[Switch]GAL4/UAS-LacZ* flies and (B) induction of lacZ expression in all tissues following RU486 feeding demonstrates the adult restricted expression of the P[Switch] element. (C) Activation of SOD2 expression in *Sod2ⁿ²⁸³* with the *Tub P[Switch]* driver extends the mean adult life span of these flies from less than 24 hrs to about five days. Further extension of *Sod2ⁿ²⁸³* adult survival is possible by keeping the SOD2 activated flies under hypoxia (5% O₂). These flies now survive up to 21 days (mean life span). Neither hypoxia nor RU486 feeding alone significantly influences the survival of *Sod2ⁿ²⁸³* adults. (D) Evidence from western blot analysis of SOD2 expression in *Sod2ⁿ²⁸³* following RU486 feeding. (E) Suppression of SOD2 expression in adults by activating a UAS-SOD2IR (*Sod2RNAi*) with the *Tub P[Switch]GAL4* driver leads to a significant reduction in adult life span. (F) Significant suppression of SOD2 expression in *Sod2IR*.

for some means of tissue protection, which led to the finding of disproportionate autophagy in *Sod2ⁿ²⁸³* larval tissues. In light of our data, we consider it the possibility that ROS-mediated induction of autophagy provides protection of larval tissues against oxidative damage as has been shown in other experimental systems.³⁸ While prior studies have indicated a possible connection between autophagy and oxidative stress^{20,24} here we show that autophagy is indeed induced in pre-adult tissue in vivo in response to a high ROS environment. Thus, *Sod2ⁿ²⁸³* could offer

a model for exploring the cellular mechanism of oxidative stress-mediated autophagic induction.

According to this model, SOD2 protection is clearly essential for adult viability. To examine this possibility further, we activated SOD2 expression in newly eclosed *Sod2ⁿ²⁸³* adults and observed a modest improvement in survival. The failure to observe more robust improvement in adult viability could reflect limitations in the GeneSwitch system (e.g., lag in feeding response time) or it could reflect in vivo limitations of the model itself.

In terms of the biological efficacies of free radicals in vivo, an interesting corollary is emerging between the protection offered by SOD2 and a second parallel pathway for ROS protection system in *Drosophila*, which consists of Thioredoxin reductase and GSH. Loss of either of these later enzymes causes severe developmental defects both in mice and *Drosophila* alike.³⁹⁻⁴² One can also include in this list the complexI mutant that is known to cause high ROS production leading to serious developmental defects.¹⁵ Why then loss of SOD2 is principally related to the adult survival only? We propose that SOD2 loss of function effects are principally superoxide mediated, where as complex1, GSH and Thr have an active SOD enzyme system available that is capable of generating all kinds of ROS (H₂O₂, OH⁻ etc.,) at a much faster pace so the damaging effects are far more potent. Admittedly we need to prove this hypothesis further by measuring specific ROS production during development and during adult life.

Materials and Methods

Making SOD2^{-/-} oocytes and fat body cells. Fly stocks carrying *P[w[+mW.hs] = FRT(w[hs])]G13* insertion at 42B were recombined with *Sod2ⁿ²⁸³* to create *FRT42B Sod2ⁿ²⁸³* chromosome. Somatic recombination was induced in females of the genotype *hs-FLP; FRT42B Sod2ⁿ²⁸³/FRT42B OvoD1* following activation of the Flipase enzyme with heat shock (1 hour at 37°C) applied during the early larval stage. Once mature, eggs that are laid by this female should be *Sod2ⁿ²⁸³* because all homozygous and heterozygous *OvoD1* eggs will degenerate.⁴³ Mature females were crossed with males, wild-type or *Sod2^{n283/+}*. Thus the effects of germ line depletion of SOD2 activity was monitored by counting the number of adults produced from the *Sod2ⁿ²⁸³* eggs. Three cohorts each of experimental and control crosses were set up carrying five males and five females in each cohort.

To make *Sod2^{-/-}* clones in the fat body tissue, *Sod2ⁿ²⁸³* was recombined with the *FRT42D* and somatic recombination was induced during second larval instar with heat shock.

Tissue preparation and immuno staining. Dissected tissues were incubated in DHE reagent (Dihydroxy Ethidium; Invitrogen Inc.,) to detect in vivo superoxide generation. 30 uM of DHE was dissolved in Schneider's medium.¹⁵ For BrdU labeling tissues were incubated in BrdU solution (75 ug of BrdU in 1 ml PBS) for 45 minutes followed by detection with an anti-BrdU antibody (1:200 in PBSA). Caspase activation and nuclear lamin was detected with an activated caspase antibody (Cell Signaling at

1:100 in PBSA), Anti-LaminDmo antibody (DSHB, Iowa State University). For Phalloidin staining, dissected fat bodies were incubated in Phalloidin (Sigma), washed and mounted. For the purpose of embryo staining, flies of the genotype *Sod2ⁿ²⁸³/CyO, GFP* were mated inter se. Embryos were collected and processed for antibody staining. Tissues were mounted with Vectashield (Vector Laboratories) and observed under either a confocal microscope (Nikon Model: EZ-C1 Eclipse) or regular fluorescence microscope (Olympus). Tissue histochemical studies were performed with a minimum of ten individuals per experiment. As a routine practice all microscopic observations were confirmed by observing a specific effect at least in ten different fields.

Improving the adult viability of Sod2ⁿ²⁸³. SOD2 expression was turned on in *Sod2ⁿ²⁸³; UAS-SOD2/TubulinP[Switch]Gal4* adult flies by keeping them in a media spread with RU486 solution (Sigma M8046; 5 mg/ml).⁴⁴ We put our best efforts to transfer them to RU486 medium within ~1 hour after eclosion. Surviving adults were counted each day. Data analysis was done in Prism Software (V3.02 Graph Pad Incorporated). *Tubulin-P[Switch]* is a generous gift from Scott Pletcher lab. The *UAS-Sod2* overexpression and *UAS-Sod2IR* lines were obtained from John Phillips' lab.

Western immunoblot analysis. Performed according to previously published procedures.^{45,46}

Determination of eclosion ratio. A *Sod2ⁿ²⁸³/CyO, Twist-GFP* stock was constructed. All homozygous *Sod2ⁿ²⁸³* embryo, larva and pupa from this stock are GFP negative where as the heterozygotes appeared as GFP positive. Eclosion ratios were compared between homozygote null (*Sod2ⁿ²⁸³*), heterozygotes (*Sod2ⁿ²⁸³/CyO*) and KG06854R control by collecting 250 larvae from each genotype and the number of larvae successfully transitioning to the pupal and adult stages were recorded. *KG06854R* shares the same genetic background as *Sod2ⁿ²⁸³*.⁴⁷ Chi-square independence test was performed at 95% confidence interval by feeding the data into the University of Kansas web site <http://www.people.ku.edu/~preacher/chisq/chisq.html>.

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