Human SOD1 ALS Mutations in a *Drosophila* Knock-In Model Cause Severe Phenotypes and Reveal Dosage-Sensitive Gain- and Loss-of-Function Components

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ABSTRACT Amyotrophic Lateral Sclerosis (ALS) is the most common adult-onset motor neuron disease and familial forms can be caused by numerous dominant mutations of the copper-zinc superoxide dismutase 1 (SOD1) gene. Substantial efforts have been invested in studying SOD1-ALS transgenic animal models; yet, the molecular mechanisms by which ALS-mutant SOD1 protein acquires toxicity are not well understood. ALS-like phenotypes in animal models are highly dependent on transgene dosage. Thus, issues of whether the ALS-like phenotypes of these models stem from overexpression of mutant alleles or from aspects of the SOD1 mutation itself are not easily deconvolved. To address concerns about levels of mutant SOD1 in disease pathogenesis, we have genetically engineered four human ALS-causing SOD1 point mutations (G37R, H48R, H71Y, and G85R) into the endogenous locus of *Drosophila* SOD1 (*dsod*) via ends-out homologous recombination and analyzed the resulting molecular, biochemical, and behavioral phenotypes. Contrary to previous transgenic models, we have recapitulated ALS-like phenotypes without overexpression of the mutant protein. *Drosophila* carrying homozygous mutations rendering SOD1 protein enzymatically inactive (G85R, H48R, and H71Y) exhibited neurodegeneration, locomotor deficits, and shortened life span. The mutation retaining enzymatic activity (G37R) was phenotypically indistinguishable from controls. While the observed mutant *dsod* phenotypes were recessive, a gain-of-function component was uncovered through dosage studies and comparisons with age-matched *dsod* null animals, which failed to show severe locomotor defects or nerve degeneration. We conclude that the *Drosophila* knock-in model captures important aspects of human SOD1-based ALS and provides a powerful and useful tool for further genetic studies.

KEYWORDS ALS; SOD1; *Drosophila*; motor neuron

AMYOTROPHIC Lateral Sclerosis (ALS) is characterized by progressive loss of upper and lower motor neurons (MNs) leading to paralysis, and death in afflicted individuals within 3–5 years after diagnosis, on average. The mechanisms of disease pathogenesis leading to the exclusive demise of MNs remain unclear and there is no effective therapy. Over 50 genes have been linked to ALS, suggesting large genetic

heterogeneity of the disease mirroring a diverse range of clinical presentations (Abel *et al.* 2012; Sreedharan and Brown 2013; Leblond *et al.* 2014). The first ALS-associated mutations were found in the superoxide dismutase 1 (SOD1) gene (Rosen *et al.* 1993). SOD1 encodes a small protein of 153 amino acids (16 kDa), constitutes ~1% of the cytoplasmic protein, and is expressed ubiquitously (Pardo *et al.* 1995). Functional SOD1 is a homodimer and catalyzes the conversion of superoxide radicals to hydrogen peroxide. Recent data suggest SOD1 also acts as a transcription factor and upregulates genes involved in oxidative stress response (Hu *et al.* 2009; Tsang *et al.* 2014; Bunton-Stasyshyn *et al.* 2015). Strong evidence indicates that SOD1 mutations produce toxic gain-of-function properties at the protein level. The vast majority of the >150 SOD1 mutations identified in patients

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show dominant inheritance patterns, and disease severity correlates with aggregation potential of mutant protein rather than loss of enzymatic activity (Abel *et al.* 2012; Saccon *et al.* 2013).

Animal models containing mutant ALS-associated transgenes have been important tools for understanding disease pathogenesis. To date, most transgenic models generated in multiple species express mutant human SOD1 (hSOD1) in a genetic background containing the endogenous wild-type SOD1 gene in rodents (Gurney *et al.* 1994; Trotti *et al.* 1999; Kato 2008), in *Drosophila* (Watson *et al.* 2008; Bahadorani *et al.* 2013) and in other model organisms (Joyce *et al.* 2011). The majority of these models recapitulate characteristics of ALS, including progressive motor deficits, paralysis, MN degeneration, and early lethality (McGoldrick *et al.* 2013). However, ALS-like phenotypes in these animals are highly dependent on transgene expression levels and severity of phenotypes correlate with level of protein overexpression (Gurney *et al.* 1994; Alexander *et al.* 2004; Wang *et al.* 2009a). Furthermore, overexpression of wild-type hSOD1 (hSOD1^{wt}) is sufficient to recapitulate some ALS phenotypes such as mitochondrial dysfunction, axonal degeneration, and premature MN death (Jaarsma *et al.* 2000; Ezzi *et al.* 2007; Graffino *et al.* 2013). These results suggest mutant phenotypes are sensitive to gene dose and/or SOD1 protein levels. Extrapolating information gained from these disease models to human ALS is therefore difficult.

While mutations in other genes cause ALS, the pathways leading to SOD1-mediated toxicity are still poorly understood and it is unclear to what extent pathogenic mechanisms are shared between the various forms of familial ALS (fALS). Intriguingly, cytoplasmic SOD1 inclusions have been reported in ALS patients irrespective of SOD1 mutations (Gruzman *et al.* 2007; Bosco *et al.* 2010; Forsberg *et al.* 2010), strengthening the hypothesis that there may be a common mechanism for neurodegeneration in ALS and emphasizing a critical role for SOD1 regarding the general pathogenesis of the disease. Uncovering how mutations in SOD1 ultimately lead to the dysfunction and the ultimate death of MNs may shed light on how ALS develops and progresses in all patients, regardless of sporadic or familial modes of transmission.

To create an ALS model in which mutant SOD1 protein is expressed at endogenous levels, we have used ends-out homologous recombination (HR) as a gene replacement strategy to introduce relevant ALS-associated mutations at conserved residues of the *Drosophila* *sod1* (*dsod*) gene. Similar strategies have been used in flies to successfully model other diseases (Sun *et al.* 2012; Schutte *et al.* 2014). In this study, we show *dsod* G85R, H71Y, and H48R mutants exhibit recessive ALS-associated phenotypes including a shortened life span, progressive paralysis, and degeneration of MNs. We also demonstrate that aspects of these phenotypes represent a gain of function through comparisons with SOD1 null (*dsod*^{null}) alleles. Using *Drosophila* as a genetically tractable model organism, we have developed an additional tool which can be used to uncover relevant ALS-related cellular responses.

Materials and Methods

Drosophila strains

Drosophila were raised at a constant 25°, on standard corn-meal molasses food and under 12 hr day/night cycles. The *dsod*^{X-39} and *dsod*^{G51S} lines (n1 or n108) were ordered from Bloomington *Drosophila* Stock Center (BDSC) (stock numbers 24492 and 24490, respectively). The names of the dSOD mutations are based on the human SOD1 amino acid numbering system throughout this work. The *dsod*^{X-16} line was a kind gift from W. C. Orr from Wayne State University.

HR and CRISPR

We performed ends-out HR to create three independent lines of *dsod*^{WTLoxP}, *dsod*^{G37R}, *dsod*^{H48R}, *dsod*^{H71Y}, and *dsod*^{G85R} using previously reported methods (Staber *et al.* 2011). Briefly, we used the ends-out targeting vector p[w25.2] that contains the *mini white* marker (*white*⁺), a selectable red eye color, flanked by *LoxP* sites for subsequent removal by Cre recombinase (Figure 1). Homology arm 1, corresponding to exon 1 of *dsod*; and homology arm 2, corresponding to exon 2 of *dsod*; were cloned and sequenced in pTOPO (Life Technologies) and then shuttled into the multiple cloning sites of the vector to generate p[w25-*dsod*], which was then introduced into the *Drosophila* genome by standard *P*-element transgenic methods (Genetic Services). Full targeting region coordinates were chromosome 3L: 11,103,794–11,108,715 (4922 nt). All targeting was done in a *w*¹¹¹⁸ background, as previously described, and performed in a wild-type chromosome 3 background (Gell and Reenan 2013). A full list of cloning, mutagenic, and sequencing primers can be found in Supplemental Material, Table S3. Targeting was performed to generate multiple independent targeting events that incorporate (G37R, H48R, H71Y, and G85R) or exclude engineered mutations (*WTLoxP*). All targeted animals have a *LoxP* “scar” of 72 nt.

After generating the transgenic flies, white or mosaic-eyed females were collected from heat-shocked vials and then crossed with *yw;ey-Flp,noc^{scd}/CyO* males and only red-eyed female progeny were selected for additional validation. Targeted alleles were validated by PCR amplification, using primers outside the region of targeting and primers specific to the *white*⁺ marker. Following recombination, the *white*⁺ minigene selection cassette was removed via genetic crosses, which introduced Cre recombinase. All targeted alleles were sequenced to verify that no unintended mutations were introduced. It is important to note here that all of the targeted alleles contain a natural polymorphism at the site 1013 having a C instead of an A, which leads to the N98K missense mutation in human amino acid numbering system (N96K in *Drosophila* dSOD numbering system), which is also referred as *dsod*^{fast} allele in the literature (Phillips *et al.* 1995). The *dsod*^{G51S} allele that is generated through EMS mutagenesis does not contain this polymorphism, and it is the *dsod*^{slow} allele. *dsod*^{fast} and *dsod*^{slow} alleles do not result in a phenotypic change in *Drosophila*, they are named after their differential mobility on a native polyacrylamide gel (Lee *et al.* 1981; Hudson *et al.* 1994; Phillips *et al.* 1995). All three knock-in lines from each line were

Figure 1 ALS-associated mutations introduced into *dsod*. Mutations G37R, H48R, H71Y, and G85R were introduced into *dsod* by ends-out HR. (A) Alignment of hSOD1 and dSOD show amino acid changes were at conserved residues. Black bars are mutations linked to familial ALS, orange residues are conserved between species, gray residues are similarly charged domains between organisms. Asterisk (*) shows the targeted residues. (B) Sequencing chromatograms confirm the presence of targeted mutations. Orange, G37R (GGC → CGC); blue, H48R (CAC → CGC); green, H71Y (CAT → TAC); red, G85R (GGC → CGC). (C) Targeted mutations and the intronic *LoxP* site did not interfere with alternative splicing of *dsod* messenger RNA. *dsod* full-length ~500-bp and *Gapdh* gene-specific primers were used to amplify the region of interest from total RNA isolates.

backcrossed to w^{1118} , a white-eyed genetic background, for five generations. *Drosophila* stocks were kept as heterozygotes using third chromosome balancers. Heterozygous *dsod* alleles that were used in the experiments were generated by crossing mutant lines to the wild-type line *WTLoxP*.

To create *dsod*^{null} alleles, CRISPR/Cas9 was used to remove the complete *dsod* ORF (Genetivision, Houston, TX). Primers used to create target sequences were 5'CTTCGACGAAATTCGCAAGTAGAAT and 5'AAACATTCTACTTGCGAATTCGTC for guide RNA 1 (gRNA1); and 5'CTTCGGCATTATTTGGGGAATTC and 5'AAACGGAATCCCCAATAATGCC for gRNA2. Two independent lines were analyzed and reported herein.

Eclosion assay

For the eclosion percentages, we set up at least 12 vials of cohorts of 2–3 heterozygous males and virgin females at 25° on standard cornmeal molasses food and under 12 hr day/night cycles. The parents were transferred to a new vial every day. The heterozygous *dsod* alleles were balanced over a third chromosome balancer: *TM3,GFP,ser,w⁺* (BDSC stock number 4534). When the progeny started eclosing, heterozygous progeny having red eyes due to the balancer were counted and homozygous progeny having white eyes due to lack of the balancer which eclosed or stuck in the pupal case were counted. The progeny carrying homozygous balancers died early in development due to a recessive lethal marker on the balancer chromosome. The progeny from each vial were counted until the number of homozygous mutants reached 200. Then, the eclosion percentages were calculated by (total number of eclosed homozygous adults)/(total number of homozygous pupal cases) × 100. The results of 12 vials were averaged and Fisher's exact test was performed.

Survival assay

For the survival assay, parental flies were raised at 25° on standard cornmeal molasses food and under 12 hr day/night

cycles. The parental flies were collected from population-density-controlled broods (2–3 males and females in each vial) to avoid any confounding effects due to overcrowding. The parents were allowed to mate and lay eggs for 2 days before being transferred to fresh food twice. Three trials of survival analysis were performed for each genotype. For the heterozygous genotypes, to eliminate maternal effects on the *dsod* mutant allele, in trial 1 the mutant allele was passed from the mother and in trial 2 the mutant allele was passed from the father. The third trial was either identical to either the trial 1 cross or the trial 2 cross. The offspring from these parents were collected over a period of 24 hr and sorted by sex. A total of 12 males and 12 females were kept in vials containing standard cornmeal molasses food. For each genotype, multiple replicate vials were set up so the total sample size was 200–300 for each sex. Flies were transferred onto fresh food three times a week by blinded undergraduate researchers. The number of deaths was recorded. Survival assays were either performed at 18 or 25°. Once all the flies were dead, log-rank tests were performed for statistical analysis.

H₂O₂ feeding assay

All chemicals were delivered to *Drosophila* in instant food (Nutri-Fly Instant, Genesee, 66–118). Instant food is prepared based on the manufacturer recipe. For each vial, 2 g instant food was dissolved in 5 ml Milli-Q water or the chemical solution. For each bottle, 21 g instant food was dissolved in 50 ml Milli-Q water or the chemical solution.

A 2% concentration of hydrogen peroxide (H₂O₂) was diluted from 30% stock (Fisher Chemical H325-100) and delivered to 1-day-posteclosion *Drosophila* in vials. Each vial contained 50 males or 50 females. Three trials of H₂O₂ survival analysis were performed for each genotype and sex. As in survival analysis, for the heterozygous genotypes to eliminate the maternal effects on the *dsod* mutant allele, in trial

one the mutant allele is passed from the mother and in trial two the mutant allele is passed from the father. Trial three was identical either to the trial one cross or the trial two cross. The number of deaths was recorded every 8 hr by blinded undergraduate researchers. Once all the flies were dead, log-rank test was performed for statistical analysis.

Larval motility assay (manual version)

The larval motility assay was performed as previously described in Batlevi *et al.* (2010). Larvae were selected during the wandering third instar stage. They were washed in 1× PBS and placed on a 1% agarose plate made with 0.5% TBE (100- × 15-mm petri dish) and were allowed 1 min to acclimate. The plate was placed on a 1- × 1-mm square grid and the larvae were allowed to crawl for 2 min. The total number of squares a larva crossed was counted by blinded undergraduate researchers. We considered a square to be crossed when the larvae's posterior end crossed a line and the total number of squares crossed was counted. Dunnett's test following one-way ANOVA was used to compare the experimental group to the wild type.

Larval motility assay (computational version)

In the computational larval motility assay, five larvae were allowed to crawl on a 22-cm diameter dish filled with 1% agarose in H₂O. The larvae were videotaped for 1.5 min and videos were analyzed using a Matlab program that calculates total distance traveled for each larva. A total of >30 larvae were used per group. If two larvae collided, the data were discarded in case it altered behavior. Experiments used either midthird instar larvae or wandering third instar larvae. Wandering third instars were identified as fully grown larva that had exited the food but not inverted their anterior spiracles. Midthird instars were pulled from the food ~24 hr before wandering. Tukey's honest significant difference (HSD) test was used to calculate significance.

Adult climbing assay (negative geotaxis assays)

A total of 10 adult male or female flies at the appropriate age were placed into a vial without food and negative geotaxis assays were conducted in three trials with each group consisting of 20 vials for each sex and genotype. The flies were gently tapped to the bottom of a vial and allowed to climb for 5 min. The number of flies reaching above a 75% mark (5 cm) of the total cylinder length in 5 min was recorded and the average calculated. Dunnett's test following one-way ANOVA was used to compare the experimental group to the wild type.

Western blotting

For all the denaturing gels that are shown in the figures, protein samples were homogenized in Leammli buffer (Bio-Rad, Hercules, CA) and β-mercaptoethanol, and run out on a 4–20% gradient gel (Bio-Rad Mini-Protean-TGX, 456-1093). For the mutant tissue that shows trace amounts of dSOD protein on denaturing gels (dSOD^{G85R/G85R}, dSOD^{H71Y/H71Y}, dSOD^{G51S/G51S}), various protein extraction solutions were assayed, e.g., radioimmunoprecipitation assay buffer in com-

bination with proteinase inhibitor cocktails, but no significant improvements were observed. For adult tissue the abdomen region was discarded and for larval tissue intestines were removed. Unless otherwise specified, one adult fly or one larva was homogenized in 50 μl sample buffer and a 10 μl sample was run on 100 V. Samples were transferred to nitrocellulose membrane for 1 hr at 4°. For developmental time-point Western blots, the total protein amount was equalized between different samples via Coomassie Protein Assay using the manufacturer instructions (Thermo Scientific, 1856209). Rabbit polyclonal anti-dSOD antibody (a kind gift from W. C. Orr from Wayne State University) was used at 1:3000, and anti-actin (Millipore, Bedford, MA) was used at 1:50,000. Horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (Abcam ab5930) was used at 1:5000 and HRP-conjugated goat anti-rabbit secondary antibody (Jackson Immuno 111-0350144) was used at 1:10,000. Immunoreactive bands were visualized by ECL detection reagent (Genesee, Amersham ECL Reagent, 84-817). Nonsaturated bands were quantified on ImageJ (National Institutes of Health) and expressed as a ratio in relation to the internal reference actin. At least three biological replicates were quantified.

For high-salt buffer protein extraction experiments, the following buffers have been tried: (i) 750 mM NaCl, 50 mM Tris-HCl (pH 7.5), 10 mM NaF, 5 mM EDTA; (ii) 750 mM NaCl, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, proteinase inhibitor cocktail (Roche), 0.1% Triton X-100; (iii) 5% SDS, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 175 mM NaCl; (iv) 5% SDS, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 175 mM NaCl, 8 M urea; (v) 750 mM NaCl, 50 mM Tris-HCl (pH 8.8), 10 mM NaF, 5 mM EDTA; (vi) 750 mM NaCl, 50 mM Tris, 10 mM NaF, 5 mM EDTA; and (vi) Bio-Rad ReadyPrep Protein Extraction Kit (Soluble/Insoluble, 163-2083). The extraction conditions were similar to the methodology that was reported previously (Watson *et al.* 2008). Briefly, five male adults (abdomen discarded) were homogenized in 100 μl high-salt buffer. Then, 30 μl of the protein sample was removed and run as “total” protein sample. The rest of the protein sample was spun at 10,000 g for 30 min in 4°. Then, 30 μl of the protein sample was removed and run as “supernatant” protein sample. The pellet was resuspended in 100 μl of high-salt buffer and spun at 10,000 g for 30 min at 4° twice. Finally, the pellet was resuspended in 50 μl high-salt buffer and run as “pellet” sample.

The semi-denaturing detergent agarose gel electrophoresis assay was performed as described before in Halfmann and Lindquist (2008) and Cushman-Nick *et al.* (2013).

Native PAGE

For nondenaturing gels, protein samples from two adult flies (abdomens discarded) were homogenized in 50 μl Native Sample Buffer (Bio-Rad, 161-0738) and run out on a 10% gradient gel (Bio-Rad Mini-Protean-TGX, 456-1033) for 5–8 hr on 100 V at 4°. The following protein standards were used: prestained Isoelectric Focusing Standards (Bio-Rad, 161-0310) and nonstained Novex NativeMARK Unstained Protein Standard (Fisher Scientific, LC0725). As a positive control,

human (ENZO, 80-1642) or bovine (ENZO, ALX-202-022-UT50) SOD1 protein isolates were diluted in native sample buffer and 50 units were loaded to the well. The gel was blotted with 1:3000 dSOD antibody and the transfer conditions were the same as described in the previous *Western blotting* section. In addition, the same samples were run in SDS-PAGE denaturing gels, after adding β -mercaptoethanol.

SOD activity assay

For SOD activity assay, the same samples with native PAGE were used. Briefly, after running the gel for 5–8 hr, the gel was washed three times in distilled water. The gel was incubated for 15 min in 10 mg Nitro Blue Tetrazolium (NBT) (Sigma N5514-10TAB) and 4 mg riboflavin (Fisher BP167-50) solution. Then, the gel was incubated for 15 min in TEMED-water solution (10 μ l TEMED in 10 ml distilled water in the dark). Finally, the gel was washed three times in distilled water and imaged on a white light box until the desired contrast was reached.

Leg muscle atrophy and nerve structure analyses

For bright field leg images, each leg was removed with tweezers on a silicon (Dow Corning, Sylgard 184 silicone elastomer kit) plate. The legs were incubated in Vectashield (Fisher NC9532821) overnight at 4°. The images were taken with a Carl Zeiss (Thornwood, NY) AX10 Imager M1. *dsod*^{G85R/G85R} full-leg lengths were calculated by tracing along the midline of the femur on ImageJ. The nerve integrity of 2-week-old *dsod*^{H71Y/H71Y} legs was evaluated either based on whether they maintained nerve branches or not. Additional parameters including continuity of nerve bundle, nerve size, number of leg kinks, and lower leg morphology were scored blinded for genotypes on a scale of one, being the worst, to five, being the best.

For muscle atrophy imaging, relevant *dsod* mutants were combined with a *myosin heavy chain (mhc)-tau-GFP reporter* (BDSC: 38460): *dsod*^{WTLoxP/WTLoxP}, *mhc-tau-GFP*; *dsod*^{G85R/G85R}, *mhc-tau-GFP*; *dsod*^{H71Y/H71Y} lines were generated. Legs were dissected and fixed as described previously (Soler *et al.* 2004). Briefly, the full animal was fixed in 4% paraformaldehyde for 5 hr at room temperature. Then, the legs were dissected and continued to fix more overnight at 4°. Images were taken at the confocal microscopy as described in the microscopy section.

Tunnel assay, immunohistochemistry, and microscopy

Adult or larval brains were dissected in PBS with 0.1% Tween 20 (PBTX) on a silicon (Dow Corning, Sylgard 184 silicone elastomer kit) plate. The tissue was fixed with 4% paraformaldehyde for 20 min at room temperature on the nuator. After three rounds of quick washes in PBTX, the tissue was blocked in PBTX with 5% normal goat serum. The primary antibodies used in this study were anti-Elav and anti-Repo (Developmental Studies Hybridoma Bank), both used at 1:200; Alexa Fluor 488 and 564 secondary antibodies were used at 1:200. DAPI (Invitrogen, Carlsbad, CA) was used at 1:1000.

For the terminal deoxynucleotidyl transferase (TdT) dUTP nick end labeling (TUNEL) assay, the CF-488 TUNEL kit

(Biotium) was used according to manufacturer instructions. After Elav and Repo staining performed as described above, the tissue was refixed with 4% paraformaldehyde for 20 min at room temperature on the nuator. After three rounds of quick washes in PBTX, the tissue was reblocked in PBTX with 5% normal goat serum. Each genotype was incubated in 10 μ l TUNEL equilibrium buffer for 5 min. Then, the buffer was replaced with the enzyme solution (2 μ l TdT enzyme in 100 μ l TUNEL reaction buffer) and incubated for 2 hr at 37° in a humidifying chamber. Finally, the tissue was washed three times with PBST.

All tissue was mounted in Vectashield (Fisher NC9532821). All confocal Z-series images were obtained by LSM510 confocal microscope. Images were contrast enhanced in Adobe Illustrator. Each image shown is a representative example of $n \geq 5$ unless otherwise reported.

Data availability

The full list of *Drosophila* lines used in this study is found in Table S2. The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article.

Results

Generation of *dsod* mutant *Drosophila* alleles via HR

To develop *Drosophila* models of fALS, we knocked in G37R, H48R, H71Y, and G85R point mutations into the endogenous *dsod* locus via HR. SOD1 is highly conserved between organisms; hSOD1 and dSOD differ at 49/153 residues, and 22 of these different residues possess a similar side-chain chemistry (Figure 1, A and B) (Bertini *et al.* 1998). The mutations we selected are highly conserved across species and have varying significance for the tertiary protein structure and enzymatic activity of the SOD1 protein in humans, and the patients carrying these mutations have different disease onsets and disease progression (Juneja *et al.* 1997; Rabe *et al.* 2010; Weis *et al.* 2011; Özoğuz *et al.* 2015) (Table S1). The targeting and targeted mutations had no effect on *dsod* transcript levels or splicing (Figure 1C). Because the HR technique leaves a 72-bp *LoxP* site within the *dsod* intron, *dsod*^{WTLoxP} lines were analyzed as controls in these studies.

For mutant and control lines, we generated at least three independent targeting events. To remove any potential confounding genetic background differences, one line for each mutation was backcrossed to w¹¹¹⁸ for five generations. In addition to the alleles we created by HR, we analyzed *dsod*^{G51S}, a previously reported line that carries a point mutation as a result of EMS mutagenesis (Campbell *et al.* 1986; Phillips *et al.* 1995).

***dsod*^{G85R}, *dsod*^{H48R}, and *dsod*^{H71Y} mutants show reduced viability and fertility**

All three independent *dsod*^{G85R} lines generated by HR are homozygous lethal as pharate adults and the vast majority

Figure 2 Targeted mutations cause eclosion defects and shorten life span. (A) A representative image of *dsod*^{G85R/G85R}. (B) The wrinkled-wing phenotype of eclosed adult *dsod*^{H71Y/H71Y} flies. (C) Homozygous *dsod*^{G51S/G51S}, *dsod*^{H71Y/H71Y}, *dsod*^{H48R/H48R}, and *dsod*^{G85R/G85R} flies do not eclose in expected Mendelian ratios. The eclosion rates of heterozygote flies and *dsod*^{G37R/G37R} flies are normal. Error bars are SEM. *** $P < 0.0001$, Fisher's exact test. Eclosion defects are dosage sensitive for (D) *dsod*^{G85R} and (E) *dsod*^{H71Y} mutants. Single-copy *dsod* mutants were analyzed as *trans*-heterozygotes with *dsod*^{X-16}, an allele that deletes the first exon and promoter region. Bars are average eclosion rates from 12 different vials with at least 200 homozygous mutant progeny. Error bars are SEM. *** $P < 0.0001$, Fisher's exact test. (F) Life spans were analyzed for *dsod* mutants and control *dsod*^{WTLoxP/WTLoxP} flies and survivorship was plotted over time. Survival curves represent an average of three life-span trials. *** $P < 0.0001$, log-rank test. (G) Statistical comparisons of life-span analysis show eclosed *dsod*^{H71Y/H71Y} flies have a severely shortened life span, similar to *dsod*^{G51S/G51S}. *dsod*^{G85R/G85R} flies do not survive to adulthood, thus are excluded from this experiment. All heterozygous mutants we analyzed have a normal life span.

of these animals die with their heads everted from the pupal case and a characteristic shortening of the legs, in particular the metathoracic legs (Figure 2A and File S1). We observed rare (~1/1000) *dsod*^{G85R/G85R} homozygotes that eclosed. These escapers live less than an hour and exhibit severe paraparesis, uncoordinated locomotion, leg muscle twitching, extreme proboscis pulsing, and seizures (File S2). Similar eclosion phenotypes were seen in *dsod*^{H48R/H48R} homozygous lines but slightly more adults eclose (2.3%), but they die within 24 hr. Homozygous *dsod*^{H71Y/H71Y} mutants exhibit less severe eclosion abnormalities than *dsod*^{G85R/G85R} homozygous adults (eclosion rate: $33.33 \pm 6.00\%$) (Figure 2C) and the majority displayed an obvious wrinkled-wing phenotype (Figure 2B). Interestingly, after 2 weeks, *dsod*^{H71Y/H71Y} exhibit a locomotor uncoordination phenotype similar to that of the *dsod*^{G85R/G85R} escaper flies (File S3). The *dsod*^{G51S} allele that was generated via EMS mutagenesis, was also reported to display eclosion defects similar to those of *dsod*^{H71Y/H71Y} (Staveley *et al.* 1991; Parkes *et al.* 1998). Here, we confirm that *dsod*^{G51S/G51S} animals exhibit lower eclosion rates

($48.48 \pm 10.00\%$) (Figure 2C). We tested *trans*-heterozygote *dsod*^{G85R/H71Y} flies and found eclosion rates intermediate between the severe *dsod*^{G85R/G85R} and *dsod*^{H71Y/H71Y} alleles ($15.00 \pm 7.00\%$) (Figure 2C). Moreover, the severity of the eclosion defects for *dsod*^{H71Y/H71Y} and *dsod*^{G85R/G85R} flies were dosage dependent (Figure 2, D and E). Reducing the mutant allele copy number by 50% by placing ALS-mutant alleles over a previously characterized null mutation partially rescued the eclosion phenotype ($37.14 \pm 16.01\%$ for *dsod*^{G85R/X-16} and $42.29 \pm 4.65\%$ for *dsod*^{H71Y/X-16}). However, eclosed adults exhibit an uncoordinated behavior and early death (File S4). These data suggest that both the G85R and H71Y mutations confer at least some part of their effects via a dosage-sensitive toxic gain of function.

Eclosed *dsod*^{H71Y/H71Y} adults exhibit a severely reduced life span (12.37 ± 0.27 days), similar to that of *dsod*^{G51S/G51S} homozygotes (12.70 ± 0.97 days) (Figure 2, F and G). The viable adults of *dsod*^{H71Y/H71Y} are also infertile. Heterozygous combinations of mutant *dsod* alleles with *dsod*^{WTLoxP} as well as *dsod*^{G37R/G37R} homozygotes showed no life-span reduction

under standard laboratory conditions (Figure 2, F and G, and Figure S1).

Because SOD1 functions to convert superoxide radicals to H_2O_2 and water, we treated *dsod* mutants with H_2O_2 and assessed survival to monitor response to oxidative stress (Fridovich 1986). Treating the heterozygous adults (*dsod*^{H71Y/WTLoxP} and *dsod*^{G85R/WTLoxP}) with a superoxide radical ($O_2^{\cdot-}$) generator did not alter survival when compared to wild-type *dsod*^{WTLoxP/WTLoxP} controls (Figure S2). However, homozygous *dsod*^{H71Y/H71Y} flies were extremely sensitive to oxidative stress, consistent with previous results regarding the *dsod*^{G51S/G51S} homozygous allele (Parkes *et al.* 1998; Kirby *et al.* 2008) (Figure S2, A and B). Finally, *dsod*^{G37R/G37R} homozygotes respond similarly to *dsod*^{WTLoxP/WTLoxP} controls (Figure S2, C and D).

Severe locomotion defects in *dsod*^{G85R} and *dsod*^{H71Y} mutants

Motor dysfunction is one of the first apparent symptoms in ALS patients (Pasinelli and Brown 2006). We therefore analyzed locomotion in *dsod* mutants by examining crawling behavior in larvae and climbing behavior in adults. During late third instar larval stages, *dsod*^{H71Y/H71Y}, *dsod*^{G85R/G85R}, and *dsod*^{G51S/G51S} homozygotes crawled at almost half the rate of wild-type larvae (Figure 3A). When we reduced the mutant copy number by half, the crawling defect was suppressed completely; again supporting the notion of dominant gain-of-function toxicity (Figure 3, B and C). Homozygous *dsod*^{G37R/G37R} were indistinguishable from *dsod*^{WTLoxP/WTLoxP} controls (Figure 3A). In keeping with a gain of function, heterozygous *dsod*^{H71Y/WTLoxP} and *dsod*^{G85R/WTLoxP} larvae showed crawling defects relative to wild type; indicating a dominant component for this phenotype at this life stage (Figure 3A). Because *dsod*^{H71Y/H71Y} and *dsod*^{G51S/G51S} adults survive for reasonable lengths of time, we assessed locomotion in adults through a negative geotaxis climbing assay. Homozygous *dsod*^{H71Y/H71Y} and *dsod*^{G51S/G51S} lines show an almost-complete loss of climbing ability within 1 week of eclosion (Figure 3D for males and Figure S3A for females). All heterozygous combinations with *dsod*^{WTLoxP/WTLoxP} as well as *dsod*^{G37R/G37R} homozygotes were indistinguishable from *dsod*^{WTLoxP/WTLoxP} flies throughout course of the assay (Figure 3 and Figure S3). Thus, in the adult stage, heterozygotes for the severe alleles appear to be recessive for locomotor defects.

Muscle atrophy and denervation in *dsod*^{G85R} and *dsod*^{H71Y} mutants

Denervation of muscles by MNs in the limbs of patients is a characteristic symptom of ALS. At the terminal stages, *dsod*^{G85R/G85R} animals struggle in the pupal case while usually failing to eclose (File S1). These flies look morphologically wild type except for the fact that their legs are shorter when compared to wild-type flies (Figure 4, A and B, and Figure S4). In addition, it was clear from visual inspection of the few escaper flies that *dsod*^{G85R/G85R} and 2-week-old *dsod*^{H71Y/H71Y} animals drag their metathoracic legs (leg 3)

Figure 3 Locomotion defects are apparent in *dsod* larvae and adults. (A) Larval crawling ability is measured manually in single-blinded assays counting the total number of squares each third larva traveled in 2 min. Error bars are SEM. *dsod* mutant larvae display crawling defect in a dosage-dependent manner. $N > 40$, Dunnett's test, *** $P < 0.001$. Computational measure of (B) *dsod*^{G85R/G85R} and (C) *dsod*^{H71Y/H71Y} larval crawling behavior in a dosage-dependent manner. Tukey's HSD test, *** $P < 0.001$, $N > 33$. (D) Adult climbing ability is significantly reduced in homozygous *dsod*^{G51S/G51S} and *dsod*^{H71Y/H71Y} flies within a week of eclosion compared to *dsod*^{WTLoxP/WTLoxP}. Heterozygous *dsod*^{WTLoxP/G85R} and *dsod*^{WTLoxP/H71Y} flies show normal climbing ability when assessed within a 7-week period after eclosion. In all adult climbing tests in this figure, male flies were used. Error bars are SEM. *** $P < 0.001$, one-way ANOVA followed by Dunnett's *post hoc* test.

while walking (File S2 and File S3). We determined the overall health scores of legs from *dsod*^{G85R/G85R} flies based on nerve health, lower leg structure, and kink severity of the femur based on a scale of 1–5 (worst to best) (Materials and Methods, and Figure S5). In *dsod*^{G85R/G85R} flies, leg 3 was the most severely affected, however leg 2 and leg 1 were not as healthy as a wild-type control legs. Wild-type legs scored

Figure 4 *dsod* mutant adults show progressive degeneration of the leg MNs. (A) Bright-field image of the MN bundle in the femur of the metathoracic (leg 3) leg exhibit deformation in *dsod*^{G85R/G85R}. *N* = 10. Bar, 0.2 mm. (B) Average leg length quantification of *dsod*^{WTLoxP/WTLoxP}, *dsod*^{H71Y/H71Y}, and *dsod*^{G85R/G85R} legs in microns. All *dsod*^{G85R/G85R} legs are significantly shorter than wild type. *N* = 10. Error bars are SEM. *** *P* < 0.001, one-tailed student's *t*-test. (C) Leg degeneration is progressive in *dsod*^{H71Y/H71Y} adults. Bright-field and fluorescent-microscope images of the same metathoracic leg of 1-day-old and 14-day-old adults: *mhc-tau-GFP*; *dsod*^{WTLoxP/WTLoxP} and *mhc-tau-GFP*; *dsod*^{H71Y/H71Y}. *N* = 10. Bar, 0.2 mm. (D) Quantification of legs showing MN degeneration based on bright-field images. Leg 1, prothoracic; leg 2, mesothoracic; leg 3, metathoracic. *N* > 10. Error bars are SEM. *** *P*, 0.001, one-tailed student's *t*-test.

higher than 4.5, whereas *dsod*^{G85R/G85R} scores were 2.87 for leg 1, 2.5 for leg 2, and 1.40 for leg 3.

Next, we used an *mhc-tau-GFP* intrinsic fluorescent muscle reporter to investigate the muscle structure and condition of *dsod* mutants (Figure 4C and Figure S4). In *dsod*^{G85R/G85R} flies, the muscle appeared to be undergoing severe atrophy and the MNs surrounding the leg appear degenerated (Figure 4A and Figure S4). In *dsod*^{H71Y/H71Y}, the muscle structure and leg length were normal at eclosion (Figure 4, C and D). However, leg nerves lost efferent branches by 2 weeks in 13 out of 15 legs when these flies were near the terminal stage of their life span (Figure 4D).

MNs of *dsod* mutants do not undergo cell death and gliosis

Since MN death is a feature of ALS, we examined whether MNs died in our mutant flies (Figure 5). To visualize MNs, we used a nuclear GFP protein (*UAS-nlsGFP*) that was expressed under an MN-specific driver (*ok371-GAL4*) as well as staining nuclear DNA with DAPI. We examined both third instar larval brains and adult ventral nerve cord (specifically the T1/T2 region of the thoracic ganglia) of *dsod*^{G85R/G85R} and *dsod*^{H71Y/H71Y} and did not observe any MN cell body loss (Figure 5). Another signature of ALS, gliosis in the neuronal samples, was also not seen in larval central nervous system, adult brain, or adult ventral nerve cord as observed by staining for the glial cell marker, *Repo*, between mutants and controls (Figure S6). We also found no evidence for an increase in TUNEL staining

in mutants, further strengthening the argument for the absence of MN death in third instar larval brains and adult ventral nerve cord of ALS-mutant *Drosophila* at the stages assessed (Figure S6).

Protein levels and dismutase activity of mutant *dsod* animals

In ALS patients, hSOD1 missense mutations variously alter protein folding, protein stability, enzymatic activity, and metal-binding properties; while others maintain wild-type SOD1-like properties (Valentine *et al.* 2005). hSOD1^{G85R} lacks enzymatic activity while hSOD1^{G37R} displayed 150% activity of wild-type SOD1 protein based on previous studies, while enzymatic activity has not been characterized for H48R or H71Y mutations (Borchelt *et al.* 1994) (Table S1). With our allelic series, we asked whether the ALS-like phenotype severity correlates with the possible loss of superoxide scavenging. We measured the state of dSOD dimerization and enzymatic activity via a native gel-based SOD activity assay (Figure 6). Based on the native gel, homozygous *dsod*^{H71Y/H71Y}, *dsod*^{G85R/G85R}, *dsod*^{H48R/H48R}, *dsod*^{G51/G51S}, and heteroallelic *dsod*^{IX16/X39} control adults exhibited no detectable SOD1 activity while *dsod*^{G37R/G37R}, and all heterozygotes, were indistinguishable from wild-type Canton-S and *dsod*^{WTLoxP/WTLoxP} (Figure 6).

To determine dSOD protein levels in *dsod* mutants, we used denaturing and native polyacrylamide gels to quantify protein amounts based on immunoreactivity with a dSOD antibody.

Figure 5 Absence of MN cell body loss in *dsod*^{G85R/G85R} mutants. (A) The nuclear GFP protein (*UAS-nlsGFP*) is expressed under the *ok371* MN-specific driver (*ok371-GAL4*). The MN regions that are shown in the following pictures are highlighted in red squares. DAPI is used to label all nuclei. MNs and DAPI staining in *dsod*^{G85R/G85R} (B) third instar larval brains (*N* = 5) and (C) adult ventral nerve chord (*N* = 5) do not exhibit MN loss. Bar, 50 μ m.

Similar to SOD1 enzymatic assays, the null *dsod*^{X-16/X-39}, *dsod*^{H71Y/H71Y}, *dsod*^{G85R/G85R}, *dsod*^{H48R/H48R}, and *dsod*^{G51S/G51S} homozygotes also did not reveal any SOD1 protein immunoreactivity by SDS-polyacrylamide denaturing gel (Figure S7 and Figure S8A). These data are consistent with reports of reduced SOD1 protein amount in the nontransgenic mouse model harboring the mSOD1^{D83G/D83G} mutation (Joyce *et al.* 2015). Because mutant SOD1 forms insoluble aggregates in ALS patients and model animals, we then examined if the undetectable dSOD protein on SDS-PAGE gel is in insoluble protein fractions by extracting protein using six different

Figure 6 Superoxide dismutase activity is diminished in severe *dsod* mutants. (A) SOD1 dismutase activity was assessed by NBT after native-PAGE. Homozygotes *dsod*^{G85R/G85R}, *dsod*^{H71Y/H71Y}, *dsod*^{G51S/G51S}, *dsod*^{H48R/H48R} show dramatically reduced enzyme activity compared to *dsod*^{G37R/G37R}, heterozygous combinations, and *dsod*^{WTLoxP/WTLoxP} controls. Purified bovine SOD1 protein was used as a positive control. (B) Coomassie Blue staining of the same samples analyzed above electrophoresed on native-PAGE gel.

high-salt or urea protein extraction solutions. All of these extraction methods failed to reveal detectable dSOD protein in the insoluble fraction via SDS-PAGE (a representative gel is shown in Figure S8). Semidenaturing detergent agarose gel electrophoresis also failed to reveal higher molecular size SOD1-positive inclusions (a representative gel is shown in Figure S9).

Because the SOD1 activity assay is based on native gel electrophoresis, we performed native gel Western blot analysis to assess whether immunoreactive dSOD dimers are present. Surprisingly, the native polyacrylamide gel results revealed that dSOD dimers are present in all mutants on a nondenaturing gel at levels approaching the wild-type controls (Figure 7). Moreover, in the native gel immunoblot, dSOD positive protein species with altered motilities were detected in homozygous mutants and heterozygotes. These additional protein bands could represent misfolded dSOD or destabilized dimers yielding monomeric dSOD (Rakhit *et al.* 2007; Auclair *et al.* 2010; Liu *et al.* 2012). It is an intriguing observation that the faster migrating species of dSOD immunoreactivity seen in homozygotes, which comprises substantial amounts of dSOD immunoreactivity, is greatly diminished in heterozygotes. This observation hints at potential mutant: wild-type subunit interactions to form dimeric complexes.

Next, we attempted to discern whether the lack of detectable dSOD on an SDS-PAGE denaturing gel is a general technical artifact, or if it is related to the progression of disease pathology. To assess if ALS mutations affect dSOD protein stability or accessibility to extraction in earlier stages as dramatically as in adults, we measured protein levels of dSOD during the adult stage from all mutant lines by SDS-PAGE (Figure 8A). As previously observed in the endogenous point-mutant mSOD1^{D83R/D83R} mouse, *dsod*^{H71Y/H71Y} and *dsod*^{G85R/G85R} exhibited a significant reduction in amounts of dSOD protein. Intriguingly, in the previously designated “null or *dsod*^{-/-}” allele *dsod*^{G51S} (other names: *n108* or *n1* allele),

we observed protein expression, suggesting that the *dsod*^{G51S} point mutation did not result in a protein null expression as previously reported (Campbell *et al.* 1986; Staveley *et al.* 1991; Phillips *et al.* 1995; Parkes *et al.* 1998; Kirby *et al.* 2008; O'Keefe *et al.* 2011; Mishra *et al.* 2014).

Next, we asked whether lack of immunoreactivity in SDS-PAGE gels is consistent throughout development or whether it is stage specific. Detection of dSOD protein from homozygous *dsod*^{H71Y/H71Y} and *dsod*^{G85R/G85R} is found in L1 larvae and declines gradually during development, indicating possible maternal contribution which reduces during larval development (Figure 8, B, D, and E). Maternally derived dSOD protein is only detectable in L1 larvae, albeit at reduced levels compared to wild-type L1 larvae (Figure 8, B and C).

Toxicity associated with the *dsod*^{G85R} allele is dosage sensitive

To determine to what extent mutant *dsod* alleles produce dosage-sensitive phenotypes, we increased *dsod* copy number and assessed phenotypes. We used the original HR cassette containing the entire wild-type *dsod* locus to increase gene dose. Transformed lines containing *P[dsod*^{WTLoxP}] show dSOD protein levels are comparable to the endogenous *dsod* locus by Western blot analysis (Figure 9, A and B). Flies carrying four copies of dSOD^{wt} (*P[dsod*^{WTLoxP}]/*P[dsod*^{WTLoxP}; *dsod*^{WTLoxP}/*dsod*^{WTLoxP}) showed a reduced life span compared to two copy dSOD^{wt} controls (Figure 9, C and D; Figure S10, A and B; 38.91 ± 0.55 days for males and 49.80 ± 1.25 for females vs. 52.39 ± 0.74 days for males and 61.95 ± 0.85 for females).

We further assessed the effect on phenotype of altering the copy number of mutant *dsod* relative to wild-type *dsod*. Animals containing either one or two extra doses of wild-type *dsod* (*P[dsod*^{WTLoxP}]/*P[dsod*^{WTLoxP}; *dsod*^{G85R}/*dsod*^{G85R} or *P[dsod*^{WTLoxP}]/+; *dsod*^{G85R}/*dsod*^{G85R}) showed dose-dependent suppression of *dsod*^{G85R}/*dsod*^{G85R} lethality at eclosion (Figure S10C). One dose of *dsod*^{wt} partially rescued viability to ~70%, while two doses rescued eclosion to wild-type levels. However, flies carrying two extra wild-type *dsod* alleles in a *dsod*^{G85R} background (*P[dsod*^{WTLoxP}]/*P[dsod*^{WTLoxP}; *dsod*^{G85R}/*dsod*^{G85R}) showed a 40% decrease in life span relative to controls carrying four wild-type *dsod* alleles (28.98 ± 0.61 days for males and 39.37 ± 1.32 days for females, compared to 52.39 ± 0.74 days for males and 61.95 ± 0.85 days in females) (Figure 9, C and D; Figure S10, A and B). In all cases, flies carrying four copies of *dsod* failed to show eclosion or progressive locomotor defects regardless of whether four wild-type copies or two wild-type and two mutant copies were present (Figure S10, C, D, and E). Nevertheless, there was a general rapid loss of locomotor function in the days preceding death for *P[dsod*^{WTLoxP}]/*P[dsod*^{WTLoxP}; *dsod*^{G85R}/*dsod*^{G85R} flies (File S5).

Complete loss-of-function mutations at *dsod* support a gain-of-function toxicity component for phenotypic ALS alleles

Because mutant *dsod* alleles exhibit predominantly recessive phenotypes and lack enzymatic activity by native gel analysis,

Figure 7 Altered conformations of dSOD mutant protein. (A) Analysis by native-PAGE dSOD blot and (B) the same gel over exposed show bands consistent with dSOD dimer present on the gel in all genotypes except null. Additional immunoreactive species were present, representing possibly misfolded or monomeric dSOD (*). (C) Coomassie staining of the native gel showing total protein loaded on the gel.

we compared our *dsod* mutants with *sod*^{null} lines to determine whether any components of the phenotype might be due to loss of function. To analyze *sod*^{null}, we assessed phenotypes using a heteroallelic combination of previously reported null mutations (X-16/X-39) (Staveley *et al.* 1991; Parkes *et al.* 1998; Missirlis *et al.* 2003). A heteroallelic combination was used because homozygous *sod*^{X-16/X-16} or *sod*^{X-39/X-39} alleles showed that numerous downward-modifying mutations had clearly accumulated in the stock, severely reducing eclosion rates and viability. Extensive backcrossing of these stocks did not alleviate this problem. To overcome this obstacle, we generated new *sod*^{null} mutations via CRISPR/cas9 that removed the *dsod* ORF (see Materials and Methods) (Figure 10A). Two lines were characterized, *dsod*^{null21} and *dsod*^{null26}, which had similar eclosion ratios as originally reported for

Figure 8 Expression of mutant dSOD is diminished in third instar larvae. (A) Immunodetection of dSOD protein is reduced dramatically in *dsod*^{G85R/G85R}, *dsod*^{H71Y/H71Y}, and *dsod*^{G51S/G51S} adults compared to wild-type Canton-S or *dsod*^{WTLoxP/WTLoxP} controls. Upon overexposure, a trace amount of protein is visible. Five male flies were homogenized for this blot. dSOD protein levels were assessed throughout development and (B) constant dSOD levels were observed in *dsod*^{WTLoxP/WTLoxP}; whereas (C) dSOD protein is seen only in L1 for null transheterozygotes *dsod*^{X-39/X-16}, representing maternal contribution. In (D) *dsod*^{G85R/G85R} and (E) *dsod*^{H71Y/H71Y} lines, dSOD protein is detectable at low levels through pupal stages of development.

dsod^{X-16} and *dsod*^{X-39} alleles, which displayed complete infertility (Mockett *et al.* 2003) similar to *dsod*^{H71Y/H71Y} (Figure 10B). In contrast to *dsod*^{H71Y/H71Y}, *dsod*^{null21/null21} and *dsod*^{null26/null26} homozygotes exhibit less severe climbing and life-span defects (Figure 10, C and D). *dsod*^{null21/null21} and *dsod*^{null26/null26} displayed median life spans of 11 and 14 days respectively, and climbing defects were only apparent in 2-week-old adults. In contrast, *dsod*^{H71Y/H71Y} displayed a median life span of 7 days at 25° and the 50% of animals alive at this time point displayed a complete loss of climbing ability (Figure 10, File S6, and File S7). There were not sufficient numbers of SOD1 null flies left at 30 days to perform climbing assays. These data confirm that, while lack of dSOD does confer deleterious phenotypes in *Drosophila*, ALS-causing mutations cause more severe phenotypes, produce mutant proteins, and likely act through a combination of both loss-of-function mechanisms (superoxidase activity) and a toxic gain of function whose mechanism is unknown.

Discussion

In this study, we used HR in *Drosophila* to model fALS mutations found in human disease: *dsod*^{G37R}, *dsod*^{H48R}, *dsod*^{H71Y}, *dsod*^{G85R}, and a wild-type control *dsod*^{WTLoxP}. Generating multiple lines and assessing consistency of phenotypes is important in HR methodology because the recombinases and

enzymes used (SceI endonuclease, FLP, and Cre recombinase) can potentially introduce unintended mutations in the genome that may affect downstream analysis of the targeted alleles (O'Keefe *et al.* 2007). We also minimized any undesired background effects by backcrossing the final engineered lines to a common genetic background stock, *w*¹¹¹⁸. Thus we show that the ALS-related phenotypes described in this study result from point mutations in the endogenous *dsod* locus. Similar to the genetic architecture of ALS patients, these mutations are within the context of the endogenous *dsod* gene and mutant *dsod* alleles used in this study are not overexpressed at either transcript or protein levels. Here, we show that homozygous *dsod*^{G85R/G85R}, *dsod*^{H48R/H48R}, and *dsod*^{H71Y/H71Y} ALS-associated mutations confer eclosion defects, reduced survival, and locomotor defects in larvae and adults; potentially stemming from neuronal degeneration and retraction from adult muscles.

In humans, 95% of ALS-associated SOD1 mutations confer disease via dominant inheritance (Saccon *et al.* 2013); however, some heterogeneity exists. For example, the hSOD1^{D90A} mutation shows mostly dominant inheritance patterns but is recessive in some Scandinavian populations (Andersen *et al.* 1995, 1997). Of the *dsod* alleles we created, *dsod*^{G85R}, *dsod*^{H48R}, and *dsod*^{H71Y} displayed prominent ALS-like phenotypes only when made homozygous. While these results are

Figure 9 *dsod*^{G85R} allele causes survival defects through gain of toxic function in a dosage-sensitive manner. (A) dSOD protein levels are gene dose dependent for *dsod*^{WTLoxP} alleles but not for mutant *dsod*^{G85R} or (B) *dsod*^{H71Y} by denaturing SDS-PAGE analysis. (C) Average life span of three trials of males of transgenic lines expressing extra *dsod* on the second chromosome and controls. *dsod*^{G85R/G85R} flies die in the pupal case and do not survive to adulthood. (D) Summary statistics for life-span analysis. log-rank test was used. *dsod*^{G85R/dsod}^{WTLoxP} and *dsod*^{WTLoxP/dsod}^{WTLoxP} are not significantly different from each other. $P < 0.0001$ $P[dsod^{WTLoxP}]/P[dsod^{WTLoxP}]$; $dsod^{G85R}/dsod^{G85R}$, $P[dsod^{WTLoxP}]/+; dsod^{G85R}/dsod^{G85R}$ and $P[dsod^{WTLoxP}]/P[dsod^{WTLoxP}]$; $dsod^{WTLoxP}/dsod^{WTLoxP}$ life spans compared to wild type (*dsod*^{WTLoxP/dsod}^{WTLoxP}).

discrepant with the genetic pattern of inheritance for human fALS in general, mice carrying an endogenous mSOD1^{D83G} point mutation (Joyce *et al.* 2015) created by N-ethyl-N-nitrosourea (ENU) mutagenesis display predominantly recessive phenotypes. These phenotypes include progressive motor and behavioral deficits and loss of muscle force due to degeneration of lower and upper MNs (Joyce *et al.* 2015). Interestingly, SOD1^{null} mice do not display this phenotype, providing strong evidence that ALS-mutant SOD1 can confer both loss of SOD1 enzymatic activity, and a toxic gain of function (Joyce *et al.* 2015). We observed very similar results in *dsod* mutant flies. Most phenotypes we observed were recessive yet clearly more severe than *sod*^{null/null} mutants. These endogenous SOD1 mutants in mice and flies demonstrate that ALS-like phenotypes are achievable in model organisms without the necessity for transgenic overexpression of the mutant allele. In addition, 73% of reported cases of SOD1 mutant canines develop degenerative myelopathy (Zeng *et al.* 2014) and exhibit ALS-like symptoms only when mutant alleles were homozygous.

There are several possible explanations for the apparent recessive nature for our observed phenotypes. First, given the importance of mutant dSOD1 protein dosage in accelerating the ALS-like phenotype in transgenic SOD1-mediated ALS models (Acevedo-Arozena *et al.* 2011), it is possible that

heterozygotes may develop symptoms later in life, or would only display symptoms if they lived well beyond their normal life span. Mice, dogs, and fruit flies have relatively short life spans compared to humans. Since ALS is a late-onset disease in humans (~55 years of age in average), we speculate that extending the life span of heterozygous fruit flies and non-transgenic SOD1 animal models beyond their normal life span might be required to cause more dramatic, late-onset ALS-like phenotypes. Alternatively, there may be some second site modifier or genetic background differences present in model organisms that in wild-type form partially suppress pathogenesis, similar to what is proposed in Scandinavian populations.

A second explanation for lack of dominant inheritance in animal models involves dosage of mutant dSOD protein. The amount of disease-causing mutant protein may be insufficient to cause cellular toxicity in one dose, or one dose of wild-type dSOD confers a novel protective effect in the short life span of animal models. We addressed this possibility in several ways. We observed that lowering mutant *dsod*^{G85R} or *dsod*^{H71Y} gene copy number by half in *dsod*^{G85R/X-16} mutants rescued eclosion (Figure 2, D and E) and larval locomotion defect (Figure 3, B and C). However, adult *dsod*^{G85R/X-16} flies still exhibited very uncoordinated behavior (File S4). Therefore, expressing only one copy of *dsod*^{G85R} is clearly less severe than the two

Figure 10 Phenotype comparison between *dsod*^{H71Y/H71Y} and additional *dsod*^{null} alleles supports gain-of-function toxicity for ALS-associated *dsod* point mutations. (A) Two new *dsod* null lines were generated by removing the *dsod* ORF using CRISPR/cas9, *dsod*^{null21} and *dsod*^{null26}, resulting in no dSOD protein product on SDS-PAGE. (B) *dsod*^{null21/null21} and *dsod*^{null26/null26} lines had similar eclosion ratios as *dsod*^{H71Y}. Error bars are SEM. *** $P < 0.0001$, Fisher's exact test. (C) *dsod*^{null21} and *dsod*^{null26} lines still retain climbing ability by 2 weeks compared to *dsod*^{H71Y/H71Y}. Error bars are SEM. *** $P < 0.001$, one-way ANOVA followed by Dunnett's *post hoc* test. (D) Life span of *dsod*^{null21/null21} and *dsod*^{null26/null26} lines are significantly shorter than *dsod*^{H71Y/H71Y}. Survival curves represent an average of three life-span trials. *** $P < 0.0001$, log-rank test. (E) Summary statistics for life-span analysis.

doses of a homozygote, yet confers much of the severity of the ALS-like phenotype. As such, the phenotypes we see do not result strictly from loss of dSOD activity, but from some toxic gain of function which wild-type dSOD can counter via a protective effect, potentially by dimerization with mutant dSOD.

While the majority of the mutant phenotypes we observed were recessive, heterozygous *dsod*^{G85R/WTLoxP} and *dsod*^{H71Y/WTLoxP} late third instar larvae show locomotor defects. This transient phenotype might stem from the differential gene expression milieu or neuronal physiology between the larval stage and the adult stages. The molecular mechanisms underlying this discrepancy remain to be determined. It is also possible that further experiments with altered environmental conditions such as altered diet, stress levels, or pharmacological interventions could reveal an ALS-like phenotype for *Drosophila* heterozygous *dsod* mutant flies.

Another observation suggesting that our ALS-like symptoms are likely due to a toxic gain of function of mutant dSOD proteins are provided by the transgenic expression of wild-type *dsod* from an engineered locus expressing normal dSOD levels. In this case, doses of wild-type dSOD protein only

partially rescued *dsod*^{G85R/G85R} phenotypes (Figure 9), leading to shortened life span. Similar to human patients developing late-onset ALS, as well as exhibiting a short progression quickly followed by death, $P[dsod^{WTLoxP}]/P[dsod^{WTLoxP}]; dsod^{G85R}/dsod^{G85R}$ flies exhibited locomotor deficits for about a day before dying early (55 days posteclosion) (File S5), which is comparable to ALS patients losing locomotor function over a 1–2 year span before the need for respiratory support (Benditt and Boitano 2008). Thus, we observe that in flies that are “effective” heterozygotes, albeit with two doses each of wild-type and G85R *dsod*, a shortened life span and apparent precipitous loss of locomotor function occurs; arguing again for a toxic gain of function. These flies with four copies of *dsod* (two wild-type copies and two G85R mutant copies) may be most similar to modeling the heterozygote state found in ALS patients with SOD1 mutations.

Even excess dosage of wild-type dSOD appeared to be slightly deleterious in our study. Four copies of wild-type *dsod* ($P[dsod^{WTLoxP}]/P[dsod^{WTLoxP}]; dsod^{WTLoxP}/dsod^{WTLoxP}$) exhibited a shortened life span compared to two-dose wild-type *dsod*^{WTLoxP}/*dsod*^{WTLoxP} flies. This shortening of life span was milder compared to when two G85R alleles were expressed. Previous

studies agree with this finding and underscore the importance of SOD1 protein dosage. Upon overexpression of wild-type hSOD1 in mice, wild-type SOD1 can acquire an abnormal conformation and lead to ALS-like symptoms such as mitochondrial dysfunction, axon degeneration, premature MN death, and SOD1 aggregation (Jaarsma *et al.* 2000; Graffmo *et al.* 2013). The overexpression of hSOD1 also caused locomotion deficits in *Drosophila* (Watson *et al.* 2008). So, while copies of wild-type dSOD might be protective through dimerization with the mutant dSOD or through the restoration of cellular damage induced by oxidative stress due to loss of enzymatic activity, our study agrees with others that elevated levels of normal SOD1 can be deleterious; emphasizing the need to use models with near physiological levels of SOD1 protein.

A consensus has not been reached regarding the nature of disrupted cellular pathways in ALS pathogenesis. Although most of the SOD1 transgenic animals exhibit similar cellular responses such as apoptosis and glia activation, it is not known what proportion of these responses may stem from overexpression of the mutant protein. As one hSOD1^{G85R} transgenic mouse model suggests, in later stages there may be SOD1 protein aggregates in MNs and glial cells (Wang *et al.* 2009b). Homozygous SOD1 mutant dogs also exhibited misfolded SOD1 species (Awano *et al.* 2009; Wininger *et al.* 2011; Crisp *et al.* 2013; Zeng *et al.* 2014). On the other hand, in SOD1 mutant patient induced pluripotent stem cell models, insoluble SOD1 species were detected only after inhibiting the proteasome (Kiskinis *et al.* 2014) or using ultrasensitive methods such as immunogold staining followed by electron microscopy analysis (Chen *et al.* 2014). Homozygous mSOD1^{D83G/D83G} mice lack SOD1-positive proteinaceous inclusions, retain no detectable dismutase activity, and show very little detectable SOD1 protein by denaturing SDS-PAGE (Joyce *et al.* 2015). A lack of protein aggregation in endogenous SOD1 mouse mutants suggests that the SOD1 protein aggregation is not required for ALS pathogenesis; however, we have not determined the state of aggregation in *dsod* mutants.

Previously published *Drosophila* ALS models recapitulate some ALS-like phenotypes such as locomotion deficits, gliosis, reduced synaptic transmission, SOD1 protein aggregation, and mitochondrial defects; however, these models are based on overexpression of hSOD1 (Watson *et al.* 2008; Bahadorani *et al.* 2013). Our data in HR-generated *dsod* mutants agrees with some aspects of this previously published work including a lack of MN loss, the presence of climbing deficits, and a sensitivity to oxidative stress (Watson *et al.* 2008; Bahadorani *et al.* 2013). Our preliminary results suggest that the cell bodies of MNs remain intact even at the terminal stages of *dsod*^{G85R/G85R}. Lack of MN death despite neuromuscular deficits is not surprising because ALS is considered a “dying back” disorder in which muscle denervation precedes the death of the MN cell body (Dadon-Nachum *et al.* 2011). This phenomenon of neuromuscular-junction degeneration preceding MN cell body death was observed in transgenic mutant hSOD1-expressing mouse models (Kato 2008),

a nontransgenic mouse model (Joyce *et al.* 2015), and an early autopsy of an ALS patient who unexpectedly died from unrelated causes (Fischer *et al.* 2004). Neurodegenerative processes do appear to come into play in the later stages of a *dsod* mutant approaching adulthood in *Drosophila*. The *dsod*^{G85R/G85R} pharate adults fail to eclose and show shortened leg phenotypes (Figure 2 and Figure 4). Accompanying this phenotype, the leg nerves appear degenerated, with leg 3 exhibiting a distinctly more severe phenotype than leg 2 and leg 1 (File S1 and File S2). In *dsod*^{H71Y/H71Y}, all legs look wild type upon eclosion. However, by day 14, 87% of leg 3 nerves have lost most of the side branches diverging from the main nerve (Figure 4 and File S3). In short, there appears to be a degenerative process involving at least MNs that correlates with phenotypic severity and is progressive, and yet, does not appear to be the result of MN cell death.

Our study also addresses loss-of-function and gain-of-toxic-function questions at a protein level. The little or no detectable dismutase activity in homozygous *dsod*^{G85R/G85R}, *dsod*^{H71Y/H71Y}, *dsod*^{H48R/H48R}, and *dsod*^{G51S/G51S} mutant lines suggests a loss of SOD1 enzymatic activity in these mutants. While we cannot rule out low levels of enzymatic activity below levels of detection, SOD1 activity seen within these mutants is clearly diminished compared to either heterozygous or homozygous *dsod*^{WTLoxP/WTLoxP}. To assess protein levels of mutant dSOD proteins, we used standard SDS-PAGE followed by immunoblot with antibodies to dSOD. For phenotypic mutants (G85R, H48R, H71Y, and G51S) we observed very little dSOD protein (Figure 7 and Figure S7). *dsod*^{G37R} flies showed normal levels of dSOD protein in addition to being active enzymatically (Figure 6 and Figure 7). In parallel with our findings, SOD1 mutants show dramatically reduced protein amounts on denaturing SDS-PAGE gels in mice homozygous for an endogenous mSOD1^{D83G} point mutation (~10% of wild type in homozygotes) (Joyce *et al.* 2015) and patient-induced pluripotent stem cell-derived MNs (Chen *et al.* 2014; Kiskinis *et al.* 2014). These results have been explained by the instability of mutant SOD1 and its subsequent degradation (Kabuta *et al.* 2006). It has been previously reported that mutant SOD1, especially SOD1^{G85R}, has a decreased half-life when compared to wild-type SOD1 (Borchelt *et al.* 1994; Farr *et al.* 2011). This is in agreement with current literature suggesting that hSOD1^{G85R} monomers are unable to form dimers with each other or with mSOD1 and hSOD1 (Wang *et al.* 2009b).

Given the results of all attempts to detect dSOD mutant proteins from animals carrying phenotypic mutations, we were thus surprised by our immunoblot results using native gel electrophoresis. In all cases, mutant dSOD proteins were detected in homozygous animals. In fact, levels of protein on the blots were similar to wild-type controls, and much of the protein was found at the size of the normal dSOD dimer (Figure 7). However, in homozygous mutants, there also appeared a faster migrating species that we infer is monomeric dSOD, and this species represented a substantial amount of dSOD immunoreactivity in homozygotes. In

heterozygotes, again, roughly normal amounts of dSOD proteins are seen, but the anomalous fast migrating band is less intense. We interpret this as dimer formation between mutant and wild-type dSOD proteins. Further experiments will be necessary to confirm this speculation. Nevertheless, the data strongly suggest that all of the phenotypic mutants characterized in this study produce substantial amounts of dSOD protein, which is a prerequisite for generating a toxic gain of function. In addition, the data suggest a potential mechanism (dimerization) for alleviating phenotypes caused by homozygosity of toxic mutations. Recent research has shown that small, soluble monomers of SOD1, rather than insoluble aggregates, are likely to be the cytotoxic species causing neurodegeneration (Rakhit *et al.* 2007; Auclair *et al.* 2010; Liu *et al.* 2012).

Lastly, our newly generated *dsod^{null}* mutations confirm results from other studies in a conclusive manner (Reaume *et al.* 1996; Huang *et al.* 1997; Ho *et al.* 1998; Matzuk *et al.* 1998; Yoshida *et al.* 2000; Saccon *et al.* 2013). *dsod^{null}* mutants display eclosion defects, shortened life spans and infertility (Figure 10). However, their life span is not nearly as short as *dsod^{H71Y/H71Y}* homozygotes, and they do not show any locomotor deficits until much later in life. All of our phenotypic mutants (H48R, G85R, and H71Y) as homozygotes show undetectable levels of dSOD activity in gel assays, yet have much more severe phenotypes than the homozygote *dsod^{null/null}* mutants. We conclude from this that the phenotypes we observe in animals are a combination of both loss- and gain-of-function components. Recently, it has been strongly suggested that dominantly inherited human SOD1-based ALS is modified additionally by the loss of function of SOD1 activity, as most patients with mutations demonstrate an *in vivo* loss of activity (Saccon *et al.* 2013). Further studies will be necessary to untangle the loss- and gain-of-function contributions to disease phenotype, but our studies raise an interesting conjecture that the mutation G37R, which shows SOD1 activity but no phenotype as homozygotes, may be dominated by the gain-of-function component of the proposed mechanism. Thus, these mutations may be useful if under appropriate conditions or treatments, a phenotype can be revealed in heterozygotes.

Our *Drosophila* model provides a rich, fast, and efficient system complementary to rodent model organisms for addressing mechanisms associated with human SOD1 mutations causing ALS and for elucidating the dosage-sensitive results of SOD1-mediated ALS, as well as unraveling the contributions of loss and gain of function to mutant phenotypes. Using *Drosophila* to model ALS provides a unique system in which to assess effects on conserved molecular pathways fundamental to neuronal function and dysfunction in ALS. Moreover, the severe phenotypic consequences of some of these mutations provide an excellent motivation for unbiased forward genetic suppressor screens to identify genes that, when mutated, can reverse the effects of these human disease-causing mutations; potentially serving as the foundation for novel therapeutic approaches acting through such genes.

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