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TRP and rhodopsin transport depends on dual XPORT ER chaperones encoded by an operon

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Summary

TRP channels and G protein-coupled receptors (GPCR) play critical roles in sensory reception. However, the identities of the chaperones that assist GPCRs in translocating from the endoplasmic reticulum (ER) are limited, and TRP ER chaperones are virtually unknown. The one exception for TRPs is Drosophila XPORT. Here, we show that the *xport* locus is bicistronic, and encodes unrelated transmembrane proteins, which enable the signaling proteins that initiate and culminate phototransduction, rhodopsin 1 (Rh1) and TRP, to traffic to the plasma membrane. XPORT-A and XPORT-B are ER proteins, and loss of either has a profound impact on TRP and Rh1 targeting to the light-sensing compartment of photoreceptor cells. XPORT-B complexed *in vivo* with the Drosophila homolog of the mammalian HSP70 protein, GRP78/BiP, which in turn associated with Rh1. Our work highlights a coordinated network of chaperones required for the biosynthesis of the TRP channel and rhodopsin in Drosophila photoreceptor cells.

Introduction

Ion channels and GPCR comprise the two largest categories of plasma membrane proteins critical for neuronal signaling (Isacoff et al., 2013; Marinissen and Gutkind, 2001). Prior to insertion at the surface of sensory cells and other neurons, these integral membrane proteins must be properly folded and processed, before they can exit the ER and begin their journey through the secretory pathway en route to the plasma membrane and other destinations.

Neurons and other cells rely on multiple classes of protein chaperones that promote protein folding (Hartl et al., 2011; Kampinga and Craig, 2010). Among the most notable are the large and diverse families of heat shock proteins, which stabilize a broad array of partially unfolded proteins (Hartl et al., 2011; Kampinga and Craig, 2010). Other molecular chaperones are required for the folding of a more limited set of proteins. In the context of

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Author contributions

Z.C. performed all experiments, except for the results shown in Figures S5D–S5F and S6B–S6C. H.C.C. performed the *in vitro* experiments using S2R+ cells (Figures S5D–S5F, and S6B–S6C). C.M. and Z.C. designed the experiments, interpreted the results and co-wrote the manuscript.

sensory neurons, examples include the cyclophilin-related protein RanBP2, which acts as a chaperone for a cone opsin (Ferreira et al., 1996), TMHS—a protein required for the efficient localization of PCDH15 to stereocilia in cochlea hair cells (Xiong et al., 2012) and ODR-4, which mediates the delivery of odorant receptors to cilia in *C. elegans* (Dwyer et al., 1998).

Dissection of the molecular chaperones required for the function of membrane proteins is critical not only for clarifying the intracellular machinery required for neuronal excitability, but also for understanding the etiology of neuronal diseases resulting from defects in protein folding or trafficking. Protein mislocalization or accumulation of protein aggregates underlies a wide range of neurodegenerative diseases, ranging from Alzheimer's disease to Huntington's disease, Parkinson's disease and certain forms of autosomal dominant retinal degeneration resulting from accumulation of misfolded rhodopsin, which is the classical GPCR (Dryja et al., 1990; Soto, 2003). Chemical chaperones represent a major approach for discovering therapies to treat diseases resulting from misfolded GPCRs, many of which are trapped in the ER (Maya-Núñez et al., 2012).

Drosophila photoreceptor cells have provided insights into the folding and transport of signaling proteins that must negotiate the secretory pathway, prior to insertion in the microvillar domain, the rhabdomeres, where phototransduction takes place (Colley, 2012; Colley et al., 1995). The rhadomeres are comprised of thousands of tube-like microvilli, each of which is only 50 nanometer in width and ~1.5 micrometer in length (Hardie and Juusola, 2015; Montell, 2012). Due to the highly restricted architecture of the microvilli, insertion of phototransduction proteins into this organelle represents a major challenge.

The core machinery required for Drosophila phototransduction is turned on by light activation of rhodopsin, which sequentially leads to activation of a trimeric G-protein (Gq) and phosopholipase C (PLC) (Hardie and Juusola, 2015; Montell, 2012). The signaling cascade culminates with depolarization of the photoreceptor cells, following activation of the classical TRP channel (Hardie and Minke, 1992; Montell and Rubin, 1989), and the related channel, TRP-Like (TRPL; Niemeyer et al., 1996; Phillips et al., 1992). Folding of the main Drosophila rhodopsin (Rh1) depends on the ER proteins Calnexin (Rosenbaum et al., 2006) and NinaA (Colley et al., 1991). In addition, XPORT-A (formerly XPORT) serves as a chaperone for both Rh1 and the TRP channel (Rosenbaum et al., 2011). To date, XPORT-A represents the only chaperone identified that facilitates translocation of TRP from the ER.

Here, we show that the *xport* locus is bicistronic and encodes two unrelated single-pass transmembrane proteins, XPORT-A and XPORT-B. We found that XPORT-B was localized to the ER, and both XPORT proteins were non-redundantly required for insertion of TRP and Rh1 into the rhabdomeres. Loss of XPORT-B resulted in retention of the residual TRP in the ER, and a transient response to light, which was indistinguisable from the *trp* mutant phenotype. In contrast, loss of XPORT-B had no effect on TRPL expression. The *xport-B* mutant photoreceptor cells underwent age- and light-dependent retinal degeneration, similar to *trp* mutants. Consistent with XPORT-B functioning in a chaperone complex, we found that it interacted with HSC3, a GRP78/BiP homolog, which in turn associated with Rh1.

Results

XPORT was bicistronic and encoded unrelated transmembrane proteins

The *xport* locus is predicted to encode a second protein (CG42508) in addition to XPORT (CG4468; http://flybase.org/reports/FBrf0205930.html). For clarity, we renamed the original XPORT protein (CG4468) XPORT-A, and designated CG42508 as XPORT-B (Figure 1A and 1B). The XPORT-A protein is comprised of 116 amino acids with a single transmembrane segment near the C-terminal end, whereas XPORT-B includes 113 amino acids and one putative transmembrane segment near the N-terminus (Figure 1B). XPORT-A and XPORT-B do not share amino acid similarity, and are predicted to be type 2 and type 1 membrane proteins, respectively. Based on sequencing of cDNAs (http://flybase.org/reports/FBcl0116077.html), the two proteins are encoded by a single mRNA (Figure S1A). Thus, *xport* is bicistronic. At one time bicistronic genes were thought to be rare in animals. However, at least 115 Drosophila genes are suggested to be bicistronic (Lin et al., 2007).

The two prevailing mechanisms enabling translation of the second coding sequence in a bicistronic mRNA are the internal ribosome entry site (IRES) and the ribosomal leaky scanning mechanism (Misra et al., 2002). Most bicistronic mRNAs that use the IRES mechanism have long intercistronic regions of up to several kilobases, which typically include multiple AUGs. However, the leaky scanning mechanism most likely facilitated translation of *xport-B* since the *xport* locus fulfilled the criteria of no start codons in the intercistronic region, the sequence between the two cistrons was between 15 and 78 nucleotides (51 nucleotides) and the start codon for the second protein (XPORT-B: CAAUAUG) was an excellent match to the Drosophila Kozak sequence [(C/A)AA(A/C)AUG] (Cavener, 1987).

There are XPORT-B homologs in many insect species (Figure S1B), although there are no obvious homologs outside of insects. The insects with XPORT-B homologs belong to orders ranging from Diptera to Hymenoptera, Coleoptera, Hemiptera, and Isoptera (Figure S1B). Moreover, the bicistronic structure of the homologous *xport-A* and *xport-B* genes is preserved among all of these insects.

The original $xport^{1}$ allele has a nonsense mutation within the *xport-A* coding sequence (Rosenbaum et al., 2011). In order to explore the function of *xport-B*, we deleted the entire coding sequence by homologous recombination (Gong and Golic, 2003) (Figure 1A; $xport^{\Delta B}$). We engineered a second allele by homologous recombination that removed both *xport-A* and *xport-B* (Figure 1A; $xport^{\Delta AB}$).

To test whether XPORT-B was expressed in wild-type, we raised XPORT-B antibodies. Anti-XPORT-B detected a protein in “wild-type control” flies (w^{1118}) of the predicted molecular weight, which was eliminated in $xport^{\Delta B}$ and $xport^{\Delta AB}$, but was still present in $xport^{1}$ (~13 kDa; Figure 1C). Using head extracts and XPORT-A antibodies, we found that XPORT-A was not expressed in $xport^{1}$ and $xport^{\Delta AB}$, while it was still produced in $xport^{\Delta B}$ (Figure 1C). These data supported the prediction that the *xport* locus was bicistronic. We also generated four different genomic rescue transgenes and two cDNA rescues (Figures S2A and S2B) for the phenotypic analyses described below. We confirmed the effectiveness

of these transgenes by introducing them into the *xport*$^{\Delta AB}$ background, and probing for the XPORT-A and XPORT-B proteins on Western blots (Figure 1C).

XPORT-A is a transmembrane protein (Rosenbaum et al., 2011). To test whether XPORT-B is also a membrane protein as predicted, we prepared head extracts and performed high-speed centrifugation. In the absence of detergent, XPORT-B was exclusively in the pellet, but was in the supernatant after preparing the extracts in the presence of 1% Triton X-100 (Figure S1C), consistent with the prediction that XPORT-B was a transmembrane protein.

Disruption of *xport-B* resulted in a transient light response

Loss of XPORT-A causes a transient light response, which resembles the *trp* mutant phenotype (Cosens and Manning, 1969; Rosenbaum et al., 2011). To test whether XPORT-B also had an important role in photoreceptor cells, we performed electroretinogram (ERG) recordings, which measured the summed light responses of all the cells in the retina (Pak, 2010; Pak et al., 2012). In wild-type flies, light elicits a sustained response, while the ERG response in *trp* mutant animals is transient (Figure 2A). The *xport*^{1} mutants showed a transient ERG response (Rosenbaum et al., 2011), although we found that the decay of the light response was faster in *trp* mutants than in *xport*^{1} flies (Figures 2A and 2B). However, *xport*$^{\Delta B}$ mutants exhibited a transient ERG phenotype, which was indistinguishable from *trp* null mutants (Figures 2A and 2B). Removing both XPORT-A and XPORT-B (*xport*$^{\Delta AB}$) caused a similar transient light response (Figures 2A and 2B).

The impairments described here were not due to background mutations, since we rescued the *xport*^{1}, *xport*$^{\Delta B}$ and *xport*$^{\Delta AB}$ ERG phenotypes with genomic transgenes that expressed just *xport-A*, *xport-B* or both (Figures S2A and S2C). When we expressed *xport-B* under control of the *rhodopsin 1* promoter (*rh1*/*ninaE*), which is expressed in six out of eight photoreceptor cells (R1 to R6), we also rescued the *xport*$^{\Delta B}$ ERG phenotype (Figure S2C; *n*[*B*]), demonstrating that XPORT-B was required in photoreceptor cells. Over-expression of either XPORT-B in the *xport*^{1}, or XPORT-A in *xport*$^{\Delta B}$ failed to rescue the transient light responses (Figure S2C; *n*[*A*] and *n*[*B*]). Thus, XPORT-A and XPORT-B roles were not redundant.

In the absence of TRP, the remaining transient light response is mediated by the TRP-Like (TRPL) channel (Niemeyer et al., 1996). Flies missing both TRP and TRPL (*trpl;trp*) are unresponsive to light. TRPL is a non-selective cation channel, while TRP comprises the main pathway for light-induced Ca^{2+} influx. The transient light response in *trp* mutants is due to loss of sufficient Ca^{2+} influx to attenuate the activity of phospholipase C, and prevent depletion of PIP_2 (Hardie et al., 2001). Therefore, the transient ERG displayed by *xport*$^{\Delta B}$ suggested that this phenotype was due to defects in TRP but not TRPL function.

To determine whether the *xport* mutations affected TRP exclusively, we analyzed the *xport* alleles in *trp* and *trpl* null mutant backgrounds. We found that the ERG responses of all three *xport* alleles when combined with the *trp*^{MB} mutation were indistinguishable from *trp*^{MB} alone (Figures 2C and 2D). Thus, TRPL function was largely unaffected by loss of the XPORT proteins. In contrast, when we placed each of the three *xport* alleles in the *trpl*^{MB} background, they all showed a stronger ERG phenotype (Figures 2E and 2F). Upon removal

of TRPL and both XPORT proteins ($trpl^{MB}$;$xport^{\Delta AB}$ flies), the flies were nearly unresponsive to light (Figures 2E and 2F), indicating that TRP was strictly dependent on the dual XPORT proteins.

TRP and Rh1 expression depended on XPORT-B

To examine the extent to which TRP expression was affected in the *xport* mutants, we checked the protein levels of TRP and found that it was greatly diminished in all three *xport* alleles (~2% of wild-type levels; Figures 3A and 3G). In contrast, the concentration of TRPL in $xport^{\Delta B}$ was similar to the control, and reduced moderately in $xport^{1}$ (47.8±8.2% left) and $xport^{\Delta AB}$ (23.6±6.6% left; Figures 3B and 3G).

The major rhodopsin in fly photoreceptor cells is Rh1, which is expressed in six of the eight photoreceptor cells (R1-6) in each of the ~800 repeat units (ommatidia) of the compound eye. The level of Rh1 is decreased severely in $xport^{1}$ (Rosenbaum et al., 2011). We found that the concentration of Rh1 was reduced to 9.4 ±5.5% in $xport^{\Delta B}$ mutants, and even more dramatically in $xport^{1}$ and $xport^{\Delta AB}$ flies (1.0 ±0.1% and 1.1 ±0.4% left respectively; Figures 3C and 3G). Expression of the XPORT-B protein was not reciprocally dependent on TRP and Rh1, as the concentration of XPORT-B was comparable to the control in both trp^{MB} and $ninaE^{I17}$ (*rh1*) null mutant flies (Figures S3B and S3C). However, the concentration of XPORT-A was reduced in $ninaE^{I17}$ (*rh1*) null mutant flies (36.7 ±11.9% left; Figures S3A and S3C). Seven out of nine other photoreceptor cell enriched proteins were decreased modestly in $xport^{1}$ and $xport^{\Delta AB}$, while seven of these nine were unchanged in $xport^{\Delta B}$ (Figures 3D–3G; Figures S3D–S3I).

XPORT-B was an ER protein required for translocalization of TRP

In wild-type animals, TRP is restricted to the rhabdomeres (Montell and Rubin, 1989). In contrast, in $xport^{1}$, the level of TRP is diminished severely, and a significant proportion of the remaining TRP is mislocalized to the extra-rhabdomeral cell bodies (Figure 4A; Rosenbaum et al., 2011). We found that most of the residual TRP in $xport^{\Delta B}$ and $xport^{\Delta AB}$ was also retained in the cell bodies (Figure 4A; we increased the gain to detect TRP staining in the *xport* mutant sections). TRP binds to a PDZ scaffold protein, INAD, and these two proteins are mutually dependent on each other for rhabdomeral localization (Li and Montell, 2000; Tsunoda et al., 2001). In addition, several other signaling proteins, such as the PLC encoded by *norpA*, also depend on INAD for rhabdomeral localization (Chevesich et al., 1997). Consistent with the dramatic changes in the concentration and spatial distribution of TRP in *xport* mutant flies, NORPA and INAD were mislocalized in all three *xport* alleles (Figures 4B and S4A). The concentration of Rh1 was also greatly diminished in all three *xport* alleles. The low level of remaining Rh1 was primarily in the rhabdomeres of the R1-6 cells (Figure 4C). Similarly, we detected Rh6 in the R8 rhabdomeres of the *xport* mutants, after increasing the gain (Figure S4B). However, we did not quantify the changes in Rh6 levels since the Rh6 antibodies did not work on Western blots.

Because the XPORT-B antibodies were not effective for immunostaining, we generated transgenic flies that expressed XPORT-B fused to an HA epitope tag in photoreceptor cells. The spatial distribution of XPORT-B: :HA and TRP did not overlap (Figure 4D). Rather,

XPORT-B: :HA largely co-localized with two ER proteins, Calnexin and XPORT-A, both in the ER and in the subrhabdomeral cisternae (SRC), the latter of which are specialized smooth ER in photoreceptor cells (Figures 4E and 4F). Thus, XPORT-A and XPORT-B were localized to the subcellular regions, where TRP was retained in the *xport* mutants.

Dysfunction or mislocalization of many proteins that function in phototransduction leads to retinal degeneration (Wang and Montell, 2007). Consistent with the dramatic changes in localization and/or concentration of signaling proteins in the *xport* mutants, all three *xport* alleles showed severe retinal degeneration after 14 days (Figures 5A–5D). The deterioration of the rhabdomeres was much less severe in $xport^{\Delta AB}$ flies that were one day old (Figure 5E), or cultured under constant darkness for 14 days (Figure 5F), demonstrating that the retinal degeneration was age- and light-dependent.

Regions in TRP conferring dependency on XPORT proteins

TRP but not TRPL depends on the two XPORT proteins to achieve normal levels of expression in the photoreceptor cells. TRP and TRPL are ~49% identical over the N-terminal 810 and 817 amino acids, respectively, which span the N-terminal ankyrin repeat (AR) regions, the six transmembrane domains (TM), and the regions encompassing the TRP domain (C1) (Montell, 2001) (Figure 6A). The two proteins differ considerably in the C2 domains (residues: TRP, 811–1275; TRPL, 818–1124), which in TRP includes a C-terminal INAD binding site required for the mutual retention of TRP and INAD in the rhabdomeres (Li and Montell, 2000). The TRP and TRPL C2 domains shared only 13% identities, suggesting that this highly divergent region in TRP was the most likely candidate domain responsible for conferring dependency on XPORT-A and XPORT-B.

To identify the regions in TRP that bestowed sensitivity to the XPORT proteins, we generated transgenic flies expressing TRP-TRPL chimeric proteins (Figure 6B), and then examined the expression levels of the TRP-TRPL chimera in a $trpl^{MB};trp^{MB}$ background, in combination with the various *xport* alleles. Unexpectedly, we found that substitution of any of the most related regions of TRP (AR, TM or C1) into TRPL resulted in profound reductions in the protein levels, when either XPORT-A or XPORT-B was genetically removed (Figures 6C–6H). In contrast, substitution of the highly divergent C2 region of TRP into TRPL had no significant impact on protein levels in an $xport^{\Delta B}$ background, and led to less than a two-fold decrease in either $xport^{1}$ or $xport^{\Delta AB}$ backgrounds (Figures 6I and 6J). Consistent with this finding, substitution of the C2 region of TRPL into TRP did not protect TRP from loss of either XPORT protein (Figures 6K and 6L).

XPORT-B interacted with HSC3, a GRP78/BIP homolog

Since XPORT-B was necessary for expression of Rh1 and TRP, it was plausible that XPORT-B interacted directly with Rh1 or TRP. However, these latter interactions would be transient since XPORT-B is an ER protein, while TRP and Rh1 are rhabdomeral proteins. XPORT-B might also form a complex with XPORT-A, since both were localized to the ER and disruption of either protein led to similar phenotypes. To test these possibilities, we engineered transgenic flies that expressed XPORT-A, XPORT-B, TRP or Rh1 fused to a streptavidin binding peptide (SBP) tag, which facilitated one-step affinity purification of

each bait protein together with their complexes (Keefe et al., 2001). We prepared head extracts from the transgenic flies, and found that the tagged versions of XPORT-B, XPORT-A and TRP were expressed effectively (Figures 7A and 7B, input lanes, top row; Figure S5A, input lanes, top row). The Rh1: :SBP fusion protein was expressed at lower levels relative to endogenous Rh1 (Figure S5B, input lane, top row).

To test whether TRP, Rh1 or XPORT-A interacted with XPORT-B, we used Strep-Tactin beads to purify the four SBP-tagged proteins, and eluted each bait together with their interacting proteins using desthiobiotin. We did not detect interactions between XPORT-B and TRP (Figures 7A, TRP row; Figure S5A, XPORT-B row), or between XPORT-B and XPORT-A (Figures 7A, XPORT-A row; Figure 7B, XPORT-B row). A low level of XPORT-B eluted with the Rh1: :SBP (Figure S5B, XPORT-B row). However, we did not detect a significant level of Rh1 in the XPORT-B: :SBP complex (Figure 7A, Rh1 row).

To explore further a possible interaction between TRP and XPORT-B, we performed pulldown assays from late pupae, when TRP was actively transported through the ER (Satoh et al., 2005). However, we did not detect interactions between TRP and XPORT-B: :SBP (Figure S6A). We also expressed TRP, XPORT-A and XPORT-B in Drosophila S2 cells, and performed immunoprecipitations. Again, we did not detect interactions between XPORT-A and TRP, XPORT-B and TRP, or XPORT-A and XPORT-B (Figures S6B and S6C).

To identify XPORT-B interacting proteins, we prepared head extracts from XPORT-B: :SBP flies, purified the complexes with Strep-Tactin beads, fractioned the eluted proteins by SDS-PAGE, and visualized the proteins by silver staining. In addition to XPORT-B: :SBP (~18 kD), we detected an unknown protein of ~85 kD (Figure 7C). To determine the identities of XPORT-B: :SBP associated proteins in an unbiased fashion, we subjected the entire XPORT-B: :SBP complex purified from fly heads to mass spectrometry. Other than XPORT-B, the greatest number of peptides (194) corresponded to a protein (CG4147) of 72 kD. As a control, we performed a parallel analysis using extracts prepared from wild-type flies (Canton-S), which expressed XPORT-B, but not the XPORT-B: :SBP fusion protein. We did not isolate any CG4147 peptides from wild-type extracts. The difference between the predicted size (72 kD) and the size relative to protein markers on the Western blot (85 kD) might reflect post-translational modifications. Other than XPORT-B and CG4147, no other protein was represented by more than 59 peptides.

CG4147 encoded an HSP70 protein, HSC3 (heat shock cognate protein 3)—a Drosophila homolog of GRP78/BiP (Kirkpatrick et al., 1995). GRP78/BiP resides in the ER and functions as a chaperone to assist folding of newly-synthesized peptides, and also serves as a regulator during the unfolded protein response (Gething, 1999). To confirm that XPORT-B interacted with HSC3, we probed a Western blot containing the purified XPORT-B extracts with HSC3 antibodies. We detected HSC3 in the complex purified from the XPORT-B: :SBP transgenic animals, but not from control flies (Figure 7A, bottom row). HSC3 was also present in the Rh1: :SBP complex (Figure S5B, HSC3 row). However, we detected little HSC3 in the SBP: :XPORT-A complex (Figure 7B, HSC3 row) or in the SBP: :TRP complex (Figure S5A, HSC3 row).

To test whether HSC3 was required for expression of Rh1 or TRP, we used RNAi to knockdown *hsc3* (Ni et al., 2011) and overexpressed a dominant-negative form of the HSC3 protein in photoreceptor cells ($HSC3^{D231S}$) (Elefant and Palter, 1999). Both Rh1 and TRP levels were reduced dramatically (Figures S6D and S6E). However, the eyes expressing either the *hsc3* RNAi or $HSC3^{D231S}$ displayed severely disrupted morphology. The effects on Rh1 and TRP might have been an indirect consequence of the perturbation in eye morphology, since the concentrations of other photoreceptor cell proteins tested, such as INAD and NINAC p174 and p132, which did not depend on XPORT proteins for expression, displayed similar dramatic reductions in protein levels (Figures S6D and S6E).

Discussion

XPORT-B is an ER chaperone required for TRP translocation to the rhabdomeres

Most TRP channels, including Drosophila TRP, function primarily at the plasma membrane to promote cation influx. Yet Drosophila TRP and multiple other TRP channels are ineffectively expressed in tissue culture, largely as a consequence of getting trapped in ER. Similarly, invertebrate rhodopsins are notoriously difficult to express *in vitro*, and accumulate in the ER. These findings underscore the importance of chaperones in assisting in the folding and transport of these signaling proteins *in vivo*.

Currently, the chaperones that promote the trafficking of TRP channels through the secretory pathway are enigmatic. The only exception prior to this work was the identification of XPORT-A (Rosenbaum et al., 2011). We propose that XPORT-B also serves as a TRP chaperone. In support of this conclusion, XPORT-B is spatially localized to the ER, and in the absence of XPORT-B, TRP expression is dramatically reduced. Moreover, a large proportion of the residual TRP protein in $xport^{\Delta B}$ mutant flies remained in the ER. The concentration of TRP was reduced ~40-fold, presumably due to degradation of misfolded proteins that were unable to exit the ER and traverse the secretory pathway.

While both XPORT-A and XPORT-B are necessary ER chaperones, they are not sufficient. Introduction of either or both XPORT proteins did not improve surface localization of TRP expressed in the Drosophila S2 cell line (Figure S5E), which also expresses high levels of HSC3 (http://flybase.org/reports/FBgn0001218.html). Co-expression of both XPORT proteins also did not augment plasma membrane expression of Rh1 (Figure S5F). These findings differ from a previous study (Rosenbaum et al., 2011) that reports that XPORT-A greatly increases trafficking of TRP and Rh1 to the plasma membrane of S2 cells.

Light-dependent retinal degeneration due to effects on TRP and not Rh1

The requirement for XPORT-B as an ER chaperone is not specific for TRP, as the concentration of the major rhodopsin, Rh1, is decreased ~10 fold. We detected the residual Rh1 exclusively in the rhabdomeres. We suggest that the Rh1 that cannot translocate out of the ER in the absence of either XPORT protein is subsequently degraded. The small fraction of the Rh1 that is folded and escapes from the ER is trafficked to the rhabdomeres.

Due to the critical roles of XPORT-A and XPORT-B as protein chaperones, elimination of these proteins results in light- and age-dependent retinal degeneration. This phenotype

appears to be due to the dramatic reduction in the concentration of TRP rather than Rh1, since the retinal degeneration resulting from *trp* mutations is also light-dependent, and occurs over a time course similar to the *xport* mutants. Null *ninaE* mutations cause defects in morphogenesis, which is unaffected by light, and hypomorphic (weak) *ninaE* mutations that leave very low levels of Rh1 in the rhabdomeres do not undergo retinal degeneration (Kumar and Ready, 1995).

XPORT-B, but not XPORT-A, specifically affects TRP and Rh1

Overall, the functions of XPORT-A and XPORT-B are similar, since loss of either protein results in comparable reductions in the expression of TRP and Rh1. Because these two proteins are encoded in a bicistronic mRNA and serve analogous functions, they constitute an example of an operon in flies. However, the requirements for XPORT-A and XPORT-B are not identical.

In addition to TRP and Rh1, the only other proteins in which the levels are reduced in the $xport^{\Delta B}$ mutants are those that depend on expression of TRP. TRP binds to the PDZ scaffold protein INAD, and these two proteins are mutually dependent on each other for retention in the rhabdomeres (Li and Montell, 2000; Tsunoda et al., 2001). PLC (NORPA) and protein kinase C (INAC) also bind directly to INAD, and these interactions are required for their retention in the rhabdomeres (Chevesich et al., 1997; Huber et al., 1996; Tsunoda et al., 1997; Xu et al., 1998). Consequently, the ~two-fold decline in PLC and PKC levels in $xport^{\Delta B}$ mutants appears to be due indirectly to the dramatic reduction and/or mislocalization of TRP, which in turn affects the localization of INAD.

The concentrations of seven other proteins that do not depend on INAD for rhabdomeral retention are unchanged in $xport^{\Delta B}$ mutant photoreceptor cells. Among these seven are three transmembrane proteins: the Na^+/Ca^{2+} exchanger (CalX), a PI-transfer protein (RDGB) and TRPL channel. Thus, within the limits of this analysis, XPORT-B functions as a chaperone specifically for TRP and Rh1.

In contrast to the restricted effects resulting from loss of XPORT-B, we found that the consequences of eliminating XPORT-A were broader. Multiple proteins that did not depend on INAD for retention in the rhabdomeres were reduced ~two-fold in $xport^1$ photoreceptor cells. These included TRPL, NINAA, myosin III (NINAC) p132, myosin III (NINAC) p174 and CalX. Thus, while TRP and Rh1 were the only proteins dramatically affected in $xport^1$ flies, the concentrations of most signaling proteins were diminished.

Multiple domains in TRP confer dependency on XPORT proteins

XPORT-A and XPORT-B are required ER chaperones for two unrelated proteins: TRP and Rh1. Yet, expression of TRPL, which is highly related to TRP, was unaffected by the *xport* mutations. These findings indicate that TRP and TRPL may use distinct sequence determinants and chaperones to exit the ER. Indeed, some inwardly rectifying potassium channels also show differences in ER export signals (Ma et al., 2001; Ma et al., 2002).

To identify the domains that render TRP, but not TRPL, dependent on the XPORT proteins, we generated a series of chimeric proteins. TRP is ~49% identical to TRPL over the N-

terminal ~810 amino acids, whereas, the C-terminal region of TRP (the last 465 amino acids) is virtually unrelated, and is extended ~150 residues relative to TRPL. However, replacement of the C-terminus of TRP with TRPL did not alter the dependency on the XPORT proteins. Rather, substitution of any of three domains in TRP that was conserved with TRPL was sufficient to switch TRPL to an XPORT-dependent protein. These included a chimera that was nearly 90% TRPL, but encompassed the TRP box (C1 region) of TRP. Our findings indicate that XPORT-A and XPORT-B promote TRP expression through multiple domains of TRP.

XPORT-B and folding of nascent signaling proteins in the ER

To gain greater insights into the role of XPORT-B, we screened for proteins that complexed with XPORT-B *in vivo*. We used an unbiased mass spectrometry approach and found that the most prominent XPORT-B-associated protein was HSC3—an HSP70 protein. HSC3 is the fly homolog of an ER chaperone, GRP78/BiP, which functions in assisting the folding of nascent proteins, and is critical for the unfolded protein response. We found that HSC3 interacted with Rh1 but not TRP. Thus, HSC3 appears to link XPORT-B and Rh1. In this context, it is noteworthy that XPORT-A associates with two heat shock proteins, HSP27 and HSP90 (Rosenbaum et al., 2011).

We propose that XPORT-B functions as a co-chaperone to assist Rh1 folding, which is a prerequisite for Rh1 translocation out of the ER. During biosynthesis and trafficking through the secretory pathway, Rh1 undergoes dynamic changes in glycosylation, culminating with complete deglycosylated (Rosenbaum et al., 2014). Glycosylation and deglycosylation of Rh1 appears normal in $xport^{1}$ photoreceptor cells (Rosenbaum et al., 2011), and XPORT-B does not affect Rh1 glycosylation and deglycosylation since the residual Rh1 in $xport^{\Delta B}$ mutants is similar in size to Rh1 in control flies. Thus, the association with HSC3 supports the model that XPORT-B functions as an ER chaperone for folding of Rh1 in the ER.

XPORT-A and XPORT-B have non-redundant roles, and the two proteins do not appear to form a single complex. Thus, their specific functions in assisting in the folding and translocation of TRP and Rh1 from the ER may be distinct. In further support of this proposal, is the observation that elimination of XPORT-B has a more specific impact on TRP and Rh1 than loss of XPORT-A.

Future perspective

The conservation of the XPORT operon over a broad range of insect species that diverged up to 300 million of years ago underscores the critical requirement for the coordinate expression of XPORT-A and XPORT-B as ER chaperones. Currently, the protein chaperones required by mammalian TRP proteins are unknown. Although there are no identifiable vertebrate XPORT homologs based on primary amino acid sequence comparisons, we suggest that functionally similar type 1 and 2 membrane proteins may serve analogous functions as ER chaperones for TRP channels and rhodopsins in mammals.

Experimental Procedures

Fly stocks

We cultured flies on cornmeal-agar-molasses medium, at 25°C under 12 hr light/12 hr dark cycles. Nansi Colley provided *xport*^{1} (Rosenbaum et al., 2011). We obtained MKRS,*hsFLP*/TM6B, P[Cre], *UAS-hsc3* RNAi (TRIP.HMS00397) (Ni et al., 2011), *UAS-hsc3*^{D231S} (Elefant and Palter, 1999), and the following two MB lines (Bellen et al., 2011) from the Bloomington Stock Center: *trp*^{MB03672} and *trpl*^{MB010553}. We used isogenized *w*^{1118} as the control flies, and to PCR amplify all DNAs used to generate the *xport* alleles and transgenic flies.

Generation of *xport*$^{\Delta B}$ and *xport*$^{\Delta AB}$ mutants

To create the 0.3 kb deletion that removed the full *xport-B* coding sequence, thereby creating the *xport*$^{\Delta B}$ allele, we PCR amplified 3.3 kb and 3.7 kb genomic DNA fragments flanking the 5’ and 3’ ends of the *CG42508* coding frame, respectively, which we inserted into the pw35 vector (Gong and Golic, 2003). Transgenic donor lines were generated (Rainbow Transgenic Flies Inc., Camarillo, CA), and the transgenic insertion was mobilized as described (Gong and Golic, 2003). We confirmed the knockout by PCR analysis of genomic DNA. To generate the *xport*$^{\Delta AB}$ allele, which included a 1.0 kb deletion removing the entire *xport-A* coding sequence and most of *xport-B* (the N-terminal 73 residues of XPORT-B), we PCR amplified 2.8 kb and 3.4 kb genomic fragments, which we inserted into the pw35 vector. We obtained knockout flies, which we confirmed by PCR.

Generation of *xport* transgenic flies for rescue or immmunostaining experiments

To generate a genomic rescue construct that included the entire *xport* locus, g[$A^{+}B^{+}$], we PCR amplified a 2.5 kb genomic DNA from isogenic *w*^{1118} flies. The cloned genomic DNA fragment was subcloned into the pattB vector (Bischof et al., 2007) to generate the g[$A^{+}B^{+}$] construct. We generated three additional genomic rescue constructs, which differed from g[$A^{+}B^{+}$] as follows. The g[$A^{+}B^{-}$] construct included in-frame TAG stop codons in place of all seven methionine codons in *xport-B* (*CG42508*). The g[$A^{-}B^{+}$] construct had three in-frame TAG stop codons in place of the first three methionine codons of *xport-A* (*CG4468*). To generate g[$A^{+}B^{\Delta}$], we deleted the entire *xport-B* coding sequence. The genomic constructs were inserted into the *attp40* site by PhiC31 integrase mediated transgenesis (BestGene Inc., Chino Hills, CA).

To express the *xport-A* or *xport-B* sequences under the control of the *ninaE* (*rh1*) promoter, we PCR amplified the regions encoding *xport-A* and *xport-B* from cDNAs and inserted them 3’ to the *ninaE* promoter, to generate *n*[*A*] and *n*[*B*] constructs, respectively.

To generate XPORT-B: :HA transgenic flies, we fused the DNA sequence encoding an HA epitope tag to the 3’ end of the *xport-B* coding region, so that the sequences were in-frame. We expressed this construct under control of the *ninaE* promoter, and transgenic flies were generated by BestGene Inc.

Transgenic flies expressing XPORT-A, XPORT-B, Rh1 and TRP fused to SBP tags

To purify signaling proteins from fly heads, we generated the following transgenic flies expressing streptavidin binding peptide (SBP) epitopes tags fused to either the Nor C-termini: 1) SBP: :XPORT-A, 2) XPORT-B: :SBP, 3) Rh1: :SBP and 4) SBP: :TRP. With the exception of SBP: :TRP, which was generated using a 6.5 kb *trp* genomic fragment (Montell et al., 1985), the transgenes were derived from cDNAs and expressed in R1-6 cells under control of the *ninaE* promoter.

Streptavidin precipitation

We homogenized heads, either from adult flies or from late pupae (~90% pupal stage), and used Strep-Tactin Superflow plus beads (Qiagen) to precipitate the SBP tagged bait proteins and the associated complex. We eluted the bound proteins from the resin with desthiobiotin, fractionated the proteins by SDS-PAGE and performed Western blot analyses.

Mass spectrometry to identify XPORT-B interacting proteins

We crushed heads from wild-type (Canton-S) flies, and from $xport^{\Delta B}$ mutant flies that expressed the *xport-B: :SBP* transgene, and incubated the supernatants with Strep-Tactin Superflow plus beads (Qiagen). We eluted the XPORT-B: :SBP protein and its associated complex in buffer containing 1% Triton X-100 and 2.5 mM desthiobiotin, which we used for silver staining and mass spectrometry (Biomolecular and Proteomics Mass Spectrometry Facility, University of California, San Diego).

Generation of XPORT-A and XPORT-B antibodies

To generate XPORT-A and XPORT-B antibodies, we purified glutathione S-transferase (GST) fusion proteins (XPORT-A, amino acids 1–76; XPORT-B, amino acids 28–113) from *E. coli* BL21 using Glutathione Sepharose 4B beads (GE Lifescience), and raised polyclonal antibodies in rats (XPORT-A) and rabbits (XPORT-B) (BioLegend, Inc; formerly Covance).

Western blots

Heads from 1-day old flies were homogenized, fractioned by SDS-PAGE, transferred to nitrocellulose membranes (Bio-Rad), and probed with the indicated primary antibodies, and then with IRDye-conjugated secondary antibodies (LI-COR Biosciences). We used the LI-COR system to quantify the protein levels.

Whole mount immunostaining of fly eyes

Fly heads were fixed in PBS plus 4% paraformaldehyde, eyes were dissected and samples were incubated with primary antibodies overnight at 4 °C and secondary antibodies (Alexa Fluor Dyes, Life Technologies) overnight at 4°C. After rinsing three times in PBS + 0.1% Triton X-100, the samples were mounted in Vectashield (Vector labs) and examined using a Zeiss LSM 700 confocal microscope to achieve optical sections of 0.5 μm using a Plan-Apochromat 63× objective.

Transmission electron microscopy

We used LR white to embed hemisected heads from flies reared under a 12 hr light/12 hr dark cycle or in constant darkness. Thin sections (80 nm) prepared at a depth of 30 μm were examined by transmission electron microscopy using an electron microscope (JEOL JEM-1230). The images were acquired using a Hamamatsu ORCA digital camera (model #C4742-95, Advanced Microscopy Techniques).

Generation of transgenic flies expressing TRP-TRPL and TRPL-TRP chimera

The coding sequences for the indicated portions of TRP and TRPL were cloned into the *ninaE* [His-SBP] vector to create the following five TRP-TRPL chimera: I) TRP (1–328)-TRPL (336–1124), II) TRPL (1–335)-TRP (329–664)-TRPL (672–1124), III) TRPL (1–671)-TRP (665–810)-TRPL (818–1124), IV) TRPL (1–817)-TRP (811–1275), and V) TRP (1–810)-TRPL (818–1124).

Immunofluorescence in Drosophila S2R+ cells

We used the pAC5.1-V5-His vector (Life Technologies) to generate pAC5.1-Dendra2, pAC5.1-Dendra2: :TRP, pAC5.1-Rh1, pAC5.1-XPORT-B: :HA, and pAC5.1-FLAG: :XPORT-A. After performed transfections in Drosophila S2R+ cells, we checked TRP or Rh1 expression by immunofluorescence using rabbit anti-TRP or mouse anti-Rh1 primary antibodies and anti-rabbit or anti-mouse secondary antibodies (Alexa Fluor Dyes, Life Technologies) secondary antibodies, and acquired the images using a Zeiss LSM 700 confocal microscope.

Electroretinogram recordings

Electroretinogram (ERG) recordings were performed as previously described (Wes et al., 1999), with slight modifications. We exposed the flies to a 10 s pulse of bright orange light (~30 mW/cm^2) at a frequency of two pulses per minute. Unless indicated otherwise, we used w^+ flies. The animals were analyzed 1 day post-eclosion. We amplified the light-induced signals using an IE-210 amplifier (Warner Instruments), digitalized the responses with a Powerlab 4/30 (AD Instuments), and stored the data on a computer using LabChart 6 software (AD Instruments).

Statistical analyses

Data were presented as means ± SEMs. All error bars represent SEMs. We used one-way ANOVA with Holm-Sidak post-hoc analyses for statistical analyses. Asterisks indicate statistically significant differences (*p<0.05, **p<0.01).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Trafficking of TRP and rhodopsin depends on the bicistronic *xport* locus
- XPORT-A and XPORT-B coordinately contribute to ER exit of TRP and rhodopsin
- Loss of XPORT-A or XPORT-B mimics the *trp* ERG and retinal degeneration phenotype
- XPORT-B forms a complex *in vivo* with an HSP70 protein and rhodopsin

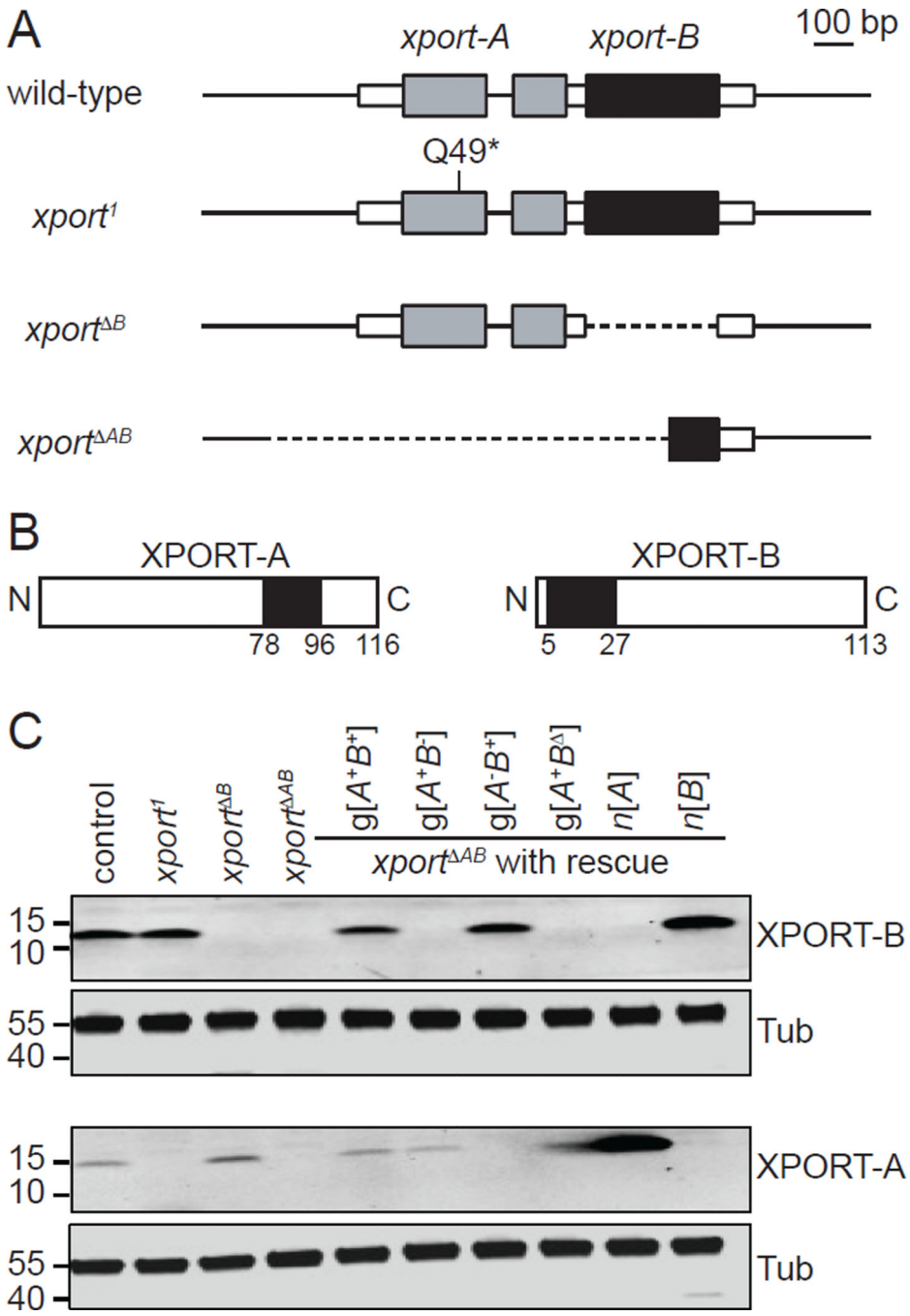

Figure 1. Coding sequences and protein expression of wild-type *xport* and mutant alleles

(A) The wild-type *xport* locus and mutations in the three mutant alleles. Wild-type *xport* encodes *xport-A* (*CG4468*) and *xport-B* (*CG42508*). Q49* indicates the premature stop codon in $xport^{1}$ (Rosenbaum et al., 2011). The genomic regions deleted in $xport^{\Delta B}$ and $xport^{\Delta AB}$ are indicated by the dash lines.

(B) The sizes and positions of the predicted single transmembrane domains (TM) in XPORT-A and XPORT-B. The numbers indicate amino acid residues.

(C) Western-blots containing head extracts from the indicated fly stocks (half a head equivalents per lane) probed with anti-XPORT-A (rat), anti-XPORT-B or anti-Tubulin (Tub). The positions of protein size markers (kD) are shown to the left.

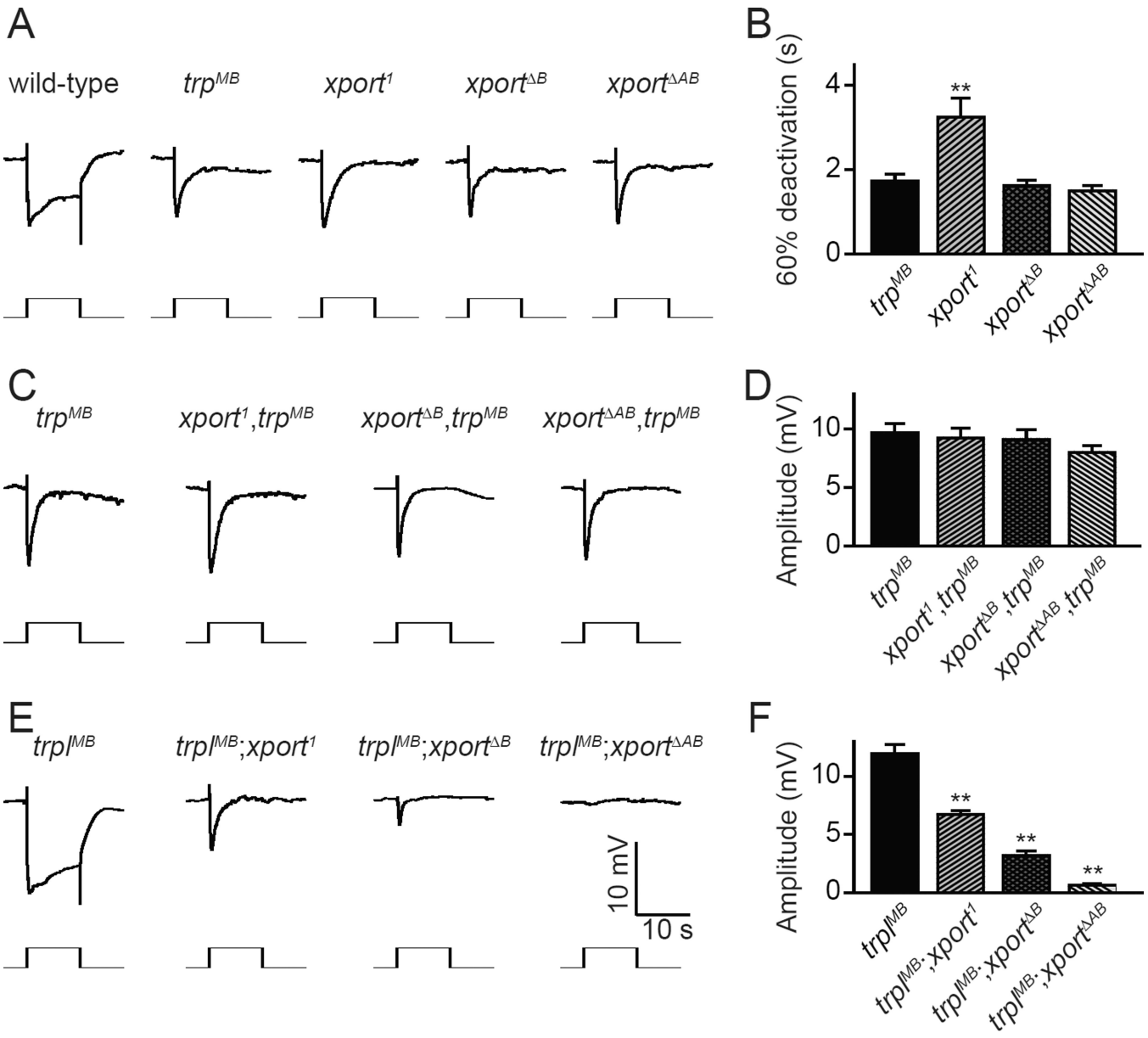

Figure 2. Disruption of *xport-B* resulted in a transient light response

(A, C & E) Electroretinogram (ERG) responses of flies of the indicated genotypes.

(B) Time required for 60% return to the baseline.

(D and F) Amplitudes of the ERG responses of the flies of the indicated genotypes. trp^{MB} refers to $trp^{MB03672}$; $trpl^{MB}$ refers to $trpl^{MB10553}$.

n≥8. The error bars represent SEMs. $^{**}p<0.01$. One-way ANOVA with Holm-Sidak post-hoc analyses.

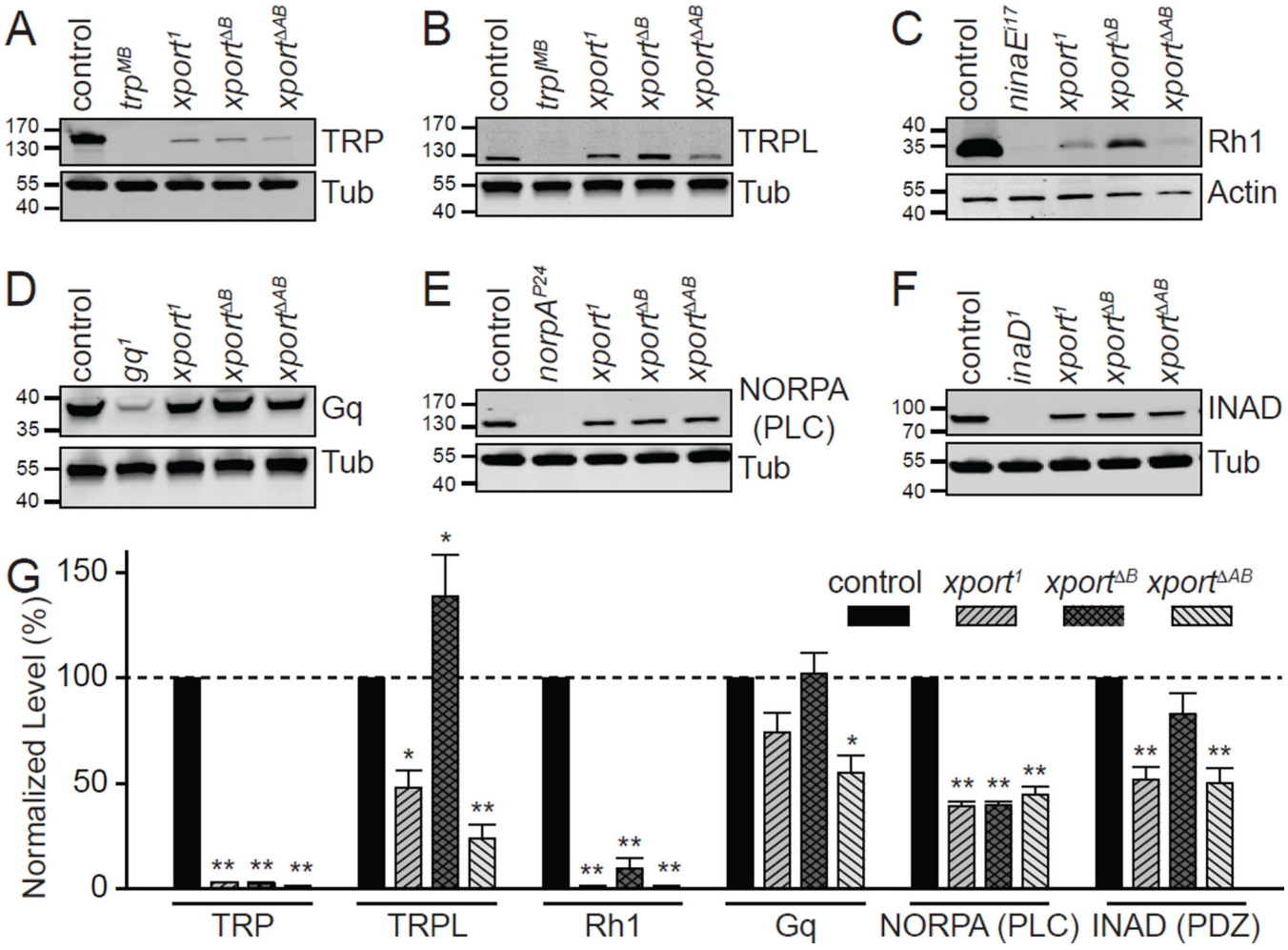

Figure 3. TRP and Rh1 were dramatically reduced in *xport* mutants

(A–F) Western blots containing extracts from control flies (w^{1118}), the indicated mutants and the three *xport* alleles (half a head equivalent/lane) probed with antibodies to the indicated proteins. The blots were also probed with either anti-Tubulin (Tub) or anti-Actin for normalization. The positions of protein size markers (kD) are shown to the left.

(G) Quantification of the relative levels of the indicated proteins from heads of the indicated flies. n=3. The error bars represent SEMs. *p<0.05; ** p<0.01. One-way ANOVA with Holm-Sidak post-hoc analyses.

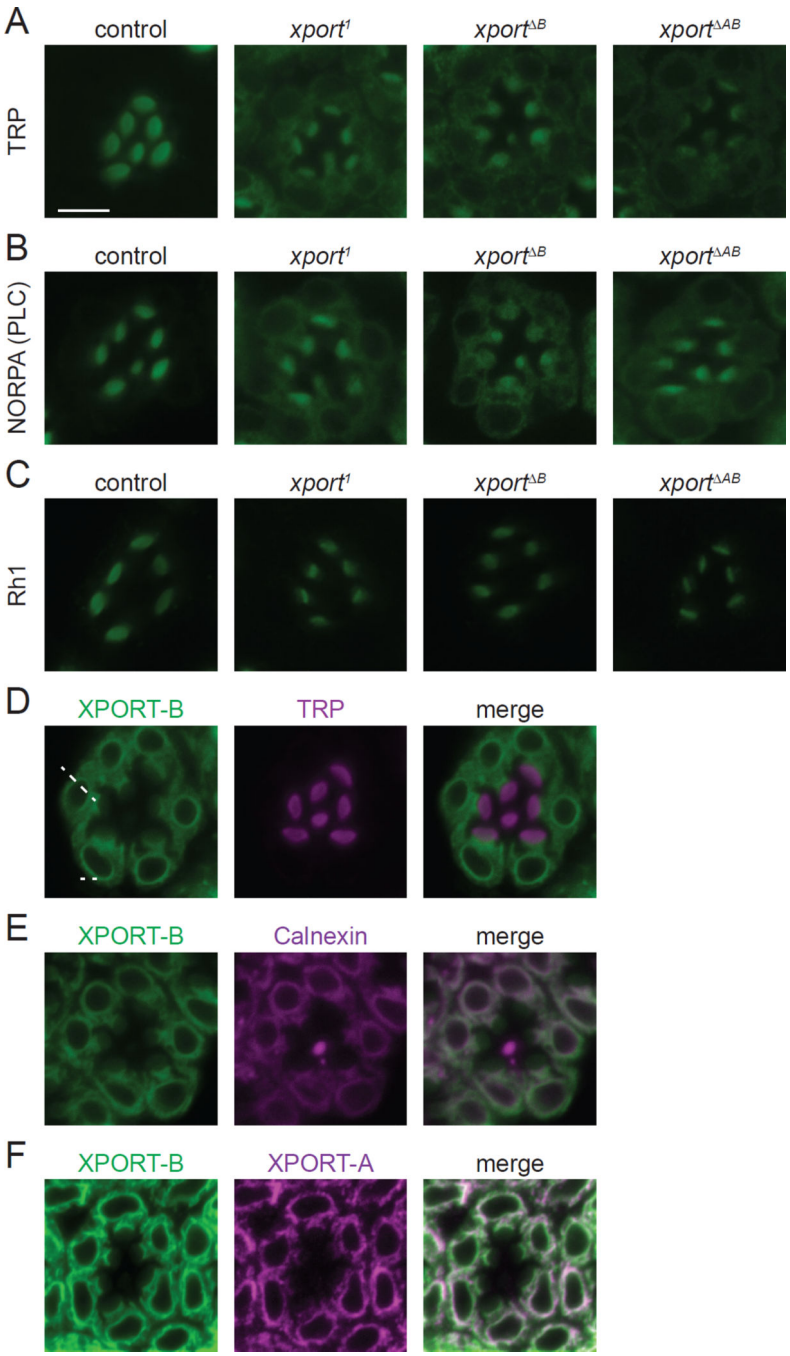

Figure 4. XPORT-B was present in the endoplasmic reticulum and required for normal TRP expression in the rhabdomeres

(A–C) Distal optical sections (R7 layer) showing single ommatidia from compound eyes from control flies and the *xport* alleles ($xport^{1}xport^{\Delta B}$ or $cn,bw;xport^{\Delta AB}$), stained with anti-TRP, anti-NORPA (PLC) and anti-Rh1.

(D–F) Distal optical sections (R7 layer) of single ommatidia from *cnbw;ninaE*[XPORT-B: :HA] flies expressing the *ninaE*[XPORT-B: :HA] transgene.

(D) Staining with anti-HA to detect XPORT-B and anti-TRP. Merge is to the right.

(E) Staining with anti-HA to detect XPORT-B and anti-Calnexin. Merge is to the right.
(F) Staining with anti-HA to detect XPORT-B and anti-XPORT-A (rabbit). Merge is to the right.
The scale bar represents 5 μm. SRC, subrhabdomeral cisternae; ER, endoplasmic reticulum.

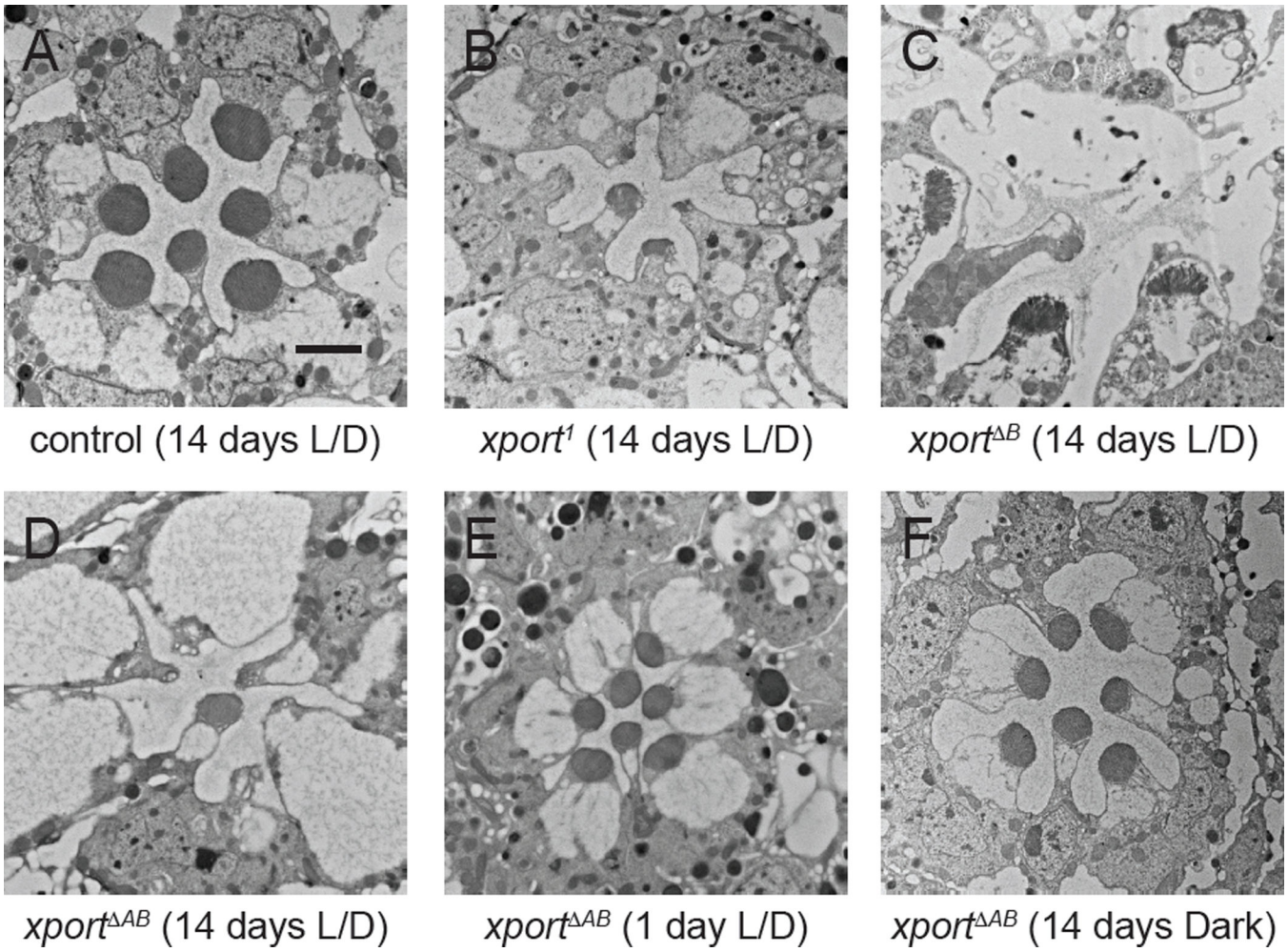

Figure 5. Retinal degeneration in *xport* mutants

Transmission electron microscopic images of single ommatidia from flies of the indicated ages (days post-eclosion) maintained under 12 hr light/12 hour dark conditions (L/D) or in the dark.

(A) Control (w^{1118}), 14 days L/D.

(B) $xport^{1}$, 14 days L/D.

(C) $xport^{\Delta B}$, 14 days L/D.

(D) *cn,bw;*$xport^{\Delta AB}$, 14 days L/D.

(E) *cn,bw;*$xport^{\Delta AB}$, 1 day L/D.

(F) *cn,bw;*$xport^{\Delta AB}$,14 days Dark.

The scale bar in (A) represents 2 μm. CB, cell body; R, rhabdomere.

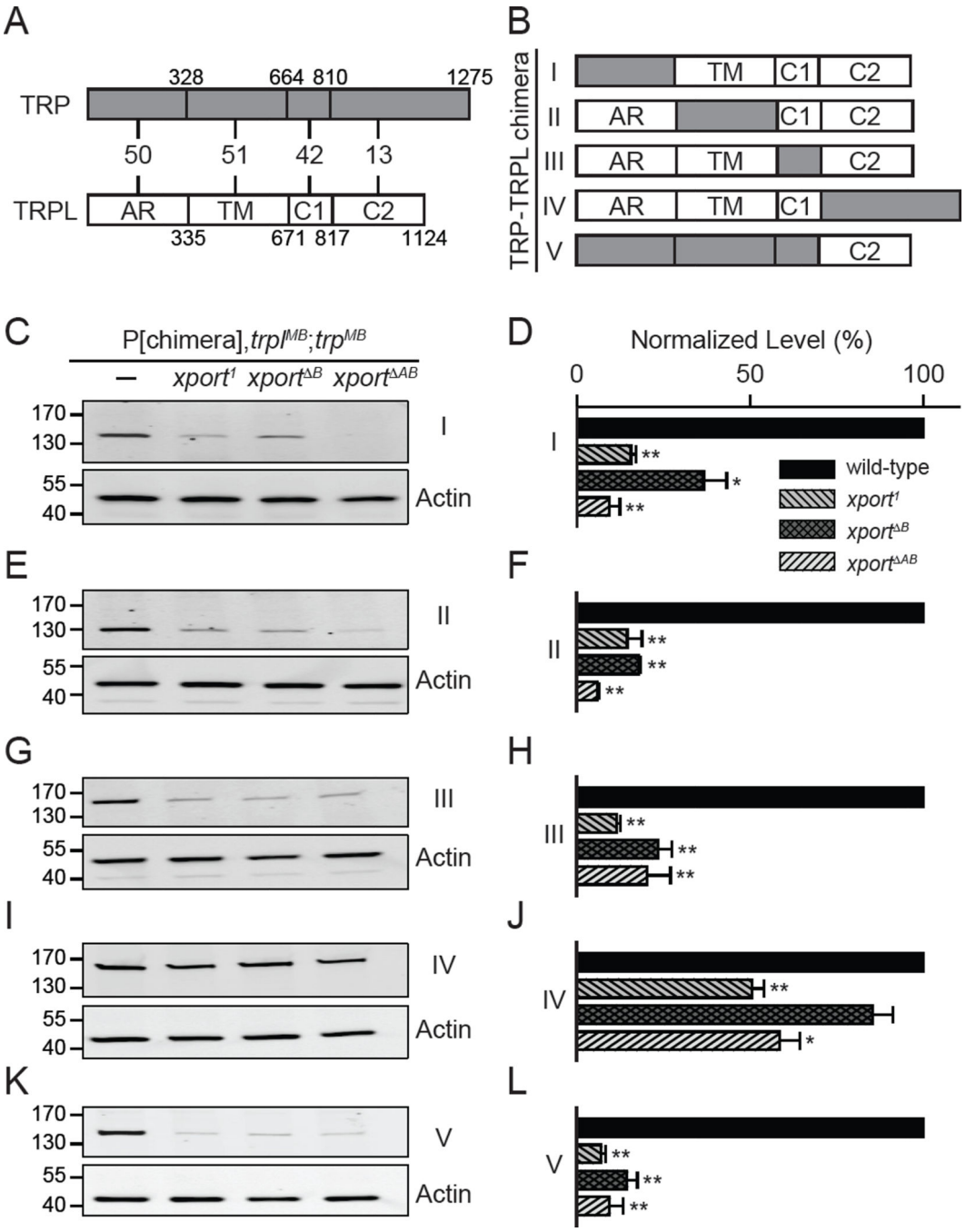

Figure 6. Multiple TRP regions conferred XPORT dependency

(A) Percent amino acid identities between TRP and TRPL across different domains are indicated between the cartoons for the two proteins. The residue numbers demarcating the different domains in TRP and TRPL are indicated above and below, respectively. Domains: AR, four ankyrin repeats; TM, six transmembrane segments; C1, the proximal C-terminal end containing the TRP domain; C2, the highly divergent C-terminal ends.

(B) TRP-TRPL chimera (I–V). TRP and TRPL sequences are indicated by the gray and white boxes, respectively.

(C, E, G, I & K) Western blots showing the expression levels of each chimera (I–V) in the *xport* mutants. Transgenes encoding each chimera, P[chimera] (I–V), which were inserted into the 2nd chromosome, were introduced into a $trpl^{MB};trp^{MB}$ background. In addition, various *xport* mutations were also introduced into the genetic background as indicated. The blots were also probed with anti-actin (Abcam, ab1801) for quantification. The positions of protein size markers (kD) are shown to the left.

(D, F, H, J & L) Quantification of TRP-TRPL chimera (I–V) levels in the *xport* mutants. The error bars represent SEM. n=3. *p<0.05; **p<0.01. One-way ANOVA with Holm-Sidak post-hoc analyses.

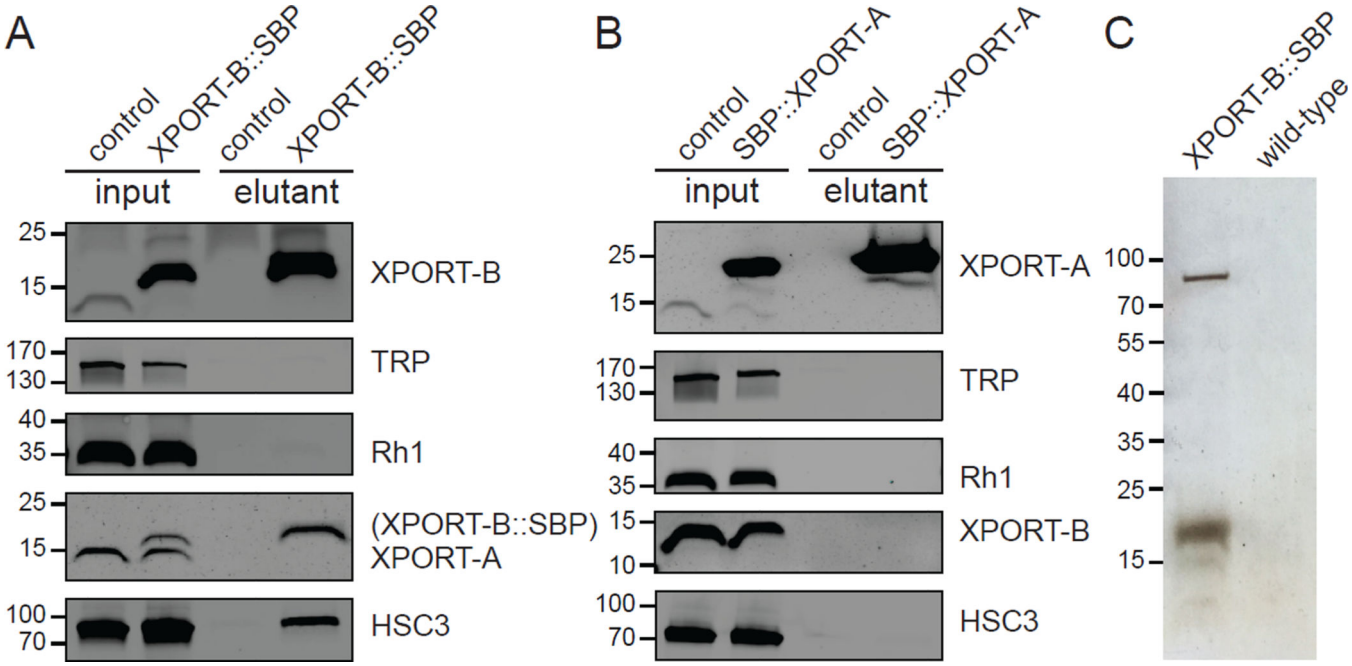

Figure 7. XPORT-B interacted with HSC3

Extracts from control flies (w^{1118}) and from transgenic flies expressing SBP tagged versions of XPORT-B and XPORT-A were incubated with streptavidin resin. The eluted proteins (elutant) were fractionated by SDS-PAGE, transferred by Western blotting, and the indicated proteins were identified with the corresponding antibodies. The "input" lanes show 20% of the proteins used to conduct the experiments shown in the "elutant" lanes. The positions of protein size marker (kD) are shown to the left.

(A) Western blot showing XPORT-B: :SBP complex probed with antibodies to the indicated proteins. The rat polyclonal XPORT-A antibodies also recognized XPORT-B: :SBP because the GST: :XPORT-A antigen used to generate these antibodies included a tandem peptide sequence (Prescission cutting site), which was also contained in XPORT-B: :SBP.

(B) Western blot showing SBP: :XPORT-A complex probed with antibodies to the indicated proteins.

(C) The XPORT-B: :SBP complex was purified from *xport-B: :SBP* flies in an $xport^{\Delta B}$ mutant background and mock purified from wild-type flies (Canton-S) using streptavidin resin. The eluted proteins were fractionated by SDS-PAGE and detected by silver staining.