Baldspot/ELOVL6 is a conserved modifier of disease and the ER stress response

**ELOVL6* modifies ER stress

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ABSTRACT

Endoplasmic reticulum (ER) stress is an important modifier of human disease. Genetic variation in genes involved in the ER stress response has been linked to inter-individual differences in this response. However, the mechanisms and pathways by which genetic modifiers are acting on the ER stress response remain unclear. In this study, we characterize the role of the long chain fatty acid elongase Baldspot (ELOVL6) in modifying ER stress and disease. We demonstrate that loss of *Baldspot* rescues degeneration and reduces IRE1-dependent *Xbp1* splicing and cell death in a *Drosophila* model of retinitis pigmentosa and ER stress ($Rh1^{G69D}$). Dietary supplementation of stearate bypasses the need for Baldspot activity. Finally, we demonstrate that *Baldspot* regulates ER stress across different tissues and induction methods. Our findings suggest that *ELOVL6* is a promising target in the treatment of not only retinitis pigmentosa, but a number of different ER stress related disorders.

INTRODUCTION

Phenotypic heterogeneity is common in simple and complex diseases and is the driving force behind the Precision Medicine Initiative [1-4]. Genetic variation among individuals accounts for much of this heterogeneity, but the identity and nature of modifying genes or variants is largely unknown [4, 5]. These modifying variants are often cryptic and may not influence the physiology or visible phenotypes of healthy individuals, but can alter the expression of disease phenotypes. Understanding the role of modifiers and the pathways in which they function will enable the development of patient-specific therapeutic approaches.

One such process is the response to endoplasmic reticulum (ER) stress, which occurs when misfolded proteins accumulate in the lumen of the ER. This activates the Unfolded Protein Response (UPR) in an attempt to return the cell to homeostasis. Failure to do so will eventually result in apoptosis [6]. Conditions of chronic ER stress lead to excess apoptosis, tissue degeneration, and dysfunction. In rare cases, primary mutations of key ER stress pathway components cause Mendelian syndromes [7-11]. More commonly, ER stress can exacerbate disease, including obesity [12], neurological disease [13], retinal degeneration [14], and some cancers [15]. Experimental genetic or pharmacological manipulation of ER stress levels can alter disease phenotypes, suggesting that inter-individual differences in ER stress responses may influence disease severity. Indeed, human [16], mouse [17], and *Drosophila* [18, 19] show extensive genetic variation in their response to ER stress. Understanding the role of genetic variation in modulating ER stress pathways may provide more therapeutic targets and improve the accurate identification of high risk patients.

In a previous study, we crossed the $Rh1^{G69D}$ model of retinitis pigmentosa (RP) into the *Drosophila* Genetic Reference Panel (DGRP) to identify genetic modifiers of this disease [19]. In this model, retinal degeneration is induced by overexpression of misfolded rhodopsin in the developing larval

eye disc [20], resulting in chronic ER stress and apoptosis. The DGRP is used to study the genetic architecture underlying complex traits. It is comprised of approximately 200 inbred strains, such that each strain represents a single, wild-derived genome. Thus, the DGRP captures genetic variation that is present in a natural, wild population. Importantly, we have the whole genome sequence of each strain, allowing phenotype-genotype studies [21]. The degree of degeneration induced by this model varied substantially across the strains of the DGRP. Using an association analysis, we identified a number of promising candidates that modify this RP phenotype and have putative roles in ER stress [19].

In this study, we demonstrate that *Drosophila Baldspot,* one of the candidate RP modifiers identified in our genetic variation screen, is both a disease modifier of ER stress-induced retinal degeneration and a more general modifier of ER stress across different physiological contexts. *Baldspot*, which is orthologous to mammalian *ELOVL6,* is an ER-associated long chain fatty acid elongase which catalyzes the extension of palmitate to stearate [22, 23]. We show that loss of *Baldspot* rescues retinal degeneration by reducing ER stress signaling. Loss of Baldspot elongase activity reduces IRE1/Xbp1 activity and downstream apoptosis, without affecting the RIDD activity of IRE1 or the misfolded protein load. Strikingly, in the absence of Baldspot activity, increasing levels of stearate through dietary supplementation bypasses the need for Baldspot to induce IRE1/Xbp1 signaling. Finally, we show that Baldspot regulates ER stress in a number of different genetic and chemically-induced ER stress models across multiple tissue types, making *Baldspot* a potentially wide-reaching modifier of ER stress-associated diseases. Understanding how modifier genes alter disease outcomes is the first step to developing personalized therapies.

RESULTS

A SNP in *Baldspot* is associated with *Rh1*^{G69D}-induced retinal degeneration

In previous work, we overexpressed a misfolded rhodopsin protein in the developing eye disc (*GMR-GAL4>UAS-Rh1*^{G69D}), the larval precursor to the *Drosophila* retina and eye, in the 200 strains from the DGRP [19]. Using a genome-wide association approach, we identified SNPs that are associated with degree of retinal degeneration. One of these candidate SNPs, Chr3L:16644000 (BDGP R5/dm3), is located in intron four of *Baldspot. Baldspot* is the only *Drosophila* orthologue of mammalian *ELOVL6*, a member of the very long chain fatty acid elongase family of enzymes (Figure 1A) [24]. Strains carrying the minor allele (C, 18880 ± 3010 pixels) exhibit more severe *Rh1*^{G69D}–induced retinal degeneration as compared to strains carrying the major allele (T, 21822 ± 2294 pixels) (Figure 1B; $p < 0.005$). This is also apparent when examining the range of degeneration in strains containing each of these alleles (Figure 1C). Based on these results, we hypothesized that altering the expression or activity of *Baldspot* may impact degeneration.

Loss of *Baldspot* rescues *Rh1*^{G69D}-induced retinal degeneration.

To test whether eliminating *Baldspot* expression can modify *Rh1*^{G69D}-induced retinal degeneration, we expressed an RNAi construct targeted against *Baldspot* in the *GMR-GAL4>UAS-Rh1*^{G69D} background. We found that loss of *Baldspot* expression results in partial rescue of Rh1^{G69D}-induced retinal degeneration (*Rh1*^{G69D}*/Baldspoti*) as compared to controls expressing only the Rh1^{G69D} misfolded protein and no RNAi (*Rh1*^{G69D} controls) (Figure 2A). Quantification of eye size demonstrates a significantly larger, less degenerate eye in *Rh1*^{G69D}*/Baldspoti* flies (14043 ± 960 pixels) as compared to *Rh1*^{G69D} controls (11942 ± 473 pixels) ($P = 7.4 \times 10^{-6}$) (Figure 2B). Loss of *Baldspot* on a wild-type background resulted in no change in eye size or phenotype (Figure 2C). We conclude that loss of *Baldspot* expression provides significant rescue of *Rh1*^{G69D}-induced retinal degeneration.

Loss of *Baldspot* in the *Rh1*^{G69D} eye disc reduces ER stress and apoptosis

Rescue of eye size in the *Rh1*^{G69D} model is often accompanied by reduction in apoptosis [25, 26]. Based on this and the rescue effect we observed, we hypothesized that apoptosis in the *Rh1*^{G69D} eye discs would also be reduced upon loss of *Baldspot* expression. Indeed, we found that apoptosis, as measured by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, is reduced in *Rh1*^{G69D}*/Baldspoti* eye discs as compared to *Rh1*^{G69D} controls. (Figure 2D).

Excessive ER stress in the *Rh1*^{G69D} eye discs leads to apoptosis and degeneration in the adult eyes. To determine if the decrease in apoptosis we observed was due to changes in the ER stress response, we measured *Xbp1* splicing. Under conditions that induce ER stress, *Xbp1* transcript is spliced by IRE1, one of three transmembrane sensors of misfolded proteins in the ER. The in-frame *Xbp1* transcript is translated and acts as a transcription factor, inducing genes involved in the UPR [27, 28]. The level of spliced *Xbp1* transcript is directly proportional to the degree of activation of the ER stress response. To measure IRE1 signaling and *Xbp1* splicing in the absence of *Baldspot* expression, we used a previously characterized transgenic *Xbp1-EGFP* marker [20, 29-31]. In this transgene, the 5' end of the *Xbp1* mRNA is expressed under the control of the *UAS* promoter. The 3' end of the gene, downstream of the IRE1 splice site, has been replaced with sequence encoding *EGFP*. As with the endogenous *Xbp1* transcript, the in-frame, spliced *Xbp1-EGFP* transcript, and subsequently the Xbp1-EGFP fusion protein, increases as ER stress signaling increases. Thus, EGFP signal increases as ER stress signaling increases. As previously reported, expression of *UAS-Xbp1-EGFP* in *Rh1*^{G69D} controls results in increased EGFP, indicating high levels of ER stress (Figure 2E) [20, 29-31]. When *UAS-Xbp1-EGFP* is expressed in *Rh1*^{G69D}*/Baldspoti* flies, we see a significant reduction in EGFP signal (3.8 ± 0.8 fluorescent units vs. 6.5 ± 1.1 in controls; $P = 9.6 \times 10^{-4}$), suggestive of reduced ER stress signaling (Figure 2E and F). Strikingly, the *Rh1*^{G69D}*/Baldspoti* eyes discs display no substantial change in rhodopsin

protein levels (Figure 2E), suggesting that loss of *Baldspot* expression reduces IRE1/Xbp1 ER stress signaling without affecting the accumulation or degradation of misfolded Rh1 protein.

Stearate supplementation bypasses the requirement for *Baldspot*

Increased cellular concentration of stearate is associated with increased activation of the ER stress response [32, 33]. It is likely that the modifying effect of Baldspot is linked to its fatty acid elongation activity. Baldspot converts palmitate to stearate and the absence of Baldspot function should result in lower levels of stearate. We hypothesized that lower levels of stearate underlies the rescuing effect, reduced ER stress signaling, and retinal degeneration we observed, and that this signaling could be restored if stearate were supplemented during development. To test this, we raised $Rh1^{G69D}$/*Baldspoti* and $Rh1^{G69D}$ control flies on media with or without 10% stearate and measured eye size in adults. Stearate supplementation resulted in increased degeneration and reduced eye size in $Rh1^{G69D}$/*Baldspoti* flies (14033 ± 1553 pixels) as compared to those raised on standard media with no stearate (16846 ± 1699 pixels) ($P = 1 \times 10^{-7}$) (Figure 3A). This increase in degeneration in $Rh1^{G69D}$/*Baldspoti* flies was accompanied by an increase in IRE1 activity and spliced *Xbp1*, as evidenced by an increase in Xbp1-EGFP expression in eye discs (Figure 3B). Stearate supplementation had no effect on eye size, retinal degeneration, or Xbp1-EGFP signal in $Rh1^{G69D}$ control flies (13478 ± 1197 pixels vs. 12873 ± 1006 pixels) (Figure 3 A-B), likely because of the high level of ER stress signaling already present. High stearate levels are sufficient to induce ER stress signaling and bypasses the need for Baldspot activity.

***Baldspot* is a general modifier of ER stress signaling**

Because *Baldspot* is ubiquitously expressed (www.flyatlas2.org/), it is possible that *Baldspot's* modifying role in ER stress is generalizable across different contexts and tissues. We first tested whether Baldspot modifies ER stress in another tissue. Because wing size is a visible and easily scorable trait, we used the *MS1096-GAL4* driver to express $Rh1^{G69D}$ in the developing larval wing

disc (wing-*Rh1*^{G69D}). The Rh1^{G69D} protein acts as a misfolded protein, inducing ER stress only in that tissue. The wing disc gives rise to the adult wing, and perturbations in cellular homeostasis in this tissue result in small, misshapen wings [34]. Expression of wing-*Rh1*^{G69D} produces a phenotype similar to a vestigial wing (Figure 4A). When the RNAi construct targeted against *Baldspot* was concurrently expressed in the wing disc (wing-*Rh1*^{G69D}*/Baldspoti*), this vestigial phenotype was partially rescued. The adult wings showed an increase in size and an increase in unfolding upon eclosion (Figure 4A). Similar to what we observed in the eye discs, this partial rescue was associated with a decrease in the level of Xbp1-EGFP in the wing disc (Figure 4B). It appears that loss of *Baldspot* modifies IRE1/Xbp1 signaling across different tissues and is not eye disc-specific.

To test whether Baldspot modifies ER stress signaling by pharmacological induction, we used tunicamycin to induce ER stress. Tunicamycin inhibits N-linked glycosylation in the ER and results in massive misfolding, ER stress, and a robust UPR [35]. Treatment with tunicamycin in *Drosophila* larvae results in significant developmental lethality. We acutely exposed 2nd instar control larvae (*Tub-GAL4*, no RNAi) and larvae with ubiquitous knockdown of *Baldspot* (*Tub-GAL4>Baldspot* RNAi) to tunicamycin or control DMSO and scored survival to pupation. To account for any larval lethality due to loss of *Baldspot*, we normalized the survival of larvae on tunicamycin to the DMSO treatment. Strikingly, larvae lacking *Baldspot* were less susceptible to ER stress-induced lethality than control larvae (Figure 4D), as evidenced by increased survival to pupation. The modifying effect of *Baldspot* expression on the ER stress response is generalizable across different tissues and methods of ER stress induction.

***Baldspot* does not regulate IRE1 RIDD function**

Because our data indicates that Baldspot regulates Ire1/Xbp1 signaling, we next tested whether Baldspot regulates another key IRE1 function: Regulated IRE1 Dependent Degradation of

mRNAs (RIDD) [36-38]. In addition to *Xbp1* splicing, activated IRE1 is also important for the degradation of a number of ER-associated transcripts through RIDD. To test if RIDD activity is affected by loss of *Baldspot* expression, we measured levels of known RIDD target mRNAs, under ER stress, in control cells and tissues with or without *Baldspot* expression. We employed S2 cell culture and treated S2 cells with dsRNA targeting either *Baldspot* or *EGFP* as a control. This treatment resulted in a near complete reduction in *Baldspot* transcript levels (~7.8% of control; P = 1.3×10^{-9}) (Figure 5A). We used Dithiothreitol (DTT) to induce ER stress in S2 cells. DTT is a reducing agent that breaks disulfide bonds in proteins, resulting in misfolded proteins and a strong ER stress response [39]. As expected, treatment of cells with DTT increased *Xbp1* splicing ~3-fold, indicative of ER stress. In agreement with our *in vivo* studies in the $Rh1^{G69D}$ fly, this splicing was significantly reduced in the absence of *Baldspot*, with only ~2-fold increase (P = 0.023), indicating that IRE1 signaling is disrupted (Figure 5B).

We found that under DTT-induced ER stress, there was a similar degree of reduction (~81% of control) in RIDD target expression in the cells treated with DsRNA against *Baldspot* or control DsRNA against EGFP (Figure 5C). Non-RIDD targets, such as *Actin5C*, were unaffected (Figure 5C). It appears that loss of *Baldspot* preferentially affects IRE1/Xbp1 signaling, while RIDD function remains intact.

DISCUSSION

The ER stress response has the potential to be an important modifier of human disease. ER stress is activated in a wide variety of diseases ranging from Mendelian degenerative diseases to complex metabolic disorders [14]. The role of the ER stress response is complex and in some cases, a stronger response is beneficial and in other cases, a strong response underlies the disease. In some forms of retinitis pigmentosa, like the focus of this study, loss of vision is a direct result of the ER stress-induced cell death [40]. Neurodegenerative diseases such as Parkinson's,

Alzheimer's, Amyotrophic Lateral Sclerosis (ALS), and prion disease are all associated with the accumulation of cytoplasmic misfolded proteins that can activate a secondary ER stress response and subsequent cell death [15]. In diabetes and obesity, misfolded proteins and the ER stress response are at least partially responsible for the cellular dysfunction that is a hallmark of the disease state [41]. In some cancers, however, cells selectively upregulate ER stress pathways associated with survival, while inhibiting those associated with cell death [42]. Genetic variation that alters the degree to which the ER stress response is activated may modify disease in the population.

We and others have shown that genetic variation can influence how and to what extent the ER stress response is activated. Genetic variation in *Drosophila* [18, 19], mouse [17], and human cells [16] can impact the expression of genes involved in ER stress pathways. Furthermore, this variation in expression has been linked to differences in the UPR and the downstream activation of cell death [17-19]. It is apparent that cryptic genetic variation drives these extensive inter-individual differences in the ER stress response [17]. Stress or disease conditions reveal the effect of this otherwise silent genetic variation. The same variation that drives differences in this important stress response might also influence disease heterogeneity. Indeed, modulating the ER stress response is an effective way of modulating disease outcome in models of human disease. [43-48].

In this study we show that loss of *Baldspot/ELOVL6* activity can affect the outcome of retinal degeneration and has the potential to be a broader regulator of ER stress-associated diseases. As a 16-to-18 carbon fatty acid elongase in the ER membrane, this enzyme is responsible for increasing the concentration of C18 stearate, a saturated long chain fatty acid [24, 33]. Increases in stearate concentrations in the ER membrane can induce and enhance ER stress through the activation of IRE1/Xbp1 [32, 33]. Here, we show that reducing the activity of Baldspot/ELOVL6

reduces the degree to which the UPR and apoptosis are induced in models of ER stress. Our study suggests that Baldspot/ELOVL6 has a modifying effect on the IRE1 branch of the ER stress response. IRE1 has several roles, including catalyzing *Xbp1* signaling and RNA degradation through RIDD. Strikingly, we found that Baldspot/ELOVL6 specifically influences *Xbp1* signaling and not RIDD function. Despite utilizing the same endonuclease domain, *Xbp1* splicing and RIDD have different roles in development, cellular homeostasis, and stress responses [49]. This is also consistent with observations that the activation of various arms of the ER stress response can be alternately beneficial or detrimental, dependent upon the disease and tissue context [50]. It is unclear how these functions are partitioned by IRE1, but further studies exploring the Baldspot/ELOVL6 interaction with IRE1 may clarify the differential regulation of *Xbp1* signaling and RIDD function.

Therapeutics that reduce the activity of this key fatty acid elongase could conceivably be used to treat or delay progression in a variety of ER stress-associated diseases. In RP patients with mutations that cause the accumulation of misfolded rhodopsin, similar to our *Drosophila* model, reduction of ELOVL6 expression might reduce cell death, delaying vision loss. We present evidence that the modulation of the ER stress response through Baldspot/ELOVL6 activity may impact the severity of diseases beyond RP. Particularly intriguing is that *Baldspot/ELOVL6* can alter the ER stress response when it is induced by different conditions. Regardless of the mechanism by which ER stress is induced, inhibiting ELOVL6 activity reduces IRE1/Xbp1 signaling. In fact, in a recent study of the *db/db* mouse model of diabetes, loss of *ELOVL6* alters insulin sensitivity and the expression of ER stress genes [22]. It is likely that reducing *Baldspot/ELOVL6* expression would also alter complex diseases where ER stress contributes to degeneration and cell death. In some cases, even a small delay in degeneration could dramatically improve the quality of life.

We have identified *Baldspot/ELOVL6* in *Drosophila* as a novel modifier of the ER stress response. Its potential as a therapeutic target extends beyond RP to any number of ER stress-associated disorders. The identification of *Baldspot* in a natural genetic variation screen [19] suggests that variation in expression of this gene is well tolerated under healthy conditions and might be amenable to modulation. Identifying variable elements of the ER stress response pathway may prove to be a successful strategy to nominating new modifiers with broad applications to many ER stress-associated diseases.

METHODS

Fly stocks and maintenance

Flies were raised at room temperature on standard diet based on the Bloomington Stock Center standard medium with malt. The strain containing *GMR-GAL4* and *UAS-Rh1*^{G69D} on the second chromosome (*GMR>Rh1*^{G69D}) has been previously described [19]. The following strains are from the Bloomington Stock Center: *MS1096-GAL4* (8696), *Baldspot* RNAi (44101), control attP40 (36304). The strain containing the *UAS-Xbp1-EGFP* transgene were a gift from Don Ryoo (NYU) [20].

Eye/wing imaging

For eye and wing images, adult females were collected under CO_2 anesthesia and aged to 2-7 days, then flash frozen on dry ice. Eyes were imaged at 3X magnification using a Leica EC3 camera. Wings were dissected away from the body, then imaged at 4.5X magnification using the same camera. Eye area was measured in ImageJ as previously described [19].

Immunohistochemistry

Eye discs and wing discs were dissected from wandering L3 larvae in cold 1X PBS, then immediately transferred to cold 4% PFA on ice. Tissues were fixed in 4% PFA for 15-20 min, then

washed in 1XPAT (0.1% TritonX100) prior to blocking with 5% normal donkey serum. Tissues were stained with primary antibodies for rhodopsin (1:50, Developmental Studies Hybridoma Bank #4C5) and GFP (1:2000, Thermo-Fisher #A6455). Apoptosis was monitored using the ApopTag Red *In Situ* Apoptosis Detection Kit (Millipore #S7165). Tissues were mounted in Slowfade™ Diamond Antifade Mountant (ThermoFisher #S36967) and imaged with an Olympus FV1000 confocal microscope.

Stearate supplementation

Crosses were set up on the control, standard media alone, or standard media with 10% stearate supplementation. To make stearate supplemented media, standard media was melted in the microwave and kept at 98°C on a stir plate. Stearate (10% final concentration) was added to this media and mixed until lipids were homogenous with the media. The standard control diet received the same treatment, but no additional stearate was added. To determine retinal degeneration, flies were aged and imaged as described above. Antibody staining was performed on the eye imaginal discs of L3 larvae, also as described above.

Tunicamycin treatment

Crosses to generate the indicated genotypes were set up on egg caps containing yeast paste. L2 larvae were then treated with either 10 µg/mL Tunicamycin (diluted 1:1000 from a 10 mg/mL stock solution) or 1:1000 DMSO in Schneider's media for 30 minutes at room temperature. The larvae were then washed in 1XPBS twice and placed on solid media containing 6% yeast and 1% agar. Viability was determined by survival to pupation. Survival for each genotype was normalized to the DMSO-treated control condition. Each replicate represents ~25-60 larvae per genotype; a total 180 to 250 larvae per genotype were analyzed across all replicates.

S2 cells

DsRNA was generated using the MEGAscript™ T7 Transcription kit (ThermoFisher #AM1334), with primers for *EGFP* (F: TTAATACGACTCACTATAGGGAGACCACAAGTTCAGCGTGTCC and R: TTAATACGACTCACTATAGGGAGAGGGGTGTTCTGCTGGTAGTG) and *Baldspot* (F: TTAATACGACTCACTATAGGGAGAATCCGCCCAGGTTCATCTCG and R: TTAATACGACTCACTATAGGGAGAGTCACATCTCGCAGCGCAAC). S2 cells were treated with DsRNA against *EGFP* (as a control) or against *Baldspot* at a density of approximately 2 x 10^6 cells/mL in a 24-well plate. Cells were incubated with DsRNA for 4-7 days before treatment with 0.5 mM DTT for 60 min for *Xbp1* splicing or 1.0 mM DTT for 20 min for RIDD target analysis. Control cells were treated with DMSO. RNA was isolated from cells using Trizol/chloroform extraction and used to generate cDNA. Knockdown of *Baldspot* was confirmed using qPCR (primers: F: TGCTGGTCATCTTCGGTGGTC and R: ACGCAGACGGAGTGGAAGAG). RIDD target levels were also confirmed by qPCR (*sparc* primers: F: AAAATGGGCTGTGTCCTAACC and R: TGCAGCACAATCTACTCAATCC). *Xbp1* splicing was evaluated from the cDNA using PCR (primers for *Xbp1:* F: TCAGCCAATCCAACGCCAG and R: TGTTGTATACCCTGCGGCAG). The spliced and unspliced bands were separated on a 12% acrylamide gel and the proportion of these bands quantified using ImageJ software.

Statistics

Statistics were calculated using R software. P-values were determined using ANOVA for eye size, fluorescence levels, and transcript levels from S2 cells. Tukey multiple testing correction was used for the fatty acid feeding experiment. Bonferroni correction was used for *Xbp1* splicing in S2 cells. A pairwise T-test was performed for larval tunicamycin treatment. A cutoff of P = 0.05 was used for significance.

Acknowledgements

We thank Drs. Aylin Rodan and Carl Thummel for use of equipment and sharing reagents. We thank Emily Coelho for helpful comments.

Funding

This research was supported by an NIH/NIGMS R35 award (1R356M124780) and a Glenn Award from the Glenn Foundation for Medical Research to CYC. CYC is the Mario R. Capecchi Endowed Chair in Genetics. R.A.S.P. was supported by a NIDDK T32 fellowship (5T32DK091217).

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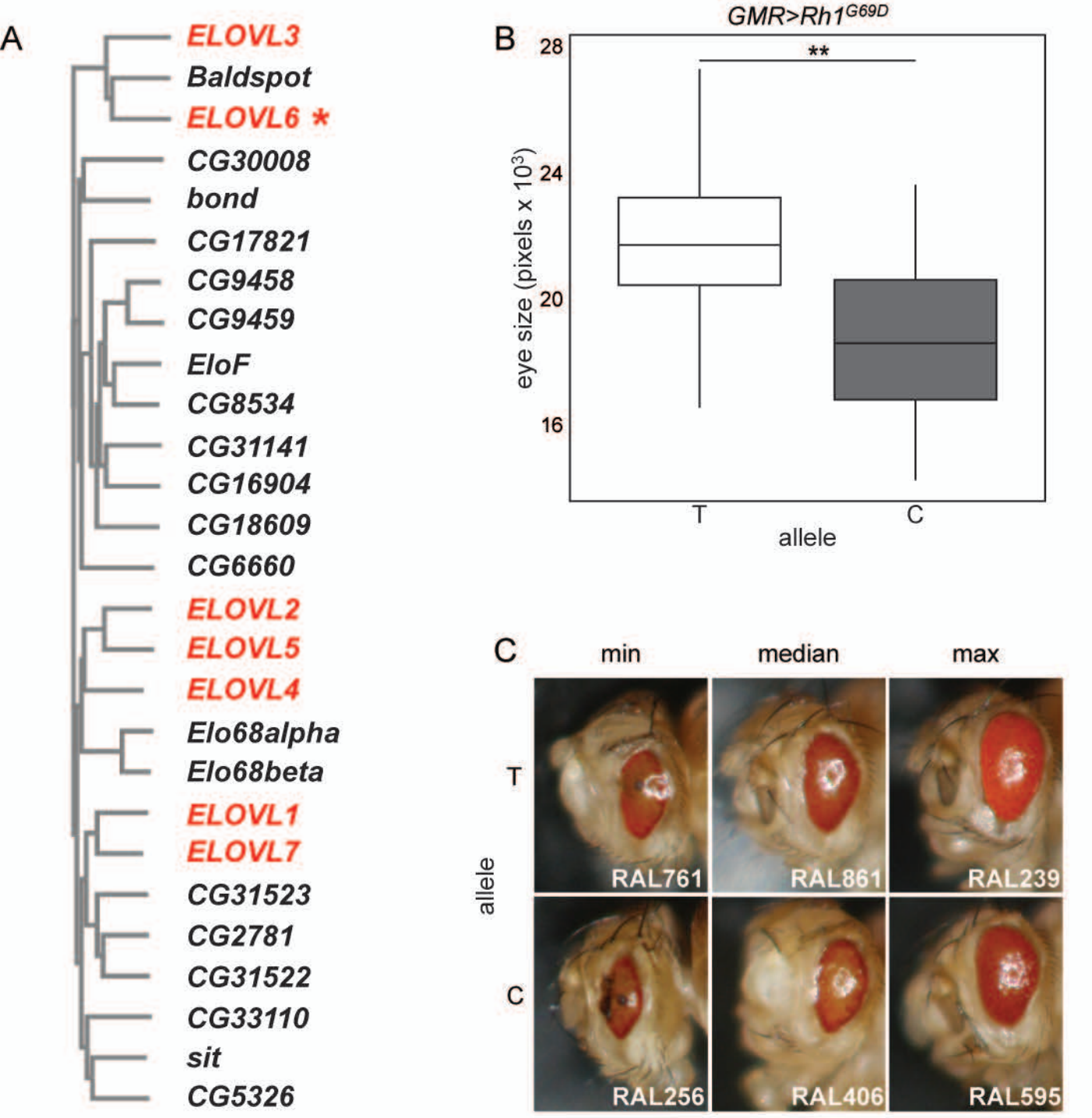

Figure 1. A SNP in *Baldspot* is associated with degeneration in a model of retinitis pigmentosa. A. *Baldspot* is the only *Drosophila* orthologue of mammalian *ELOVL6* (*). Phylogeny of long chain fatty acid elongases shows the relationship between *Drosophila* (black) and human (red) genes. **B.** The 3L:16644000SNP in *Baldspot* (BDGP R5/dm3) is associated with $Rh1^{G69D}$–induced degeneration in the DGRP. $Rh1^{G69D}$ DGRP eye size is plotted by allele identity. Strains carrying the minor C allele (18880 ± 3010 pixels) have significantly smaller eyes than those carrying the major, T allele (21822 ± 2294 pixels). **C.** Images from $Rh1^{G69D}$ DGRP strains that carry either the T or the C allele. Images were chosen from strains representing the range of eye sizes for the two alleles. All data from B and C were taken from Chow et.al 2016. ** $P < 0.005$

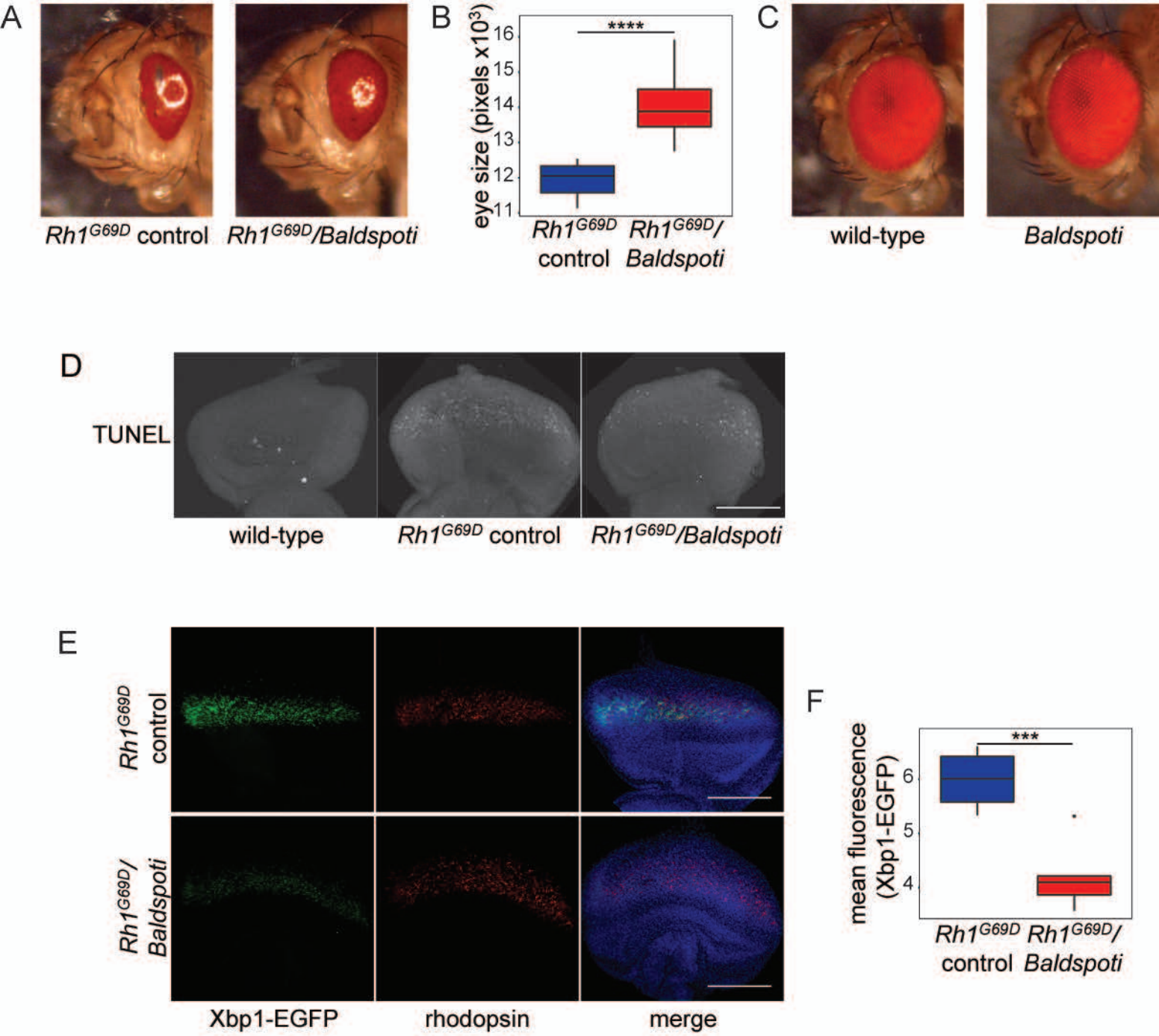

Figure 2. Loss of *Baldspot* in the *Rh1^{G69D}* eye disc alleviates ER stress and apoptosis. A. Degeneration caused by overexpression of *Rh1^{G69D}* is partially rescued by RNAi knockdown of *Baldspot* (*Rh1^{G69D}/Baldspoti* vs *Rh1^{G69D}* controls). **B.** This rescue was significant when eye size was quantified, as indicated by a larger eye in *Rh1^{G69D}/Baldspoti* flies (14043 ± 960 pixels vs. controls 11942 ± 473 pixels). **C.** Loss of *Baldspot* had no effect on eye phenotype in an otherwise wild-type background. **D.** *Rh1^{G69D}/Baldspoti* eye discs also display reduced apoptosis levels compared to *Rh1^{G69D}* controls, as measured by TUNEL staining. **E.** *Rh1^{G69D}/Baldspoti* eye discs had reduced expression of Xbp1-EGFP as compared to *Rh1^{G69D}* controls, while rhodopsin levels were unchanged. Eye discs were dissected from wandering L3 larvae expressing *Rh1^{G69D}* and *UAS-Xbp1-EGFP*, stained for rhodopsin and GFP as an indicator of ER stress, and counterstained with 4',6-diamidino-2-phenylindole (DAPI). F. Xbp1-EGFP levels are significantly reduced in *Rh1^{G69D}/Baldspoti* eye discs (3.8 ± 0.8 fluorescent units) as compared to *Rh1^{G69D}* controls (6.5 ± 1.1 fluorescent units). Fluorescence was quantified using mean fluorescence from the image stack as calculated in Image J. Scale bars = 0.1 mm. *** $P < 0.0005$, **** $P < 0.0001$.

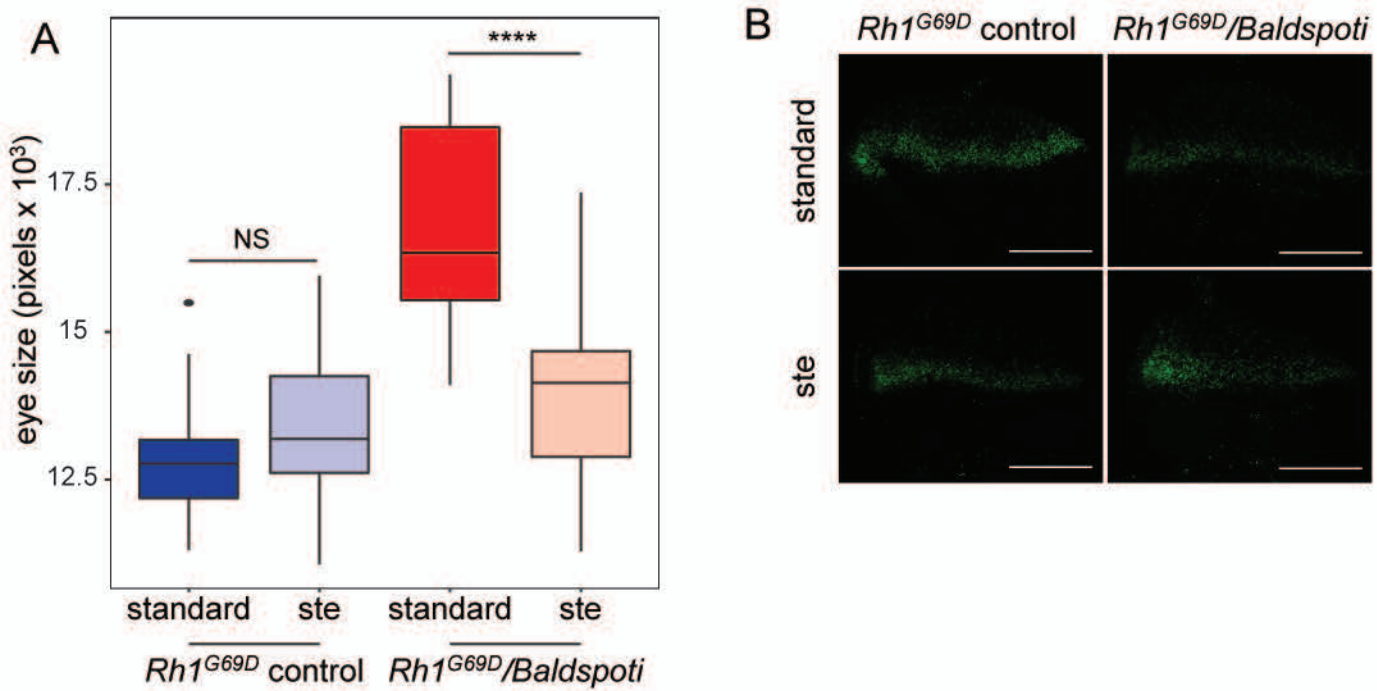

Figure 3. Stearate supplementation increases degeneration and activation of ER stress pathways in *Rh1^{G69D}/Baldspoti* flies. A. Eye size was significantly reduced in *Rh1^{G69D}/Baldspoti* flies (*Baldspot* RNAi) when they were fed 10% stearate (14033 ± 1553 pixels) as compared to standard media (16846 ± 1699 pixels). Eye size was not significantly changed in *Rh1^{G69D}* control flies (No RNAi) fed 10% stearate (13479 ± 1197 pixels) as compared to the standard media (12873 ± 1006 pixels). **B.** The 10% stearate diet also increased Xbp1-EGFP levels in eye discs from *Rh1^{G69D}/Baldspoti* flies to a similar level seen in *Rh1^{G69D}* controls. Scale bars = 0.1 mm. **** $P < 0.0001$.

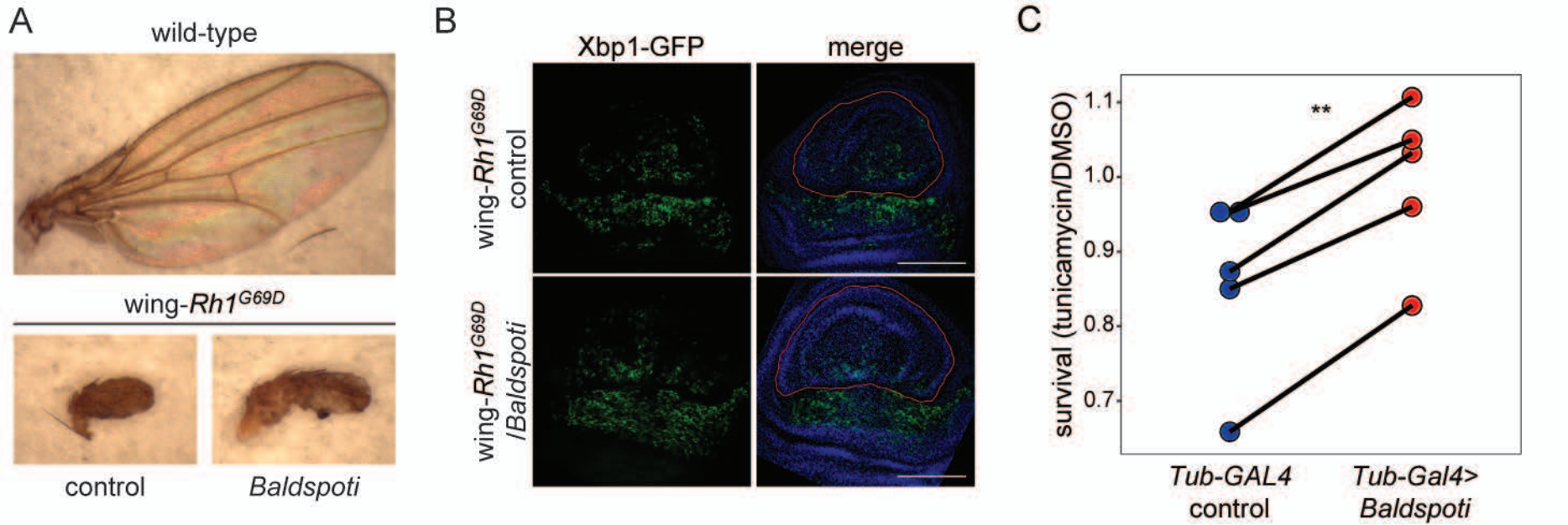

Figure 4. *Baldspot* is a general modifier of the ER stress response. A. Loss of *Baldspot* partially rescues the adult vestigial wing phenotype caused by expression of *Rh1*^{G69D} in the wing disc (wing-*Rh1*^{G69D}*/Baldspoti* vs wing-*Rh1*^{G69D} controls). **B**. Wing discs from wing-*Rh1*^{G69D}*/Baldspoti* flies had lower Xbp1-EGFP expression, as compared to wing discs from wing-*Rh1*^{G69D} controls. Wing discs were dissected from wandering L3 larvae expressing *Rh1*^{G69D} and *UAS-Xbp1-EGFP* and stained for GFP and DAPI as a counterstain. The wing disc is outlined in red in the merged image. **C.** Larvae with ubiquitous knockdown of *Baldspot* are significantly more resistant to Tunicamycin-induced ER stress than control larvae. L2 larvae were treated with tunicamycin or DMSO and survival to pupation was calculated as a measure of resistance to ER stress. Five representative experiments are shown. Scale bars = 0.1 mm. ** $P < 0.005$.

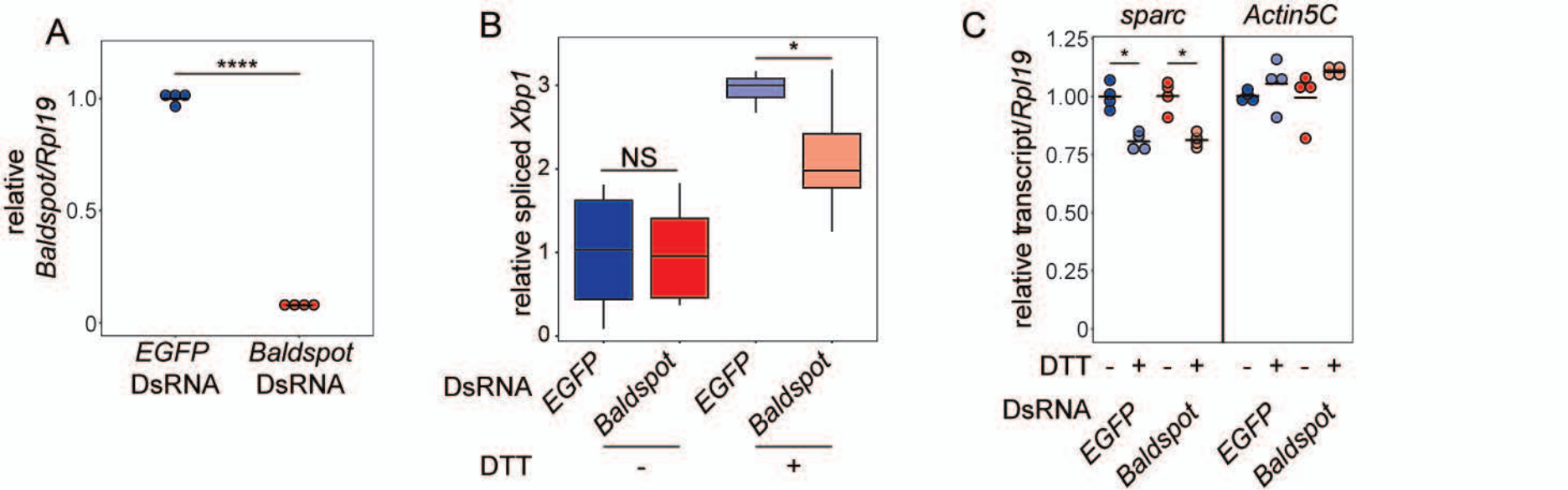

Figure 5. Baldspot regulates *Xbp1* splicing but not RIDD activity of IRE1. A. S2 Cells treated with DsRNA targeted against *Baldspot* had near complete reduction in *Baldspot* transcript levels (7.8%) as compared to control cells treated with DsRNA against *EGFP*. **B.** DTT treatment increased *Xbp1* splicing in control cells (2.96-fold increase), while splicing was reduced in cells treated with DsRNA against *Baldspot* (2.12-fold increase). **C.** Levels of the RIDD target *sparc* are reduced in control cells treated with DTT when compared to DMSO-treated controls (~81% of controls). This reduction is unaffected by treatment with *Baldspot* DsRNA. *Actin5c* transcript levels were unaffected by DTT treatment. * $P < 0.05$, **** $P < 0.0001$.