Survival Following Traumatic Brain Injury in *Drosophila* Is Increased by Heterozygosity for a Mutation of the NF-κB Innate Immune Response Transcription Factor Relish

Laura C. Swanson,*,† Edna A. Trujillo,‡,§ Gene H. Thiede,* Rebeccah J. Katzenberger,* Evgenia Shishkova,§, Joshua J. Coon,‡,§,**,†† Barry Ganetzky,‡‡ and David A. Wassarman*,1**

*Department of Medical Genetics, †Medical Scientist Training Program, School of Medicine and Public Health, ‡Department of Chemistry, College of Letters & Science, §National Center for Quantitative Biology of Complex Systems, **Department of Biomolecular Chemistry, School of Medicine and Public Health, ‡‡Department of Genetics, College of Agricultural and Life Sciences, University of Wisconsin-Madison, Madison, Wisconsin 53706, and ††Morgridge Institute for Research, Madison, Wisconsin 53706

ORCID IDs: 0000-0002-9438-7546 (L.C.S.); 0000-0002-8551-9199 (E.A.T.); 0000-0001-6097-6097 (E.S.); 0000-0002-0004-8253 (J.J.C.); 0000-0001-5418-7577 (B.G.); 0000-0002-7042-9561 (D.A.W.)

ABSTRACT Traumatic brain injury (TBI) pathologies are caused by primary and secondary injuries. Primary injuries result from physical damage to the brain, and secondary injuries arise from cellular responses to primary injuries. A characteristic cellular response is sustained activation of inflammatory pathways commonly mediated by nuclear factor-κB (NF-κB) transcription factors. Using a *Drosophila melanogaster* TBI model, we previously found that the main proximal transcriptional response to primary injuries is triggered by activation of Toll and Imd innate immune response pathways that engage NF-κB factors Dif and Relish (Rel), respectively. Here, we found by mass spectrometry that Rel protein level increased in fly heads at 4–8 hr after TBI. To investigate the necessity of Rel for secondary injuries, we generated a null allele, Rel^{del}, by CRISPR/Cas9 editing. When heterozygous but not homozygous, the Rel^{del} mutation reduced mortality at 24 hr after TBI and increased the lifespan of injured flies. Additionally, the effect of heterozygosity for Rel^{del} on mortality was modulated by genetic background and diet. To identify genes that facilitate effects of Rel^{del} on TBI outcomes, we compared genome-wide mRNA expression profiles of uninjured and injured +/+, +/Rel^{del}, and Rel^{del}/Rel^{del} flies at 4 hr following TBI. Only a few genes changed expression more than twofold in +/Rel^{del} flies relative to +/+ and Rel^{del}/Rel^{del} flies, and they were not canonical innate immune response genes. Therefore, Rel is necessary for TBI-induced secondary injuries but in complex ways involving *Rel* gene dose, genetic background, diet, and possibly small changes in expression of innate immune response genes.

KEYWORDS *Drosophila melanogaster*; innate immunity; traumatic brain injury; proteomics; gene expression

TRAUMATIC brain injury (TBI) affects 1.7 million people in the USA annually and is one of the leading causes of death and disability worldwide (Dixon 2017; Maas *et al.* 2017). Despite its prevalence, treatments have not yet been developed that effectively reduce consequences of TBI, which can include cognitive impairment, emotional lability, post-traumatic epilepsy, and death (Zaloshnja *et al.* 2008; Bazarian *et al.* 2009; Diamond *et al.* 2015; Tortella 2016; Juengst *et al.* 2017; DeWitt *et al.* 2018; Ng and Lee 2019). Neurologic damage following TBI results from both primary and secondary injuries. Primary injures are inflicted by mechanical forces on the brain that compress, stretch, displace, or tear blood vessels, neurons, and glia. In contrast, secondary injuries result from activation of cellular and molecular pathways in the minutes to years following the initial physical insult and can occur at and beyond the site of the primary injury (Pearn *et al.* 2017; Ng and Lee 2019). Primary injuries are

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[1]Corresponding author: Department of Medical Genetics, School of Medicine and Public Health, University of Wisconsin-Madison; Room 4262, Genetics/Biotechnology Bldg., 425-G Henry Mall, Madison, WI 53706. E-mail: dawassarman@wisc.edu

mainly mitigated through preventive measures such as helmets, but it remains to be determined how best to ameliorate secondary injuries (Hatton 2001; Somayaji *et al.* 2018).

Neuroinflammation is an immediate cellular response to TBI that plays both pathological and protective roles (Simon *et al.* 2017). This dichotomy is illustrated by studies of transcription factors of the nuclear factor-κB (NF-κB) family, which are major transcriptional activators of inflammatory genes. Direct inhibition of NF-κB in neurons through genetic means increases acute mortality and neurological deficits following TBI in mice (Mettang *et al.* 2018). In contrast, indirect inhibition of NF-κB by a variety of pharmacological agents, including metformin, ghrelin, resveratrol, ethylpyruvate, curcumin, allyl isothiocyanate, omega-3 polyunsaturated fatty acid, and pioglitazone, improves outcomes following TBI in mice or rats (Laird *et al.* 2010; Su *et al.* 2011; Feng *et al.* 2016; Chen *et al.* 2018; Tao *et al.* 2018; Caglayan *et al.* 2019; Deng *et al.* 2020; Shao *et al.* 2020). Adding further complexity to the neuroinflammatory response, individual transcriptional targets of NF-κB, including cytokines and chemokines, differentially contribute to TBI outcomes (Shohami *et al.* 1997; Scherbel *et al.* 1999; Sullivan *et al.* 1999; Ziebell and Morganti-Kossmann 2010; Di Battista *et al.* 2016). Therefore, NF-κB is a prime candidate target for TBI therapies, but development of maximally effective therapies will require further investigation of the functional relationship between NF-κB activation and other factors that affect progression of secondary injuries.

Drosophila melanogaster encodes three NF-κB homologs, Dorsal (Dl), Dorsal-related immunity factor (Dif), and Relish (Rel) (Lemaitre and Hoffmann 2007; Ganesan *et al.* 2011). Dl functions in formation of dorsal-ventral polarity in embryos, whereas Dif and Rel function in cellular and humoral innate immunity in defense against pathogen infection. Dif functions in the Toll pathway and Rel functions in the Immune-deficiency (Imd) pathway to activate transcription of genes such as antimicrobial peptide (AMP) genes that produce resistance to infection. While the Toll and Imd pathways have beneficial effects in the context of infection, chronic activation of either pathway can promote neurodegeneration (Tan *et al.* 2008; Chinchore *et al.* 2012; Petersen *et al.* 2012; Petersen and Wassarman 2012; Cao *et al.* 2013; Kounatidis *et al.* 2017). Additionally, chronic activation of the Imd pathway through a variety of means reduces lifespan, whereas mutation of *Rel* increases lifespan and suppresses neurodegeneration (Cao *et al.* 2013; Kounatidis *et al.* 2017). In mammals, Toll-like receptor (TLR)/Interleukin-1 receptor (IL-1R) pathways and the Tumor necrosis factor-α receptor (TNFR) pathway are homologous to the Toll and Imd pathways, respectively (Tanji and Ip 2005). Studies of TLR and TNFR pathways in mammalian TBI models provide an equally complex picture as studies of NF-κB. Knockout of *TLR2* or *TLR4* in mice improves neurological function following TBI, whereas knockout of *TNFR* in mice increases lesion volume following TBI (Sullivan *et al.* 1999; Zu and Zha 2012; Laird *et al.* 2014; Jiang *et al.* 2018; Shi *et al.* 2019). Because innate immune pathways are evolutionarily conserved between flies and mammals, studies of flies, which have a simpler immune system and a more extensive experimental toolbox than rodents, may provide novel insights into roles that NF-κB-mediated TLR and TNFR pathways play in determining TBI outcomes (Dhankhar *et al.* 2020).

We and others have developed fly TBI models to investigate the pathways that control development of secondary injuries following TBI (Katzenberger *et al.* 2013, 2015a, 2015b; Barekat *et al.* 2016; Sen *et al.* 2017; Lee *et al.* 2019; Putnam *et al.* 2019; Sanuki *et al.* 2019; Shah *et al.* 2019; Saikumar *et al.* 2020). Our model uses a spring-based instrument called a High-impact trauma (HIT) device to inflict physical trauma by rapid acceleration–deceleration forces (Katzenberger *et al.* 2013, 2015a,b,c, 2016; Fischer *et al.* 2018; Swanson *et al.* 2020). Strikes from the HIT device affect both the fly head and body, but the main pathologies appear to be primarily driven by injuries to the brain. Flies that have sustained a TBI share behavioral and physiologic characteristics with mammals, including temporary incapacitation, ataxia, transient hyper-glycemia, intestinal barrier dysfunction, progressive neurodegeneration, and reduced lifespan. Additionally, like mammals, the innate immune response in flies is rapidly and persistently activated following TBI (Katzenberger *et al.* 2013, 2015a,b; Barekat *et al.* 2016; Sanuki *et al.* 2019). Expression of AMP genes substantially increases within 30 min after TBI and is sustained for >24 hr (Katzenberger *et al.* 2016). Furthermore, AMP gene expression contributes to TBI outcomes, as mutation of some individual AMP genes can suppress or enhance outcomes (Swanson *et al.* 2020).

Here, we show that *Rel* is a dose-dependent modifier of TBI outcomes. Proteomics analysis revealed that the relative abundance of Rel protein increased in fly heads shortly after a primary injury. Genetic analysis revealed that heterozygosity, but not homozygosity, for a null mutation of *Rel* (Rel^{del}) reduced detrimental consequences of TBI. Finally, gene expression analysis differentiated transcriptional targets of the Toll and Imd pathways following TBI and identified gene expression changes that may underlie the beneficial effects of heterozygosity for Rel^{del}.

Materials and Methods

Fly lines and culturing

Flies were maintained at 25° on cornmeal molasses food containing 30 g Difco granulated agar (Becton-Dickinson, Sparks, MD), 44 g YSC-1 yeast (Sigma, St. Louis, MO), 328 g cornmeal (Lab Scientific, Highlands, NJ), 400 ml unsulfured Grandma's molasses (Lab Scientific), 3.6 L water, 40 ml propionic acid (Sigma), and tegosept (8 g methyl 4-hydroxybenzoate in 75 ml of 95% ethanol) (Sigma). In Figure 4, flies were fed water by placing 200 μ liters of water on a filter paper disk at the bottom of a vial. w^{1118} flies used for proteomics analyses were also used in our prior

analyses of TBI (Katzenberger *et al.* 2013; 2015a; 2016). Rel^{E20} flies and w^{1118} flies to which Rel^{E20} flies were backcrossed, were provided by Stanislava Chtarbanova (University of Alabama, Alabama). Drosophila Genetic Reference Panel (DGRP) lines were obtained from the Bloomington Stock Center (Mackay *et al.* 2012).

Fly head collection for proteomics analysis

For proteomics analysis of fly heads after TBI, 0- to 7-day-old male w^{1118} flies were subjected to four strikes from the HIT device, with 5 min between strikes. Following injury, flies were transferred to cornmeal molasses food at 25° until the time of collection: immediately (0), 0.5, 1, 2, 4, 6, 8, 12, 16, and 24 hr. As controls, 0- to 7-day-old w^{1118} male uninjured flies were cultured under the same conditions and collected at the same time points. Fly heads were removed from bodies by freezing flies in liquid nitrogen, vortexing the frozen flies, and separating heads from bodies by passing them through a sieve. Head samples from injured and control uninjured flies were stored at −80° until all samples were ready for analysis. Each sample contained ∼200 heads, and three independent samples were prepared for each condition.

Preparation of fly heads for proteomics analysis

Fly head samples were resuspended in 200 μl of 6 M guanidine hydrochloride in 100 mM tris(hydroxymethyl)-aminomethane (Tris) pH 8.0 and maintained at 4°. Rupturing of heads was performed by bead-beating using 2.8-mm metal beads and four rounds of beating at 30 Hz for 5 min with 1 min of rest between each round at 4°. Cell lysis occurred with two cycles of heating at 100° for 5 min followed by 5 min of cooling at ambient temperature. The protein concentration of each sample was determined by the BCA Protein Assay Kit (Pierce, Rockford, IL). Proteins were precipitated by addition of methanol up to a final concentration of 90% by volume followed by centrifugation for 20 min at 15,000 × *g*. Supernatant was removed, and protein pellets were air-dried at room temperature.

Protein digestion followed previously developed protocols from Hebert *et al.* (2014) and Richards *et al.* (2015) with the following specific changes. Protein pellets were resuspended in 50 μl of lysis buffer containing 8 M urea, 100 mM Tris (pH 8.0), 10 mM TCEP, and 40 mM chloroacetamide to denature, reduce, and alkylate proteins. Sonication for 10 min ensured that proteins were in solution. The resuspended protein solution was diluted to 1.5 M urea with 100 mM Tris (pH 8.0) and vortexed for 30 sec. Endoproteinase LysC was added at a 100:1 ratio, trypsin was added at a 50:1 protein to enzyme mass ratio, and samples were incubated for 12 hr at room temperature. After incubation, each sample was prepared for desalting using a 96-well Strata polymeric reversed phase 10-mg SPE (styrene divinylbenzene) cartridge. Cartridge wells were primed with 1 ml of acetonitrile (ACN) then followed by 1 ml of 0.1% trifluoroacetic acid (TFA). Each sample was acidified with TFA to a final pH of 2.0 or less and then centrifuged for 15 min at 2000 X g, spinning all nonprotein material to the bottom of the vial. Acidified samples were loaded on to the cartridge, washed with 1 ml of 0.1% TFA, and eluted with 600 μl of 80% ACN, 0.1% TFA into a clean 96-well plate to be dried. Eluted peptides were vacuum dried and resuspended in 0.2% formic acid. Peptide mass was assayed with the NanoDrop Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

LC-MS/MS analysis

For each analysis, 1 μg of peptides was loaded onto a 75 × 360 μm (ID × OD) 30-cm-long column with an integrated electrospray emitter packed with a 1.7-m-particle-size C18 BEH column (Waters, Milford, MA) (Shishkova *et al.* 2018). The mobile phases used were as follows: phase A consisted of 0.2% formic acid, and phase B consisted of 0.2% formic acid in 70% ACN. Peptides were eluted with a gradient of acetonitrile increasing from 0 to 55% B over 100 min followed by a 1-min increase to 100% B, 2 min wash at 100% B, 3 min descend to 0% B and a final 10 min of equilibration in 100% A for a total of a 120-min analysis.

Eluted peptides were analyzed with an Orbitrap Fusion Lumos Tribrid Mass Spectrometer (Thermo Fischer Scientific, Waltham, MA). Survey scans were performed at a resolution of 240,000 in the range of m/z 300 to 1,350 and 1e+06 automatic gain control (AGC) target using quadrupole isolation of 0.7 Da for data-dependent tandem MS (MS/MS) scans. MS/MS scans were collected using the top-speed mode with 1-sec cycle and dynamic exclusion of 15 sec on precursors with charge states 2 to 4. Isolated precursors were fragmented by higher-energy collisional dissociation with a normalized collisional energy of 25%. Mass analysis of product ions was performed in the ion trap using the "turbo" scan rate over the mass range of 150–1400 m/z with a maximum inject time of 17 ms and the normalized AGC target of 3e+4 (Trujillo *et al.* 2019).

Analysis of proteomics data

Thermo RAW files were processed using MaxQuant (version 1.6.0.16) (Tyanova *et al.* 2016). Searches were performed against a target decoy database of *D. melanogaster*, including isoforms (UniProt; downloaded August 8, 2019), using the Andromeda search engine (Tyanova *et al.* 2016). Quantitation was performed as label-free quantitation (LFQ) with an LFQ minimum ratio of 1. "Match between runs" option was enabled. Search parameters included fixed modification for carbamidomethylation of cysteine residues, variable modification for oxidation of methionine, N-terminal acetylation, and a maximum of two missed cleavages. The peptide spectral match false-discovery rate (FDR) and protein FDR were both set to 1%.

Using RStudio (v 1.2.5033), LFQ values and protein intensities for each sample were $\log_2$ transformed and filtered to retain proteins that had no missing values across three replicates and fulfilled a >50% cutoff of measurements across all the samples. A total of 277,020 protein measurements were obtained from the fly heads, resulting in 4860 unique

proteins with an interreplicate coefficient of variation of 14.49% for 20 different conditions that differed in time as well as TBI status. Normalization across the three batches was performed by transforming each protein by the mean average of each respective protein at time zero by condition. The average and standard error of the mean (SEM) were calculated from normalized protein LFQ values across the three replicates. Due to biological variability among the fly replicates, we did not correct *P*-value calculations and instead looked for the proteins that had a clear change in abundance between uninjured and injured flies across the time points. Fold changes were determined for each time point and are relative to control time zero. We employed a partial least square discriminant analysis using R (pls package) to reveal the top 25 discriminating proteins between control and TBI samples regardless of time point.

Generation of Rel^{del} by CRISPR/Cas9 gene editing

A *Rel* null allele was generated by CRISPR/Cas9 homology directed repair using the pHD-DsRed-attP vector (Plasmid #51019; Addgene) as the donor template (Gratz *et al.* 2014). One-kilobase left and right homology arms were generated immediately flanking the *Rel* cleavage sites (5′ cleavage site: −99 bp; 3′ cleavage site: +4219 bp, relative to the transcription start site). The homology arms were amplified via PCR with 20-bp overhangs homologous to the pHD-DsRed-attP vector, and the vector was amplified via PCR with 20-bp overhangs homologous to the homology arms. PCR products were purified with the Wizard SV Gel and PCR cleanup system (Promega, Madison, WI). The editing plasmid was constructed by Gibson assembly of the four DNA fragments (Gibson *et al.* 2009). Synthetic guide RNA (sgRNA) plasmids were generated using the pU6-3 gRNA vector generously provided by the O'Connor-Giles lab (Brown University). Guide sites were selected using flyCRISPR optimal target finder (Gratz *et al.* 2014). Both sgRNAs had no predicted off-targets. sgRNA plasmids were constructed by amplifying sgRNAs and the pU6-3 gRNA vector by PCR with 20 bp overhangs. PCR products were purified with the Wizard SV Gel and PCR cleanup system (Promega). sgRNA plasmids were assembled using the Kinase Ligase Dpn (KLD) mix (New England Biolabs, Ipswich, MA). sgRNA plasmid sequences were confirmed by sequencing. Plasmid DNA was prepared for injection by a mini- or midiprep kit (Qiagen, Hilden, Germany) and was injected into vas-Cas9(II) embryos (Stock #56552; Bloomington Drosophila Stock Center) by BestGene. Genetically identical control lines were developed from the injection stock. Positive transformants were identified through the fluorescent DsRed eye marker. Cas9 was removed from the stocks by backcrossing to the injection stock that did not contain Cas9.

Mortality and lifespan assays

Flies were injured using a HIT device as described by Katzenberger *et al.* (2013). Flies were injured by four strikes at 10-min intervals with the spring deflected to 90°. Except where stated otherwise, TBI was inflicted using this protocol. All vials contained 60 flies (30 males and 30 females) at 1–7 days old. We used a 1- to 7-day age range, as opposed to a smaller age range, because it was easier to collect a sufficient number of flies for the experiments, and we previously found that sample-to-sample variability of the MI_{24} of 1- to 7-day-old flies was similar to that of smaller age ranges (Katzenberger *et al.* 2013). The average mortality at 24 hr for each uninjured fly line did not exceed 2% on food and 5% on water, in line with previous studies using this model (Katzenberger *et al.* 2013, 2015a, 2016; Fischer *et al.* 2018). The exception was the *Rel^{E20}* line, which had an average uninjured mortality of 4.3% on food and 8.7% on water.

Lifespan was determined using 2- to 8-day-old flies that survived 24 hr following TBI. At least four vials containing 20 flies (10 males and 10 females) were examined per condition, and each condition was independently repeated three times ($n \geq 120$ per sex). Vials were maintained at 25° and scored for survival every 3 days. Percent survival was averaged among vials for each condition.

qRT-PCR analysis

Total RNA was extracted from 20 uninjured or injured whole male flies recovered on food for 0, 2, 4, 6, or 8 hr following TBI. RNA extraction was performed using Trizol (Invitrogen, Carlsbad, CA), according to a modified protocol described by Bogart and Andrews (2006). RNA purification was performed using the RNeasy Mini Kit and RNase-Free DNase (Qiagen, Hilden, Germany). For each sample, 1 μg of RNA was reverse transcribed using the iSCRIPT complementary DNA synthesis kit (Bio Rad, Hercules, CA). Quantitative PCR was performed using iTaq Universal SYBR Green SuperMix (Bio Rad) and the Bio Rad CFX96 Real-Time PCR Detection System. Biological replicates of each condition were performed in triplicate, and technical replicates were performed in duplicate. Primer sequences are shown in Supplemental Material, Table S6.

Statistical analyses

All data are presented as means ± SEM. A two-way ANOVA with Tukey's *post hoc* analysis was used to compare MI_{24} outcomes of *Rel* mutants to controls on food and water (Figures 4, A and B). A two-tailed Student's unpaired *t*-test was used to compare MI_{24} outcomes of RAL/+ and RAL/*Rel^{del}* flies (Figure 4C). Statistical differences in survival were quantified using a log-rank test (Figure 5 and Figure S2). For comparisons across multiple groups for qRT-PCR a two-way ANOVA followed by a Tukey's *post hoc* test was applied ($\alpha = 0.05$, number of comparisons = 2) (Figure 3C and Figure 6). Statistical analysis of the qRT-PCR time course of control expression compared to +/*Rel^{del}* flies was performed using a Student's unpaired *t*-test for each time point (Figure 7). All statistical analyses were performed using GraphPad Prism 8.

RNA-seq

mRNA isolation from whole flies, construction of mRNA libraries, high-throughput sequencing, and analysis of RNA-seq data were performed as described in Katzenberger *et al.* (2016).

Data availability

All flies and reagents used in the study will be made publicly available. Proteomics data sets are available at ProteomeXchange via the PRIDE database (accession number PXD021869). RNA-seq data sets are available at GEO repository (accession number GSE157102). Figure S1 shows Multidimensional scaling (MDS) analysis of RNA-seq data sets described in Figure 8 and Table 1. Table S2 contains statistical analyses of data in Figure 5 that are summarized in Table S1. Table S3 lists genes categorized in Figure 8, A and B. Table S4 lists genes categorized in Figure 8, C and D. Table S5 lists genes categorized in Figure 8, E and F. Table S6 contains the sequence of primers used for qRT-PCR analysis. The authors affirm that all other data necessary for confirming the conclusions of the article are present within the article, figures, and tables. Supplemental material available at figshare: https://doi.org/10.25386/genetics.13072988.

Results

Proteomics analysis identifies proteins whose expression discriminates between uninjured and injured fly heads

We used bottom-up proteomics analysis to investigate longitudinal changes in fly head protein expression following TBI. In this study, time and injury were the two factors taken into consideration for identifying biological processes that are perturbed during repair and degeneration associated with TBI. Whole fly heads from 0- to 7-day-old male w^{1118} flies were collected across 10 time points ranging from immediately following injury to 24 hr after injury. In addition, all time points included uninjured control flies of the same age, sex, and genotype, providing a proteomics abundance baseline for comparison. The Material and Methods section supplies a detailed description of how flies were injured, heads were collected, and proteins were extracted and analyzed. In brief, flies were injured, fly heads were collected from uninjured and injured flies by freezing them in liquid nitrogen and decapitation by vortexing; head tissues and cells were disrupted by bead-beating and heating, and proteins were extracted with methanol and enzymatically digested to peptides. Peptides were identified using nanoflow liquid chromatography coupled to tandem mass spectrometry (nLC-MS/MS), and quantified using LFQ (Cox *et al.* 2014).

A partial least squares discrimination analysis (PLS-DA) model revealed which proteins were responsible for the distinction between samples of uninjured and injured fly heads, regardless of time after TBI (Figure 1A). The list of proteins that drove separation between the two groups was enriched for those belonging to mitochondrial bioenergetics processes, neurosecretory cells, neuronal injury, markers of oxidative stress, and innate immune response pathways. The top 25 proteins in Component 1 of the PLS-DA included Rel and two other innate immune response proteins: Drs (Drosomysin) – an AMP; and GNBP-like3 (gram-negative bacteria binding protein-like3) – a pattern recognition receptor (Figure 1B). Thus, the proteomics analysis identified a protein expression signature that distinguishes uninjured from injured heads during the 24 hr after TBI.

Volcano plots of the data showed that the amount of only a few proteins was significantly different between uninjured and injured heads (Figure 1, C–E). We defined significance as having a $\log_2$ fold change >1 and P-value < 0.05. At 4, 6, and 8 hr after TBI, amounts of two, three, and nine proteins, respectively, were significantly altered between uninjured and injured fly heads. Rel was the only protein whose abundance was significantly changed at all three of these time points. Thus, TBI is associated with small, transient changes in protein expression in the head. Analysis of Rel expression over the 24-hr time course showed that its expression rapidly increased between 1 and 2 hr, peaked at 6 hr, and gradually declined thereafter (Figure 2). This time course is similar to the time course of expression of AMP genes as well as secondary injuries that lead to early mortality following TBI, suggesting that Rel is necessary for these events (Katzenberger *et al.* 2015a, 2016).

Generation of a null allele of Rel

To assess the necessity of Rel for secondary injuries following TBI, we used CRISPR/Cas9 gene editing by homology directed repair to generate a null allele, Rel^{del} (Figure 3A). The rationale behind generating a new *Rel* mutant line was to maintain a uniform genetic background between control and *Rel* mutant flies, since genetic background significantly influences outcomes of TBI (Katzenberger *et al.* 2013; 2015a, 2016). We deleted the coding region of *Rel* in a control fly line and replaced it with *DsRed*, which encodes a red fluorescent protein that we used to monitor the genotype of Rel^{del} flies. We confirmed the genotypes of $+/Rel^{del}$ and Rel^{del}/Rel^{del} flies by PCR analysis of genomic DNA extracted from whole flies (Figure 3B). In addition, qRT-PCR analysis of RNA extracted from whole flies showed that *Rel* mRNA expression was reduced in $+/Rel^{del}$ flies, although it did not reach statistical significance ($P = 0.11$), and eliminated in Rel^{del}/Rel^{del} flies that were either uninjured or injured ($P < 0.0001$) (Figure 3C). These data indicate that Rel^{del} is a null allele. Expression of *Rel* was significantly increased in injured relative to uninjured $+/+$ and $+/Rel^{del}$ flies at 4 hr after injury, based on a Student's *t*-test ($P = 0.012$ and 0.017, respectively). However, when this comparison was analyzed together with *Rel* expression from uninjured and injured $+/Rel^{del}$ and Rel^{del}/Rel^{del} flies using a two-way ANOVA and Tukey's *post hoc* test, it did not meet the threshold for significance (Figure 3C). Nevertheless, these findings are in agreement with the proteomics data collected from fly heads that demonstrated an increase in the amount of Rel protein 4–8 hr after injury (Figures 1 and 2).

$+/Rel^{del}$ flies are resistant to TBI-induced mortality

To investigate a role for Rel in acute outcomes of TBI, we measured the mortality index at 24 hr (MI_{24}), which represents the percent mortality of injured flies normalized to the

Table 1 Gene targets of innate immune response pathways in injured and uninjured flies

Gene	Direct or indirect targets of non-Imd pathways after TBI[a]	Direct targets of the Imd pathway after TBI[b]	Direct or indirect targets of the Imd pathway in the absence of injury[c]	Direct or indirect targets of the Imd pathway after TBI[d]
AttA	AttA		AttA	
AttB	AttB		AttB	AttB
AttC	AttC		AttC	AttC
AttD		AttD		AttD
CecA1			CecA1	CecA1
CecA2		CecA2	CecA2	CecA2
CecB		CecB		CecB
CecC		CecC		CecC
Def		Def	Def	Def
DptA		DptA	DptA	DptA
DptB	DptB		DptB	DptB
Dro	Dro		Dro	Dro
Drs	Drs		Drs	
Mtk	Mtk		Mtk	
BomBc1 (IM23)	BomBc1		BomBc1	
BomS1 (IM1)	BomS1		BomS1	
BomS4 (CG18107)		BomS4		
BomBc2 (CG15067)	BomBc2		BomBc2	
BomT3 (CG5778)	BomT3			
BomBc3 (CG5791)	BomBc3			
BomS5 (CG15065)	BomS5			
BomT2 (CG16836)	BomT2			
BomS3 (IM3)	BomS3			
BomT1 (CG43202)	BomT1			
IM2	IM2			
IM4 (Daisho1)	IM4			
IM14 (Daisho2)	IM14			
IM33		IM33		
TotA	TotA			
TotC	TotC			
TotM	TotM		TotM	TotM
TotX	TotX			
Listericin	Listericin		Listericin	Listericin
PGRP-LB	PGRP-LB			
PGRP-SA	PGRP-SA			PGRP-SA
PGRP-SB1	PGRP-SB1			PGRP-SB1
PGRP-SC1b			PGRP-SC1b	PGRP-SC1b
PGRP-SC2				PGRP-SC2
PGRP-SD	PGRP-SD		PGRP-SD	
Pirk	Pirk			Pirk
GstD1				GstD1
GstD2	GstD2		GstD2	GstD2
GstD3			GstD3	GstD3
GstD4		GstD4		GstD4
GstD5	GstD5		GstD5	GstD5
GstD6			GstD6	GstD6
GstD7				GstD7

[a] Genes upregulated in injured relative to uninjured flies for +/+, +/Rel^{del}, and Rel^{del}/Rel^{del} genotypes (Figure 8E).
[b] Genes upregulated in injured relative to uninjured flies for +/+ and +/Rel^{del} genotypes (Figure 8E).
[c] Genes downregulated in uninjured Rel^{del}/Rel^{del} flies relative to uninjured +/+ flies (Figure 8B).
[d] Genes downregulated in injured Rel^{del}/Rel^{del} flies relative to injured +/+ flies (Figure 8D).

percent mortality of uninjured flies 24 hr following TBI. The MI_{24} is a reproducible measure, and it correlates with other consequences of TBI, including incapacitation and intestinal barrier permeability as well as the median lifespan of uninjured flies (Katzenberger *et al.* 2013, 2015a; Fischer *et al.* 2018). While mortality following TBI in flies should not be equated with mortality following TBI in mammals, the molecular and cellular events that lead to mortality in flies are likely to be conserved in mammals and to contribute to the pathophysiology of TBI in mammals.

We examined the MI_{24} of injured 1- to 7-day-old mixed-sex control flies (+/+), homozygous *Rel* mutant flies (Rel^{del}/Rel^{del}), and heterozygous *Rel* mutant flies (+/Rel^{del}) that were generated by crossing +/+ and Rel^{del}/Rel^{del} flies. Flies were

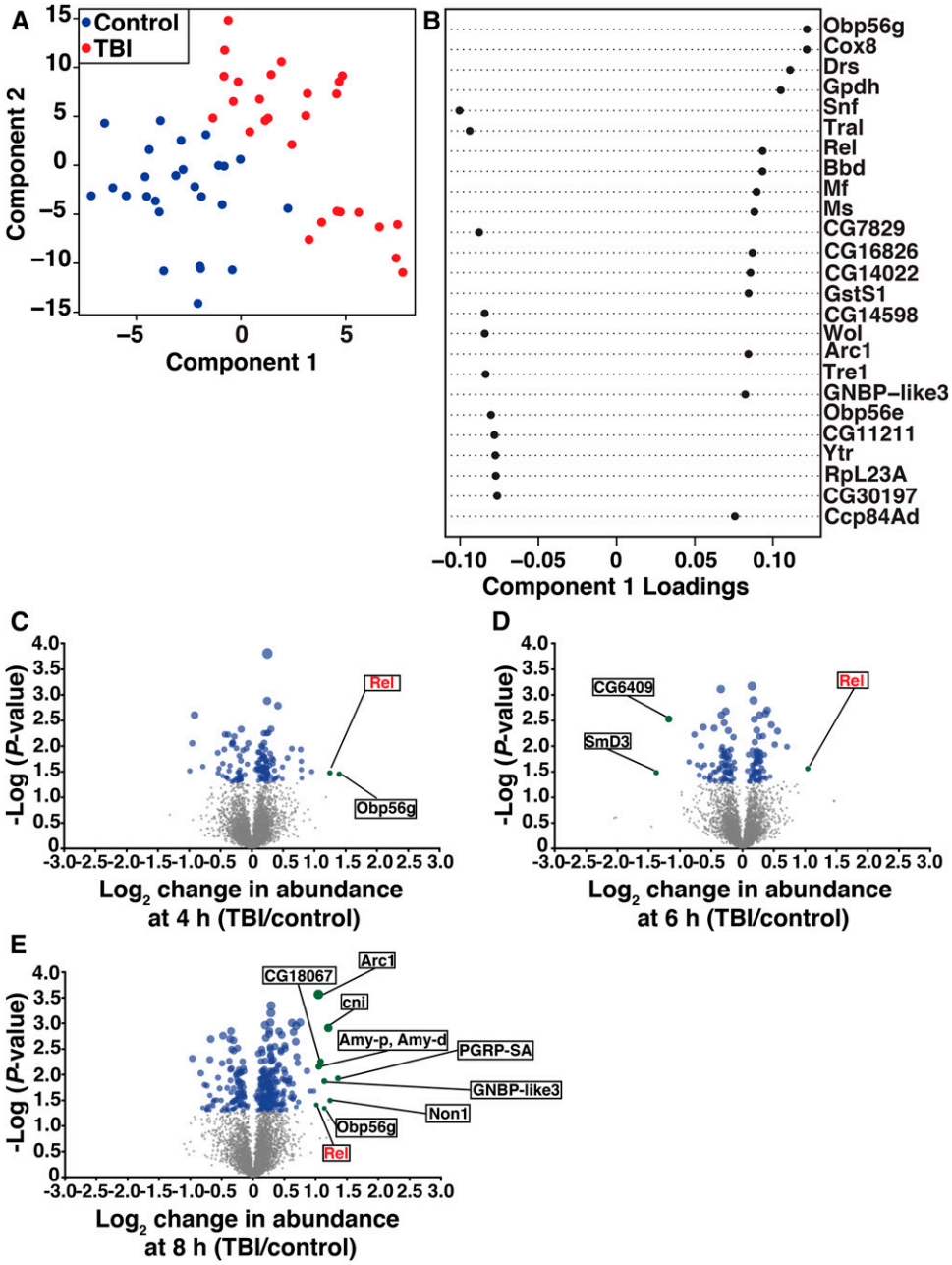

Figure 1 Proteomics analysis of fly heads following TBI. (A) PLS-DA of protein expression in head of uninjured (control) and injured (TBI) 0- to 7-day-old male w^{1118} flies. Each dot represents an independent sample at 0, 0.5, 1, 2, 4, 6, 8, 12, 16, or 24 hr after TBI. (B) The top 25 genes that drove Component 1 separation. Volcano plots of protein abundance differences and *P*-values between uninjured and injured flies at (C) 4, (D) 6, and (E) 8 hr after TBI, respectively. Blue dots indicate proteins that met the $P < 0.05$ significance cutoff, green dots indicate proteins that met both the $P < 0.05$ significance cutoff and the twofold change cutoff, and gray dots indicate proteins that met neither cutoff.

injured and incubated for 24 hr at 25° with cornmeal molasses food (hereafter referred to as food). +/+ and Rel^{del}/Rel^{del} flies had a similar MI_{24}, whereas $+/Rel^{del}$ flies had a significantly lower MI_{24} than +/+ flies (Figure 4A). The same effects on the MI_{24} were observed with another *Rel* allele Rel^{E20}, which is likely to be a null allele because it lacks a translation start codon due to imprecise excision of a *P* element (Figure 4B) (Hedengren *et al.* 1999). Relative to control w^{1118} flies, the MI_{24} was not affected for homozygous Rel^{E20}/Rel^{E20} flies and was significantly lower for heterozygous $+/Rel^{E20}$ flies. These data indicate that *Rel* is a dose-dependent enhancer of secondary injuries that promote early mortality following TBI – loss of a single copy of *Rel* is sufficient to reduce this mortality. To determine the effect of diet on the ability of heterozygosity for *Rel* to reduce mortality following TBI, we repeated the MI_{24} assay with flies fed water (*i.e.*, fasted) instead of food following TBI. In accord with our prior findings, for every genotype (+/+, +/*Rel*, and *Rel*/*Rel*) and both *Rel* alleles (Rel^{del} and Rel^{E20}), flies fed water had a lower MI_{24} than flies fed food (Katzenberger *et al.* 2015a, 2016). However, for both Rel^{del} and Rel^{E20}, neither homozygous nor heterozygous flies fed water had a significantly altered MI_{24} relative to their respective control flies (Figure 4, A and B). These data suggest that a diet of food following TBI induces a *Rel*-dependent secondary injury mechanism, but water (*i.e.*, starvation) does not. To

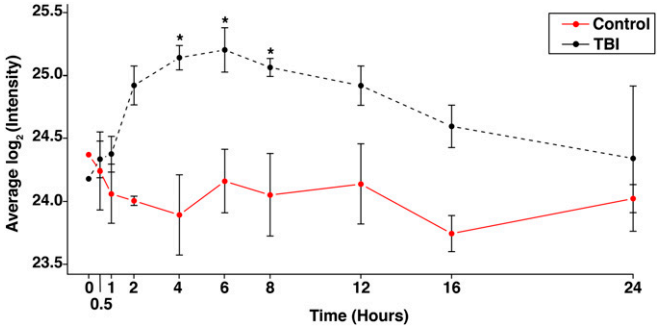

Figure 2 Rel protein expression increases in heads at 4–8 hr following TBI. Average expression of Rel protein in uninjured (control) and injured (TBI) flies at times after TBI normalized to time 0. Error bars indicate the SEM of three independent samples. $*P < 0.05$.

determine if the ability of heterozygosity for Rel^{del} to reduce the MI_{24} extends to genetic backgrounds beyond the one in which the Rel^{del} mutation was generated, we crossed the Rel^{del} mutation into different genetic backgrounds. We crossed females from +/+ or Rel^{del}/Rel^{del} lines to males from nine genetically diverse RAL lines from the Drosophila Genetic Reference Panel (DGRP) collection of ~200 inbred, fully sequenced fly lines from a natural population (Mackay *et al.* 2012). The nine lines were selected because they represent the range of MI_{24}s of the DGRP collection (Katzenberger *et al.* 2015a, 2016). We injured 1- to 7-day-old mixed-sex F1 progeny and determined the MI_{24}. The nine RAL/+ control flies had MI_{24}s that ranged from 41 to 58, which is not as large as the 7 to 58 range for the nine parental RAL/RAL lines, most likely because rather than having distinct genomes, half of each RAL/+ genome is shared, resulting also in loss of homozygosity for any unique recessive alleles affecting the MI_{24} in each RAL line (Katzenberger *et al.* 2015a). Moreover, five of the nine RAL/Rel^{del} flies had a significantly lower MI_{24} than their respective RAL/+ control flies. There was no correlation between the MI_{24} and the ability of heterozygosity for Rel^{del} to suppress mortality. The fact that heterozygosity for Rel^{del} reduced early mortality following TBI to different extents in different genetic backgrounds indicates that the protective effect of the Rel^{del} mutation is modified by polymorphisms in the genetic backgrounds.

+/Rel^{del} flies have improved lifespan following TBI

To investigate a role for Rel in chronic outcomes of TBI, we determined the lifespan of +/+, +/Rel^{del}, and Rel^{del}/Rel^{del} flies that were injured at 1 to 7 days old and survived 24 hr following TBI on a food diet. Female and male flies were injured and maintained together in vials but were scored separately for survival. In all cases, the lifespan of injured flies was significantly shorter than the lifespan of uninjured flies of the same sex and genotype (Figure 5 and Tables S1 and S2). In the absence of injury, female but not male +/Rel^{del} flies had a small but significant increase in lifespan compared to +/+ flies (Figure 5A and Table S2). In contrast, male but not female Rel^{del}/Rel^{del} flies had a small but significant decrease in lifespan compared to +/+ flies. Moreover, following TBI, both female and male +/Rel^{del} flies but not Rel^{del}/Rel^{del} flies had a significantly longer lifespan than +/+ flies (Figure 5B and Table S2). The median lifespan of injured female and male flies increased 32% and 15%, respectively (Table S1). These data indicate that in addition to promoting secondary injuries leading to early mortality after TBI in a dose-dependent manner, *Rel* also contributes to long-term consequences of TBI such as reduced lifespan in a dose-dependent manner. Thus, loss of one copy of *Rel* is sufficient to improve long-term survival following TBI.

NF-κB factors in the Toll pathway have increased expression in +/Rel^{del} flies following TBI

Given that Rel directly activates transcription of Imd pathway target genes, including AMP genes, and that TBI activates the Imd and Toll pathways, we hypothesized that improved TBI outcomes in the +/Rel^{del} flies involved altered expression of Imd and possibly Toll pathway components (Ganesan *et al.* 2011; Katzenberger *et al.* 2013; Kleino and Silverman 2014; Myllymäki *et al.* 2014; Katzenberger *et al.* 2015a, 2016). Using qRT-PCR, we measured expression of *Dorsal-related immunity factor* (*Dif*) and *dorsal* (*dl*), which encode NF-κB transcription factors in the Toll pathway, and AMP gene targets of the Imd and/or Toll pathway (*AttC*, *DptB*, *Drs*, and *Mtk*) in uninjured and injured +/+, +/Rel^{del}, and Rel^{del}/Rel^{del} whole male flies after 4 hr recovery on food (Lemaitre *et al.* 1997). Among the three genotypes, only +/Rel^{del} flies had a significant increase in *Dif* and *dl* expression following TBI, and the amount of *Dif* and *dl* expression in injured +/Rel^{del} flies was significantly higher than in injured +/+ flies (Figure 6, A and B). In addition, TBI caused a significant increase in expression of all four AMP genes in +/+, +/Rel^{del}, and Rel^{del}/Rel^{del} flies (Figure 6, C–F). The overall level of expression of three of the AMP genes was lower in Rel^{del}/Rel^{del} flies than in +/+ flies, but it was not lower in +/Rel^{del} flies. Thus, heterozygous *Rel* mutant flies are distinguished from control flies and homozygous *Rel* mutant flies by increased expression of NF-κB factors in the Toll pathway but not by altered expression of AMP genes.

Since expression of AMP genes changes over time after TBI, we analyzed +/+ and +/Rel^{del} flies at other time points (Katzenberger *et al.* 2016). In addition to 4 hr post-TBI, we analyzed 2, 6, and 8 hr because our prior data indicate that during the 24 hr following TBI, secondary injury-inducing events primarily occur at 2–8 hr (Katzenberger *et al.* 2015a, 2016). The increase in *Dif* and *dl* expression that occurred in +/Rel^{del} flies at 4 hr was transient, as expression of these genes was similar between +/+ and +/Rel^{del} flies at 2, 6, and 8 hr (Figure 7, A and B). In contrast, expression of AMP genes was similar between +/+ and +/Rel^{del} flies over the entire 8-hr time course (Figure 7, C–F). Thus, reduced mortality and increased lifespan of +/Rel^{del} flies following TBI is not a result of attenuated AMP expression but may entail a

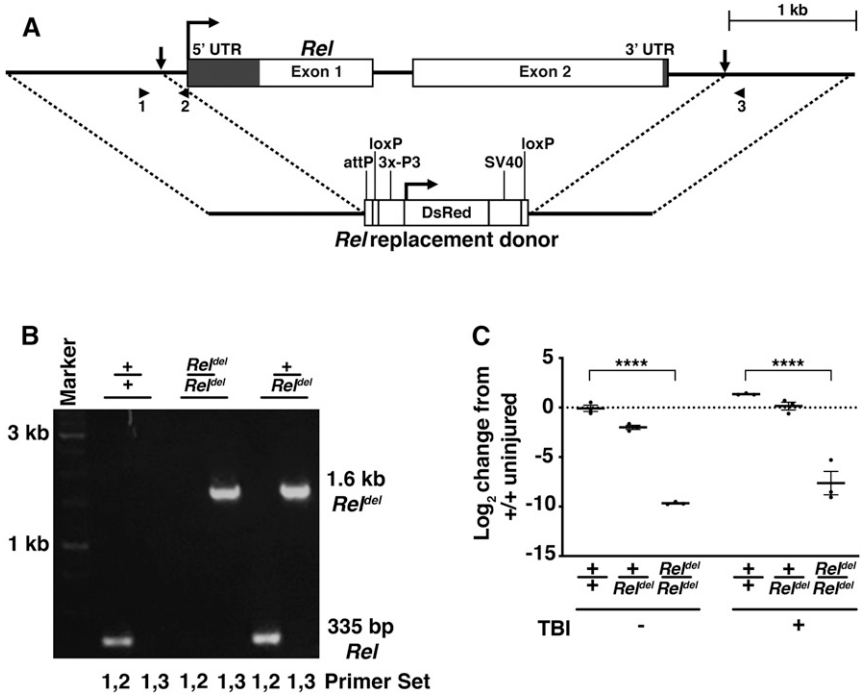

Figure 3 Generation of a null allele of *Rel* by CRISPR/Cas9 gene editing. (A) Diagrams of the wild-type *Rel* genomic locus and the *Rel* replacement donor that was used to generate the Rel^{del} allele. Horizontal arrows indicate transcription start sites, vertical arrows indicate sites targeted by sgRNAs for cleavage by Cas9, numbered arrowheads indicate the direction and identity of primers used for PCR in panel B, and dotted lines indicate ~1-kb homology arms on either side of the *DsRed* gene that were used for homology directed repair. The *DsRed* gene is described in more detail in Gratz *et al.* (2014). (B) PCR analysis of the *Rel* locus using genomic DNA from +/+, $+/Rel^{del}$, and Rel^{del}/Rel^{del} flies. Primer sets refer to primers indicated in panel A. (C) qRT-PCR analysis of *Rel* mRNA from +/+, $+/Rel^{del}$, and Rel^{del}/Rel^{del} flies that were collected 4 hr following TBI (+) or not (−) subjected to TBI. The amount of *Rel* mRNA was normalized to the amount of *RpL32* mRNA. Significance was determined from ΔCt values by a two-way ANOVA with Tukey's *post hoc* test. Error bars indicate the SEM of three independent samples. $^{****}P < 0.0001$.

transient increase in expression of NF-κB factors in the Toll pathway.

Gene expression profiles of uninjured $+/Rel^{del}$ flies and +/+ flies are very similar

To investigate why $+/Rel^{del}$ flies had less severe consequences following TBI, we used high-throughput RNA-seq to identify gene expression differences among +/+, $+/Rel^{del}$, and Rel^{del}/Rel^{del} flies before and after TBI. We analyzed mRNA from whole, 1- to 7-day-old, uninjured and injured, male flies at 4 hr after the primary injury. Multidimensional scaling (MDS) analysis revealed tight clustering of data sets from the four biological replicates of each of the six conditions, indicating high reproducibility of the RNA-seq data sets (Figure S1). In addition, the MDS analysis revealed that data sets from uninjured and injured flies were dissimilar, that data sets from +/+ flies were distinct from Rel^{del}/Rel^{del} flies, and that data sets from $+/Rel^{del}$ flies were more similar to data sets from +/+ flies than from Rel^{del}/Rel^{del} flies.

To further analyze the RNA-seq data, we defined a significant change in gene expression as a more than twofold change with a FDR $P \leq 0.05$. We hypothesized that resistance of $+/Rel^{del}$ flies to TBI outcomes could be due to differences in expression at the time of injury in $+/Rel^{del}$ flies relative to +/+ and Rel^{del}/Rel^{del} flies (Figure 8, A and B and Table S3). Alternatively, resistance to TBI could be due to differences in expression following TBI in $+/Rel^{del}$ flies relative to +/+ and Rel^{del}/Rel^{del} flies (Figure 8, C and D and Table S4) or to differences in TBI-induced changes in expression in $+/Rel^{del}$ flies relative to those in +/+ flies and in Rel^{del}/Rel^{del} flies (Figure 8, E and F and Table S5).

In terms of *Rel* expression itself, we found that it was higher in injured relative to uninjured +/+ flies, lower in uninjured Rel^{del}/Rel^{del} flies relative to uninjured +/+ flies, and lower in injured $+/Rel^{del}$ flies and Rel^{del}/Rel^{del} flies relative to injured +/+ flies (Tables S3–S5). These data are consistent with increased Rel protein expression detected in heads of injured w^{1118} flies by proteomics analysis (Figure 1 and 2) and decreased *Rel* mRNA expression in whole uninjured and injured $+/Rel^{del}$ flies and Rel^{del}/Rel^{del} flies detected by qRT-PCR (Figure 3C). Furthermore, it is important to note that the *Rel* gene is located within the large first intron of the *Nmdmc* (*NAD-dependent methylenetetrahydrofolate dehydrogenase*) gene and it is transcribed in the same direction as *Nmdmc*, but *Nmdmc* expression was not affected in $+/Rel^{del}$ flies or Rel^{del}/Rel^{del} flies relative to +/+ flies (Tables S3–S4). Thus, unintended consequences of Rel^{del} mutation on *Nmdmc* are unlikely to be responsible for the observed effects on TBI phenotypes.

In the absence of injury, expression of only 13 genes (eight upregulated genes and five downregulated genes) was different in $+/Rel^{del}$ flies relative to either +/+ or Rel^{del}/Rel^{del} flies (Figure 8, A and B and Table S3). Six of the eight upregulated genes are uncharacterized, but all five downregulated genes [*Cuticular protein 92F* (*Cpr92F*), *Vajk2*, *Vajk3*, *Adult cuticle protein 1* (*Acp1*), and *CG7221*] encode members of the CPR (cuticular protein) or CPLCP (cuticular protein of low complexity proline-rich) cuticle protein families (Cinege *et al.* 2017). Cuticle proteins and chitin are the main components of cuticle, which provides protection from the environment. Downregulation of these genes in $+/Rel^{del}$ flies may affect activation of the innate immune response following

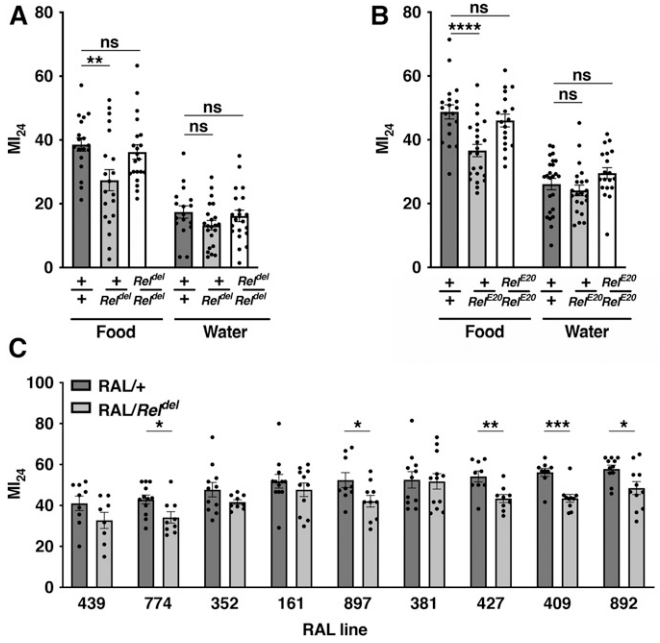

Figure 4 Survival following TBI is increased in +/Rel^{del} heterozygotes. (A) The MI_{24} of 1- to 7-day-old, mixed sex +/+, +/Rel^{del}, and Rel^{del}/Rel^{del} flies fed cornmeal molasses food (food) or water for 24 hr following TBI. (B) The MI_{24} of 1- to 7-day-old, mixed sex +/+, +/Rel^{E20}, and Rel^{E20}/Rel^{E20} flies fed food or water for 24 hr following TBI. (C) The MI_{24} of 1- to 7-day-old mixed sex F1 progeny from crosses between the indicated RAL lines and control flies (RAL/+) or Rel^{del}/Rel^{del} flies (RAL/Rel^{del}) fed food for 24 hr following TBI. All bars represent the average of ≥3 biological replicates of ≥3 technical replicates. Each dot represents an independent sample of 60 flies. Error bars represent the SEM. Significance for panels A and B was determined using a two-way ANOVA with Tukey's *post hoc* test. Significance for panel C was determined using a Student's *t*-test. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$, ns = not significant.

TBI, since breakage of the cuticle induces underlying epithelia to activate the innate immune response (Lemaitre *et al.* 1997).

In contrast to the small number of genes with altered expression in +/Rel^{del} flies, expression of 361 genes (180 upregulated genes and 181 downregulated genes) was different in Rel^{del}/Rel^{del} flies relative to +/+ and +/Rel^{del} flies, and expression of 72 genes (54 upregulated genes and 18 downregulated genes) was shared in +/Rel^{del} and Rel^{del}/Rel^{del} flies relative to +/+ flies. Many of the 361 Rel^{del}/Rel^{del}-specific genes have been characterized. Gene ontology (GO) analysis of the downregulated genes identified "antimicrobial humoral response" (FDR $P = 4.34 \times 10^{-5}$) as the most significantly enriched biological process. The set of genes uniquely downregulated in Rel^{del}/Rel^{del} flies included known direct transcriptional targets of Rel such as 10 of the 14 established AMP genes (Table 1, column 4). In addition, one other AMP gene was downregulated in both +/Rel^{del} and Rel^{del}/Rel^{del} flies. We also noted that four of the 37 Glutathione-S-transferase (Gst) genes, which play a major role in detoxification of xenobiotic and toxic compounds such as reactive oxygen species, were downregulated in Rel^{del}/Rel^{del} flies (Parkes *et al.* 1993). These data indicate that Rel activates transcription of a subset of Gst genes in uninjured flies. Unexpectedly, the set of genes upregulated in uninjured +/Rel^{del} and Rel^{del}/Rel^{del} flies relative to uninjured +/+ flies included 3 of the 10 genes (*Nazo*, *CG33926*, and *CG17264*) identified as transcriptional targets of Rel in response to picorna-like virus infection (Goto *et al.* 2018) (Table S3). Our data suggest that Rel is a repressor, not an activator, of these genes. Taken together, these data indicate that cuticular barrier function is altered in heterozygous *Rel* mutant flies, but baseline activation of the innate immune response is not altered.

Gene expression profiles of +/Rel^{del} flies and +/+ flies following TBI are very similar

At 4 hr following TBI, expression of only 10 genes (eight upregulated genes and two downregulated genes) was different in +/Rel^{del} flies relative to +/+ and Rel^{del}/Rel^{del} flies (Figure 8, C and D and Table S4). In contrast, expression of 306 genes (113 upregulated genes and 193 downregulated genes) was different in Rel^{del}/Rel^{del} flies relative to +/+ and +/Rel^{del} flies, and expression of 62 genes (44 upregulated genes and 18 downregulated genes) was shared in +/Rel^{del} and Rel^{del}/Rel^{del} flies relative to +/+ flies. Only three of the 10 genes whose expression was altered in +/Rel^{del} flies but not in Rel^{del}/Rel^{del} flies following TBI have known functions; *gammaTry* encodes a serine protease, *Lysozyme C* (*LysC*) encodes a glycoside hydrolase, and *hedgehog* (*hh*) encodes a signaling morphogen involved in segment polarity determination, stem cell maintenance, and cell migration (Daffre *et al.* 1994; Briscoe and Thérond 2013; Cao and Jiang 2018). In a mouse TBI model, activation of the hedgehog pathway reduces cerebral edema and neuronal apoptosis, suggesting that enhanced hedgehog signaling could be important for the reduced mortality of +/Rel^{del} flies following TBI. However, at 4 hr after TBI, transcriptional targets of the hedgehog pathway were not activated (Biehs *et al.* 2010; Wu *et al.* 2020).

GO analysis of downregulated genes in injured Rel^{del}/Rel^{del} flies relative to injured +/+ flies identified "antimicrobial humoral response" as the most significantly enriched biological process (FDR $P = 1.49 \times 10^{-4}$). Downregulated genes included 11 of the 14 AMP genes as well as four of the 13 peptidoglycan recognition proteins (PGRPs), which encode pattern recognition molecules that sense pathogens (Kurata 2014) (Table 1, column 5). The four downregulated PGRP genes (*PGRP-Sc1b*, *PGRP-SA*, *PGRP-SC2*, and *PGRP-SB1*) are all members of the short (S) family of PGRPs that have signal peptides and can be secreted from cells. Expression of most PGRP-S genes is upregulated by bacterial infection, whereas most long (L) family PGRP genes are constitutively expressed (Werner *et al.* 2000). In addition, seven Gst genes were downregulated in injured Rel^{del}/Rel^{del} flies, four of which were also downregulated in uninjured Rel^{del}/Rel^{del} flies. GO analysis of genes upregulated in Rel^{del}/Rel^{del} flies or in both

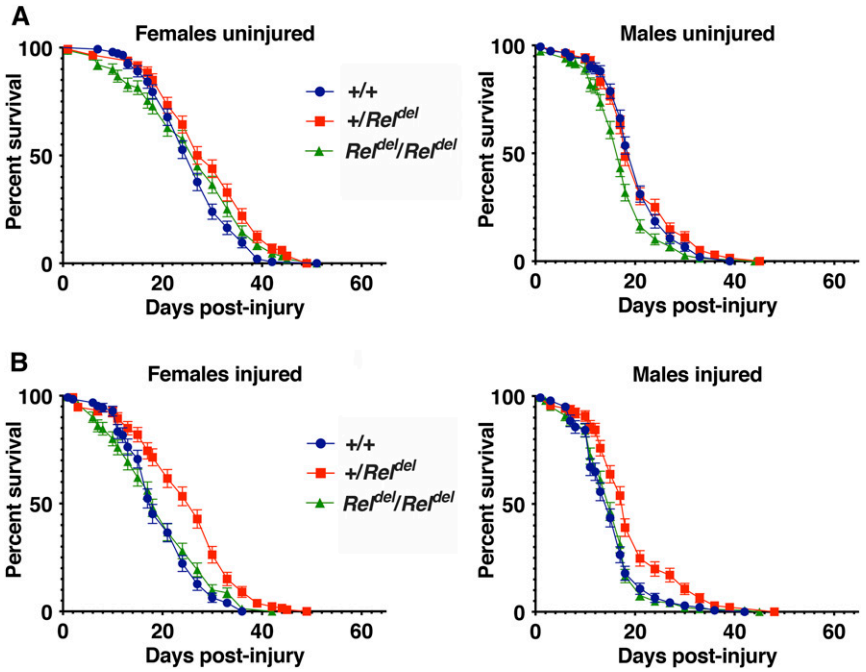

Figure 5 Lifespan following TBI is extended in $+/Rel^{del}$ heterozygotes. Percent survival of (A) uninjured and (B) injured female and male flies of the indicated genotypes starting at 2–8 days old. At least 125 flies were analyzed for each condition. Error bars represent SEM. Table S1 contains numerical values for the median and maximum lifespans, and Table S2 contains *P*-values of survival determined by the log-rank test.

Rel^{del}/Rel^{del} and $+/Rel^{del}$ flies did not identify a significantly enriched biological process. Thus, following TBI, Rel^{del}/Rel^{del} flies activate the innate immune response to a lesser degree than +/+ flies yet they share similar mortality and lifespan, suggesting that either activation of the Imd pathway is not causal for these outcomes of TBI or that genes specifically altered in Rel^{del}/Rel^{del} flies such as Gst genes counteract the beneficial effects of blocking the Imd pathway.

Comparison of uninjured and injured flies identifies which gene expression changes induced by TBI are mediated by the Imd pathway

The final way that we analyzed the RNA-seq data was to compare gene expression between uninjured and injured conditions for each of the three different genotypes: +/+, $+/Rel^{del}$, and Rel^{del}/Rel^{del}. This analysis identified effects of TBI on gene expression that were shared among fly lines as well as those that were unique to a particular genotype. Of note, genes encoding six of the nine proteins that had significantly increased expression in the proteomics analysis at 8 hr following TBI (Figure 1E) also had significantly increased mRNA expression in control flies at 4 hr following TBI (Figure 8E and Table S5).

Among the three fly lines, 429 genes were upregulated and 117 genes were downregulated following TBI (Figure 8, E and F and Table S5). Thus, at the 4 hr time point, TBI affects expression of about 4% of *Drosophila* genes. In total, 130 of the upregulated genes and nine of the downregulated genes were shared among all three fly lines. The group of nine shared downregulated genes included *Nimrod C2* (*NimC2*), which encodes a member of a family of proteins that play a role in the innate immune response by binding and phagocytosing bacteria (Kurucz *et al.* 2007). Four other members of the 10-member Nim family also had altered expression following injury: *NimB1*, *NimB2*, and *NimB3* were upregulated by injury in Rel^{del}/Rel^{del} flies and *NimC1* was downregulated by injury in $+/Rel^{del}$ flies. The significance of changes in expression of Nim genes for Rel-mediated regulation of gene expression after TBI or for suppression of TBI outcomes in $+/Rel^{del}$ flies is difficult to discern because some Nim genes were upregulated while others were downregulated. GO analysis of the 130 shared upregulated genes following TBI revealed "antimicrobial humoral response" as the most significantly enriched biological process (FDR $P = 5.55 \times 10^{-6}$). The group of shared genes included four PGRPs, a negative regulator of the Imd pathway (*pirk*), and peptide-encoding genes implicated in the innate immune response: seven AMPs, nine Bomanins (Bom), three Immune-induced molecules (IMs), four Turandots (Tots), and Listericin (Table 1, column 2) (Ekengren *et al.* 2001; Goto *et al.* 2010; Lindsay *et al.* 2018). Thus, TBI activated the innate immune response in +/+, $+/Rel^{del}$, and Rel^{del}/Rel^{del} flies. Pathways other than the Imd pathway presumably mediate TBI-induced activation of the shared genes since activation occurred in the absence of Rel (*i.e.*, in Rel^{del}/Rel^{del} flies), which is essential for the transcriptional response of the Imd pathway. Prior studies of these genes indicate that in response to pathogens the Toll pathway activates the AMP, Bom, and IM genes, and the JAK-STAT (Janus kinase-signal transducers and activators of transcription) pathway activates the Tot genes and *Listericin* (Ekengren and Hultmark 2001; Brun *et al.* 2006; Lemaitre and Hoffmann 2007; Goto *et al.* 2010; Lindsay *et al.* 2018).

Furthermore, GO analysis of the 50 genes upregulated in +/+ and $+/Rel^{del}$ flies but not in Rel^{del}/Rel^{del} flies also identified "antimicrobial humoral response" as the most significantly enriched biological process (FDR $P = 9.55 \times 10^{-4}$).

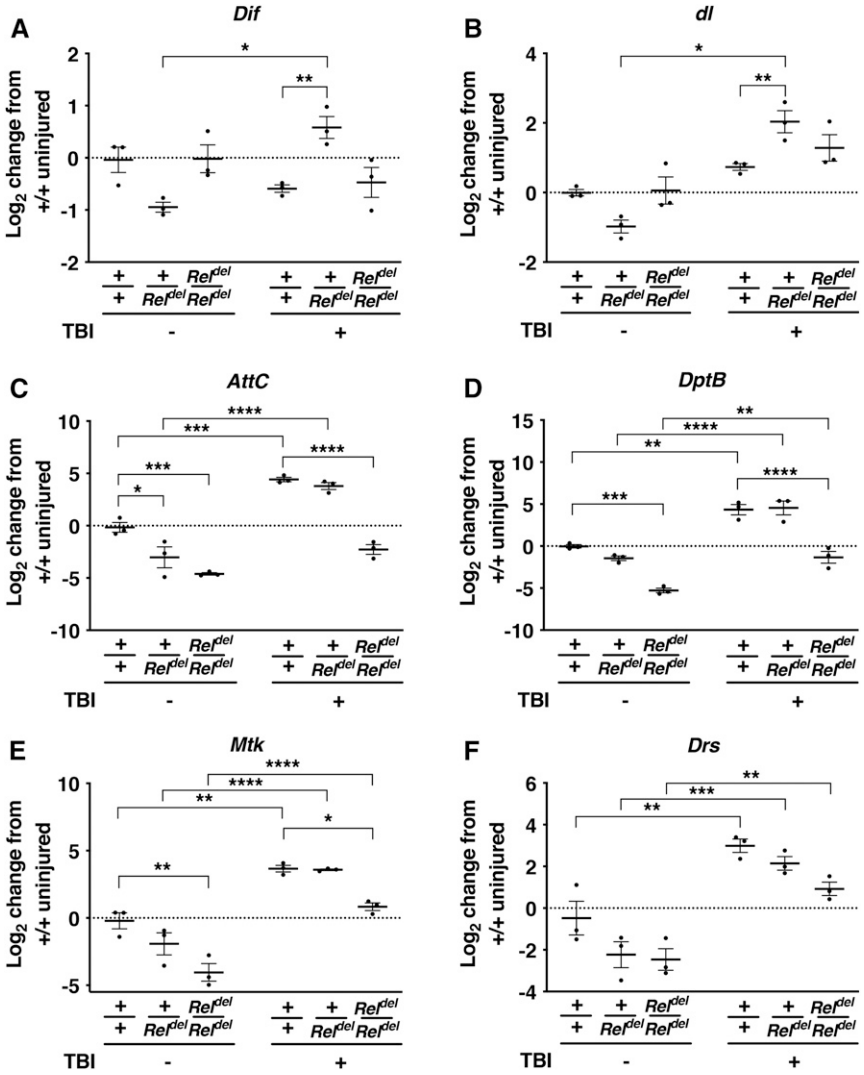

Figure 6 Expression of Toll and Imd pathway components in uninjured and injured *Rel* mutant flies. qRT-PCR analysis of (A) *Dif*, (B) *dl*, (C) *AttC*, (D) *DptB*, (E) *Mtk*, and (F) *Drs* in uninjured and injured +/+, +/Rel^{del}, and Rel^{del}/Rel^{del} whole male flies collected at 4 hr post-TBI on food. The amount of each mRNA was normalized to the expression of *RpL32*. Error bars represent the SEM of three independent samples. Significance was determined from ΔCt values by a two-way ANOVA with Tukey's *post hoc* test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.01$, $^{****}P < 0.0001$.

This group of genes included six AMPs, one Bom, and one IM but no PGRP or Tot genes (Table 1, column 3). The Imd pathway presumably mediates activation of these genes because activation did not occur in the absence of Rel (*i.e.*, in Rel^{del}/Rel^{del} flies).

Expression of cuticle and ribosomal RNA genes is uniquely affected in +/Rel^{del} flies following TBI

In addition to the shared genes described above, 206 genes were upregulated and 84 genes were downregulated in only one genotype (+/+, +/Rel^{del}, or Rel^{del}/Rel^{del}) following TBI (Figure 8, E and F and Table S5). Among the 47 genes upregulated after TBI in +/Rel^{del} flies but not in +/+ or Rel^{del}/Rel^{del} flies were eight of the 155 genes that encode structural cuticle proteins (Willis 2010). The group of altered cuticle genes included four of the five genes that were downregulated in uninjured +/Rel^{del} flies but not in uninjured +/+ or Rel^{del}/Rel^{del} flies (*Acp1*, *Vajk2*, *Vajk3*, and *CG7214*) as well as *Acp65A*, *Cpr62Bc*, *Cpr97Ea*, and *Cpr100A* (Tables S3 and S5). In addition, two genes (*Mur18B* and *CG13806*) contain a predicted chitin-binding domain. Among the 59 genes downregulated after TBI in +/Rel^{del} flies but not in +/+ or Rel^{del}/Rel^{del} flies, eight were 18S or 28S ribosomal RNA genes (Table S5). Taken together, these data suggest that increased cuticle biogenesis and decreased ribosome biogenesis protect +/Rel^{del} flies from deleterious consequences of TBI.

lncRNA genes are enriched among genes with altered expression in +/Rel^{del} or Rel^{del}/Rel^{del} flies relative to +/+ flies

In the course of our analysis of the RNA-seq data, we noted that many long noncoding RNA (lncRNA) genes had altered expression in +/Rel^{del} and Rel^{del}/Rel^{del} flies compared to equivalently treated +/+ flies. LncRNAs are noncoding RNAs >200 nt in length that serve a variety of functions, often related to regulation of gene expression (Kung *et al.* 2013). Among the 446 genes with altered expression in uninjured +/Rel^{del} and/or Rel^{del}/Rel^{del} flies relative to uninjured +/+ flies, 93 (20.9%) were lncRNAs (Figure 8, A and B and Table S3). Similarly, among the 378 genes with altered expression

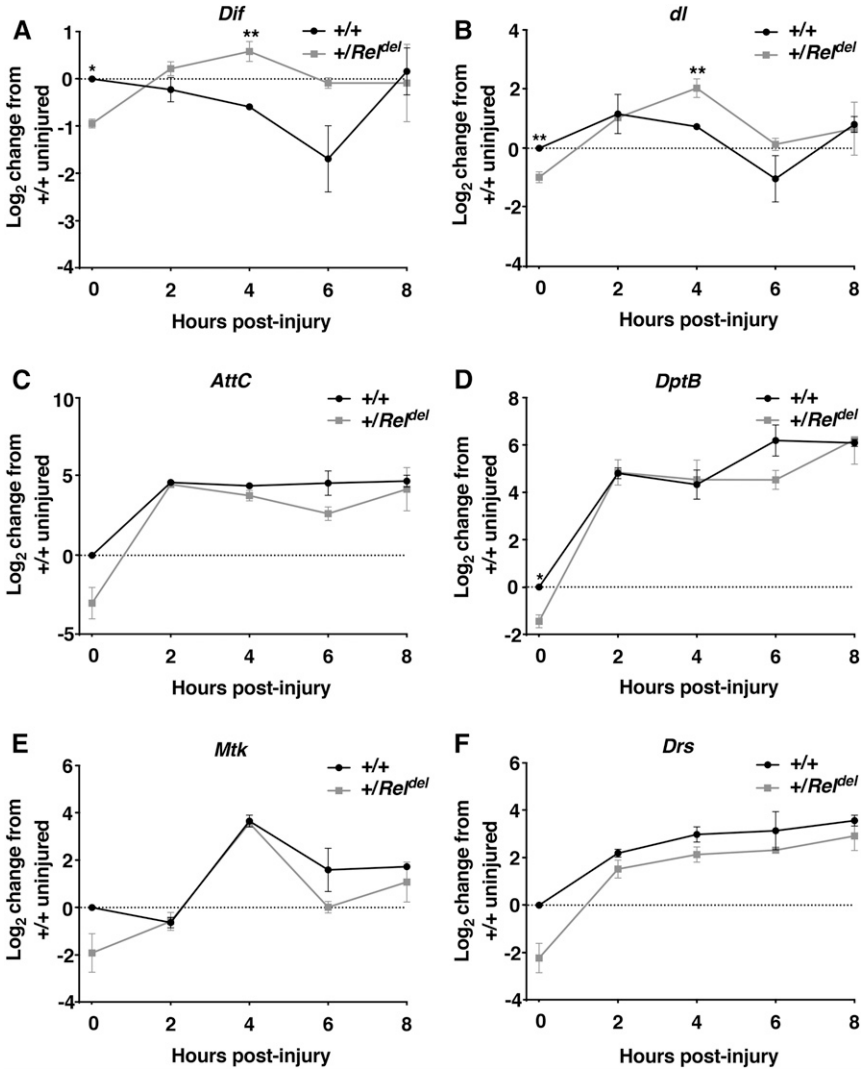

Figure 7 Expression of Toll and Imd pathway components in uninjured and injured *Rel* mutant flies. qRT-PCR analysis of (A) *Dif*, (B) *dl*, (C) *AttC*, (D) *DptB*, (E) *Mtk*, and (F) *Drs* in uninjured and injured +/+ and +/Rel^{del} whole male flies at 0, 2, 4, 6 and 8 hr after injury. The amount of each mRNA was normalized to the expression of *RpL32*. Error bars represent the SEM of three independent samples. Significance was determined from ΔCt values with a Student's *t*-test. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$.

in injured +/Rel^{del} and/or Rel^{del}/Rel^{del} flies relative to injured +/+ flies, 81 (21.4%) were lncRNAs (Figure 8, C and D and Table S4). For both uninjured and injured flies, lncRNAs were abundant among both the upregulated and downregulated genes (uninjured: upregulated (14.5%), downregulated (28.4%); injured: upregulated (23.0%), downregulated (20.7%)). Thus, lncRNA genes are a major class of Rel transcriptional targets. However, among the 546 genes with altered expression between uninjured and injured conditions for +/+, +/Rel^{del}, and Rel^{del}/Rel^{del} flies, only 2.6% of upregulated genes and 8.5% of downregulated genes were lncRNAs, a total of only 21 genes (Figure 8, E and F and Table S5). These data indicate that Rel-mediated regulation of lncRNA gene expression is not altered by TBI. Notably, NF-κB signaling in mammals is modulated by lncRNAs, with a subset of these lncRNAs regulated by NF-κB itself, suggesting feedback regulation (Hadjicharalambous and Lindsay 2019; Gupta *et al.* 2020). Our findings support this conclusion, suggesting that cross-talk between NF-κB signaling and lncRNAs is conserved between flies and mammals, but it is not appreciably influenced by TBI.

Discussion

Substantial neuroinflammation is one of the hallmarks of secondary injury following TBI in both humans and preclinical models (Lozano *et al.* 2015; Chiu *et al.* 2016; Simon *et al.* 2017). However, it remains unclear the extent to which the inflammatory response promotes tissue recovery and repair or exacerbates tissue damage and long-term sequelae (Schmidt *et al.* 2005; Loane and Kumar 2016; Sochocka *et al.* 2017). Current data support a more fluid model in which short, controlled release of pro-inflammatory cytokines and chemokines in combination with anti-inflammatory and neuroprotective factors benefit the recovery process, while prolonged, unchecked self-propagation of pro-inflammatory signaling drives tissue damage (Jassam *et al.* 2017; Simon *et al.* 2017). The balance of these two extremes would therefore dictate the degree of tissue damage and death, neurodegeneration, and

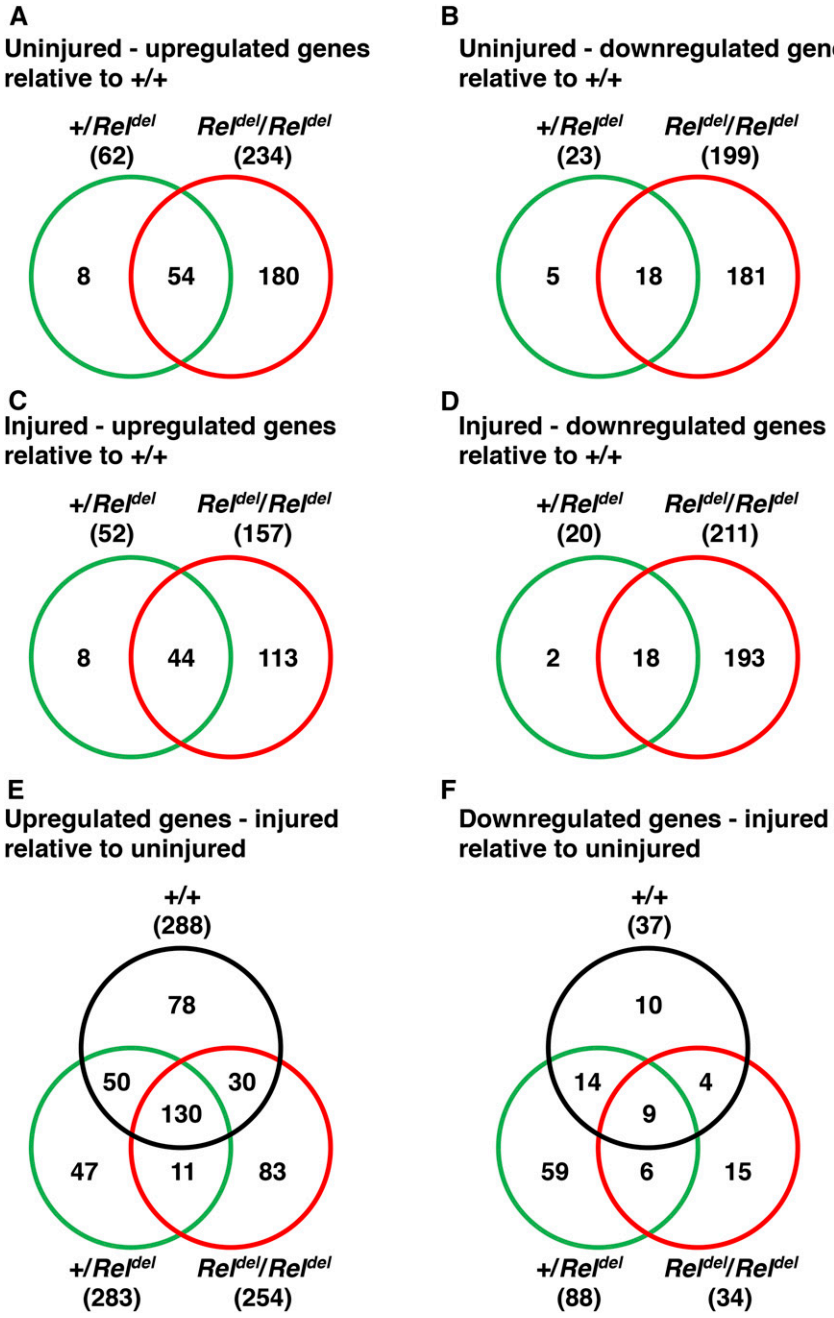

Figure 8 Venn diagrams comparing gene expression changes among uninjured and injured +/+ and *Rel* mutant flies. Genes (A) upregulated or (B) downregulated ≥twofold, $P < 0.05$ in uninjured +/Rel^{del} and Rel^{del}/Rel^{del} flies relative to uninjured +/+ flies. Genes (C) upregulated or (D) downregulated ≥twofold, $P < 0.05$ in injured +/Rel^{del} and Rel^{del}/Rel^{del} flies relative to injured +/+ flies at 4 hr after TBI. Genes (E) upregulated or (F) downregulated ≥twofold, $P < 0.05$ between uninjured and injured conditions in +/+, +/Rel^{del}, and Rel^{del}/Rel^{del} flies.

behavioral outcomes following TBI. Translating these observations into therapies that appropriately target post-TBI inflammation requires a deeper understanding of the specific mechanisms by which the NF-κB-mediated pro-inflammatory response can both benefit and hinder TBI recovery. *Drosophila* models provide an excellent means of exploring NF-κB signaling, due to their simplified, but well conserved, innate immune response pathways (Lemaitre and Hoffmann 2007).

Rel is a dose-dependent suppressor of TBI outcomes

Based on identification of Rel in proteomics analyses of fly heads following TBI (Figures 1 and 2), we examined the extent to which Rel-mediated transcription activation by the Imd pathway was responsible for short-term mortality and reduced lifespan in flies subjected to TBI. We generated a null allele of *Rel* (Rel^{del}) by replacing its entire coding sequence with *DsRed* (Figure 3). We observed that while homozygous Rel^{del} and control flies had comparable early mortality following TBI, heterozygous +/Rel^{del} flies had significantly reduced early mortality relative to control flies (Figure 4B). Heterozygous +/Rel^{del} flies also exhibited reduced early mortality following TBI in some other, but not all, genetic backgrounds (Figure 4C). Furthermore, +/Rel^{del} flies did not have reduced mortality when fed water rather than food following TBI, suggesting that heterozygosity for Rel^{del} and a diet of water (*i.e.*, fasted) reduce mortality through the same mechanism (Figure

4B). For example, they may both increase production of ketone bodies or reduce oxidative stress, which are neuroprotective following TBI (Greco *et al.* 2016; Caglayan *et al.* 2019; Bernini *et al.* 2020; Eastman *et al.* 2020). Alternatively, a diet of water may inhibit Rel activity. In support of this possibility, expression of *pirk*, which encodes a negative regulator of Imd signaling, is activated to a greater extent by a diet of water than a diet of food following TBI (Kleino *et al.* 2008; Katzenberger *et al.* 2016). These data highlight the complexity of factors that intersect with the Imd pathway to determine TBI outcomes.

Variable effects of genetic background and diet on TBI outcomes are not unique to flies. The importance of genetic background is hinted at but not firmly established in humans by association of DNA polymorphisms in about 20 genes with clinical pathologies and by studies in rodents showing that identical primary injures in different strains produce different outcomes (Fox *et al.* 1999; Tan *et al.* 2009; Dardiotis *et al.* 2010; Hoh *et al.* 2010; Reid *et al.* 2010; Dalla Libera *et al.* 2011; Al Nimer *et al.* 2013; Failla *et al.* 2015). Diet also affects the risk of mortality in TBI patients as well as other outcomes in rodent TBI models (Davis *et al.* 2008; Härtl *et al.* 2008; Greco and Prins 2013; Rubovitch *et al.* 2019; Shaito *et al.* 2020). In addition to reducing mortality, heterozygosity for Rel^{del} significantly rescued the shortened lifespan after TBI in both sexes and extended the lifespan of uninjured female flies (Figure 5). Thus, similar to what has been observed in mammals, an attenuated but not absent NF-κB-mediated injury response is sufficient to improve both acute and chronic outcomes of TBI in flies (Zhang *et al.* 2019; Deng *et al.* 2020; Long *et al.* 2020).

Suppression of TBI outcomes in heterozygous Rel mutant flies is not due to dramatically reduced expression of canonical gene targets of the Imd pathway

Since Rel is a transcription factor, we hypothesized that suppression of TBI outcomes in $+/Rel^{del}$ flies would be due to reduced expression of previously identified canonical *Rel* target genes. The gene expression analysis showed that only 23 genes uniquely changed expression in uninjured or injured heterozygous Rel^{del} mutant flies relative to equivalently treated control flies or homozygous Rel^{del} mutant flies (Figure 8, A–D and Tables S3 and S4). Among the 23 genes, none were canonical innate immune response genes. Furthermore, canonical genes such as those in the AMP, Bom, IM, and Tot families that were upregulated in injured relative to uninjured control flies were also upregulated in injured relative to uninjured heterozygous Rel^{del} mutant flies (Figure 8E and Table 1, columns 2 and 3). Thus, suppression of TBI outcomes is either not due to effects on expression of canonical innate immune response genes or is due to subtle changes in expression of these genes. Accordingly, suppression of TBI outcomes in heterozygous Rel^{del} mutant flies compared to control flies may be due to a small change in the relative amounts of Rel, Dif, and Dl NF-κB factors. Based on qRT-PCR analysis (Figure 3) and proteomics analysis (Figure 2), *Rel* expression increased in control flies following TBI, but it did not increase to the same absolute level in heterozygous Rel^{del} mutant flies following TBI (Figure 3). Furthermore, based on qRT-PCR analysis (Figures 6 and 7), *Dif* and *dl* expression increased in heterozygous Rel^{del} mutant flies following TBI, but these genes did not increase to the same absolute level in control flies following TBI. The different ratios of NF-κB factors may translate to small changes in the relative expression of individual AMP genes, shifting the balance toward AMP gene expression that collectively plays a more protective than harmful role following TBI. This possibility is strengthened by our prior finding that expression of individual AMP genes can alter both early mortality and longevity following TBI in flies (Swanson *et al.* 2020).

Alternatively, suppression of deleterious consequences of TBI may be due to one or more of the 23 genes that uniquely changed expression in uninjured or injured heterozygous Rel^{del} mutant flies. Among this group were five genes encoding cuticle proteins that were uniquely downregulated in uninjured heterozygous Rel^{del} mutant flies. The cuticle protects underlying cells from injury, so structural differences in the cuticle of heterozygous Rel^{del} mutant flies relative to homozygous Rel^{del} mutant flies or control flies may better protect them from primary injuries. Similarly, eight cuticle genes were upregulated following TBI in heterozygous Rel^{del} mutant flies but not following TBI in homozygous Rel^{del} mutant flies or control flies, suggesting that TBI-induced structural alteration of the cuticle provides better protection from secondary injuries (Figure 8E and Table S5). However, it is not obvious how altered expression of cuticle genes in heterozygous Rel^{del} mutant flies would reduce secondary injuries following TBI or increase longevity. We also hypothesized that more severe TBI outcomes in homozygous Rel^{del} mutant flies relative to heterozygous Rel^{del} mutant flies would be due either to reduced expression of protective genes or increased expression of harmful genes. The former possibility is supported by reduced expression of seven Gst genes in homozygous Rel^{del} mutant flies, which may result in reduced capacity to combat oxidative stress-induced secondary injuries following TBI (Table 1, column 5).

Rel directly or indirectly activates transcription of all AMP genes following TBI

Since transcription of Rel target genes cannot occur in homozygous Rel^{del} flies, we were able to use the RNA-seq analysis to identify genes in injured flies that are directly or indirectly activated by Rel. Innate immune response genes that were commonly upregulated in +/+, $+/Rel^{del}$, and Rel^{del}/Rel^{del} flies following TBI have to be activated independently of Rel and the Imd pathway because they were upregulated in Rel^{del}/Rel^{del} flies that lack Rel protein (Table 1, column 2). Many genes in this group are known to be activated by the Toll pathway. Conversely, innate immune response genes that were upregulated in +/+ and $+/Rel^{del}$ flies (*i.e.*, flies that express Rel protein) but not in Rel^{del}/Rel^{del} flies (*i.e.*, flies that do not express Rel protein) following TBI are likely to be

activated by Rel and the Imd pathway (Table 1, column 3). Almost all of these Imd pathway targets were AMP genes. However, these strict interpretations do not explain how expression of non-Imd pathway AMP genes were downregulated in uninjured or injured Rel^{del}/Rel^{del} flies (Table 1, columns 4 and 5). Thus, these data suggest that TBI activates both Dif via the Toll pathway and Rel via the Imd pathway to increase transcription of AMP genes, but there is cross-regulation between Dif and Rel. Rel is absolutely necessary for transcription activation of *AttD*, *CecA2*, *CecB*, *CecC*, *Def*, and *DptA*, whereas Rel augments but is not absolutely required for transcription activation of the other AMP genes by Dif. Synergy between Rel and Dif through heterodimer formation has also been shown to occur *in vitro* and in the response to pathogens (Tanji *et al.* 2007, 2010; Chowdhury *et al.* 2019). Thus, a slightly lower amount of Rel protein in flies heterozygous for Rel^{del} may reduce mortality and increase longevity following TBI by changing the amounts of NF-κB homodimers and heterodimers and consequently the amount of transcription of individual AMP genes.

Our data do not directly address the cellular source of differences in gene expression caused by heterozygosity and homozygosity for Rel^{del} in uninjured and injured flies. Fat body cells and hemocytes are possible cellular sources based on the fact that transcription of AMP genes is activated in these cells by pathogen infection (Lemaitre and Hoffmann 2007). The fat body is functionally equivalent to the human liver and is distributed throughout the adult fly body, including the head (P *et al.* 2020). Hemocytes are circulating immune surveillance cells (Parsons and Foley 2016). Expression of AMP genes is also activated in glia by mutation of *ATM* (*Ataxia-telangiectasia mutated*) in a fly model of the human neurodegenerative disease ataxia-telangiectasia (Petersen *et al.* 2012). In addition, expression of the AMP gene *Mtk* increases in glia and neurons following TBI (Swanson *et al.* 2020). Furthermore, knocking down *Rel* expression in glia or neurons extends the lifespan of flies, and knocking down *Rel* expression in glia but not neurons suppresses neurodegeneration caused by mutation of a negative regulator of the Imd pathway (Kounatidis *et al.* 2017). Thus, fat body cells, hemocytes, neurons, and glia are all candidate sources of differences in gene expression caused by heterozygosity and homozygosity for Rel^{del} in uninjured and injured flies.

In conclusion, by taking advantage of genetic and molecular tools in *Drosophila*, we have been able to demonstrate that short-term and long-term deleterious consequences of TBI are ameliorated by deleting one copy of the NF-κB transcription factor Rel. Furthermore, we have been able to begin to unravel the complex changes in gene expression patterns in heterozygous mutant flies that may underlie the protective effects of the $+/Rel^{del}$ genotype. These studies point to the possibility that small changes in expression of innate immune response genes at precisely timed intervals following TBI together with other factors can exert major effects on the severity of secondary injuries. Our findings both raise the hope for rational development of effective therapeutic intervention for TBI in humans and highlight the inherent difficulty of doing so when the interplay of multiple components in complex networks must be taken into account.

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