**Drosophila Signal Peptidase Complex Member Spase12 Is Required for Development and Cell Differentiation**

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**Abstract**

It is estimated that half of all proteins expressed in eukaryotic cells are transferred across or into at least one cellular membrane to reach their functional location. Protein translocation into the endoplasmic reticulum (ER) is critical to the subsequent localization of secretory and transmembrane proteins. A vital component of the translocation machinery is the signal peptidase complex (SPC), which is conserved from yeast to mammals and functions to cleave the signal peptide sequence (SP) of secretory and membrane proteins entering the ER. Failure to cleave the SP, due to mutations that abolish the cleavage site or reduce SPC function, leads to the accumulation of uncleaved proteins in the ER that cannot be properly localized resulting in a wide range of defects depending on the protein(s) affected. Despite the obvious importance of the SPC, in vivo studies investigating its function in a multicellular organism have not been reported. The Drosophila SPC comprises four proteins: Spase18/21, Spase22/23, Spase25 and Spase12. Spc1p, the S. cerevisiae homolog of Spase12, is not required for SPC function or viability; Drosophila spase12 null alleles, however, are embryonic lethal. The data presented herein show that spase12 LOF clones disrupt development of all tissues tested including the eye, wing, leg, and antenna. In the eye, spase12 LOF clones result in a disorganized eye, defective cell differentiation, ectopic interommatidial bristles, and variations in support cell size, shape, number, and distribution. In addition, spase12 mosaic tissue is susceptible to melanotic mass formation suggesting that spase12 LOF activates immune response pathways. Together these data demonstrate that spase12 is an essential gene in Drosophila where it functions to mediate cell differentiation and development. This work represents the first reported in vivo analysis of a SPC component in a multicellular organism.

**Citation:** Haase Gilbert E, Kwak S-J, Chen R, Mardon G (2013) Drosophila Signal Peptidase Complex Member Spase12 Is Required for Development and Cell Differentiation. PLoS ONE 8(4): e60908. doi:10.1371/journal.pone.0060908

**Editor:** Andreas Bergmann, University of Massachusetts Medical School, United States of America

**Received:** October 11, 2012; **Accepted:** March 5, 2013; **Published:** April 3, 2013

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**Funding:** This work was supported by funds to GM by the Retina Research Foundation ([www.retinaresearchfnd.org](http://www.retinaresearchfnd.org)) and funds to EHG by the National Eye Institute ([www.nei.nih.gov](http://www.nei.nih.gov)) (T32 EY007102) and the National Institutes of Health ([www.nih.gov](http://www.nih.gov)) (T32 GM008307). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

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**Introduction**

Processing by the signal peptidase complex (SPC) is critical to the localization and function of secretory and membrane proteins which must enter the endoplasmic reticulum (ER) before they can be directed to their final destination. As proteins are transferred into the ER, the SPC cleaves the signal peptide sequence (SP), an N-terminal stretch of amino acids — usually 20–30 residues in length — that directs proteins to the ER [1]. SPs possess a tripartite structure that includes a positively charged amino terminal domain, a 7–13 residue hydrophobic domain, and a hydrophilic domain that includes the cleavage site [2]. While the SP sequence is not conserved, the properties associated with each domain are static and ensure that SP-bearing polypeptides are recognized by the cell and translocated into the ER [1,3].

Signal peptidases have been extensively studied in yeast and bacteria, yet little has been done to investigate their role in multicellular organisms. In S. cerevisiae, four proteins, Sec11, Spc1p, Spc2p and Spc3p, comprise the SPC [4,5] (Table 1). Sec11 and Spc3p are required for SPC catalytic function and cell viability. Temperature-sensitive sec11 and spc3p mutants accumulate uncleaved SPC targets at non-permissive temperatures, indicating that both are required for SP cleavage [6–8]. Spc1p and Spc2p do not have catalytic function and are dispensable for SPC cleavage activity and viability at normal growth temperatures. However, over-expression of Spc1p attenuates the sec11 temperature-sensitive phenotype [5], while depletion of Spc2p at high temperatures leads to the accumulation of uncleaved protein [9], suggesting that Spc1p and Spc2p contribute to SPC function in yeast although the mechanism has yet to be identified.

In mammals, the SPC consists of five subunits: SPC18, SPC21, SPC22/23, SPC12 and SPC25 [10]. SPC18 and SPC21 have high identity to each other [11] and are homologous to Sec11 [6,12,13]. SPC22/23 is homologous to Spc3p [14–17] while SPC12 and SPC25 are homologous to Spc1p and Spc2p, respectively [5,9,18,19] (Table 1). SPC18, SPC21 and SPC22/23 are single-pass transmembrane proteins, the bulk of which reside within the ER lumen. SPC12 and SPC25 are double-pass transmembrane proteins each containing a small lumenal domain, while the N- and C-termini of both are cytosolic [11]. SPC18, SPC21, and SPC22/23 have catalytic function and the residues required for cleavage activity are localized to the ER lumen [11].
Four SPC homologs have been identified in Drosophila: Spase18/21, Spase22/23, Spase12 and Spase25 (Table 1). Spase18/21 is homologous to yeast Sec11, as well as mammalian SPC18 and SPC21 [20]. ER vesicles (microsomes) purified from Drosophila embryos and added to an in vitro translation system results in cleavage of murine myeloma light-chain IgG, demonstrating that the Drosophila SPC is functionally conserved [21].

Despite playing a key role in protein sorting, in vitro studies of SPC function in metazoans have not been reported. We have used the Drosophila eye as a model system to investigate the role of spase12 and the SPC in a higher eukaryote. The eye originates from a developmental structure called the eye imaginal disc, an epithelial monolayer of cells that begin to differentiate during the third instar larval stage. The eye continues to develop through larval and pupal stages into a highly organized array comprised of approximately 800 unit eyes (ommatidia). Each ommatidium contains eight photoreceptor cells and four cone cells enclosed by two primary, six secondary, and three tertiary pigment cells, as well as three interommatidial bristles (IOBs) [22,23]. The genetic approaches available in Drosophila, coupled with the well characterized development and structure of the eye, make it an ideal model for developmental studies.

In this report, we characterize spase12 loss-of-function (LOF) phenotypes in the Drosophila eye through clonal analysis. Our findings show that spase12 mutants are embryonic lethal, while spase12 LOF clones result in developmental defects in all tissues tested. Specifically, spase12 LOF in the Drosophila eye leads to errors in cell differentiation. Together, these data indicate that spase12 is required for viability, development, and differentiation.

## Results

### spase12 is required for development

To determine the effects of spase12 LOF on Drosophila development, we utilized three spase12 mutant alleles. spase12d4 is a lethal 4 kb deletion that removes two additional genes of unknown function: CG2006 and CG2310 (Figure 1A). spase12dd10774 carries a P-element inserted into the second exon of spase12 (Figure 1B). spase12dd24 is a 305 bp deletion generated through imprecise excision of E10774 that removes the first and second exons of spase12, as well as a portion of exon three (Figure 1B). These three alleles are embryonic lethal, recessive, and fail to complement one another.

To investigate spase12 function, we focused on the eye, which is unnecessary for viability and the development of which is well characterized. Using the ey-flp cell lethal (cl) method, clones were induced in the eye imaginal disc during the second larval instar stage and that comprise approximately 90% of the adult eye field [24]. ey ey-flp/+; FRT 82B P[+]/ cl/FRT 82B spase12dd24/ P[+]; ey ey-flp/+; FRT 82B P[+]/ cl/FRT 82B spase12dd24/ (spase12dd24) animals have a disorganized adult eye (Figure 2B–D). Additionally, loss of pigmentation is observed in spase12dd24 clones where clonal tissue appears to be a light yellow-orange color (Figure 2B) rather than the strong red P[+] color observed in spase12dd24/+ heterozygotes (Figure 2B'). This specific phenotype cannot be observed with spase12dd10774 (weak P[+] allele with light orange eye color) or spase12dd24 (w– allele) clones (Figure 2C', D').

We tested whether spase12 LOF is responsible for the observed phenotypes using rescue with both genomic DNA constructs and the Gal4/UAS system. A single copy of a 29 kb spase12 genomic rescue (spase12 GR) construct – which extends 10 kb both 5' and 3' of the spase12 locus and is intended to cover the deleted region and encompass all necessary regulatory elements (Figure 1A), rescues all known spase12d4 eye phenotypes (Figure 2E), spase12d24', a precise excision of the E10774 insertion, complements spase12d4 suggesting that phenotypes observed in spase12dd24/++ mutants are caused by the P-element insertion and spase12 LOF (Figure 2F).

### spase12 mosaic eyes have disrupted retinal structure

Adult eyes were sectioned to assay whether the disorganized appearance of spase12 mosaic eyes is indicative of a compromised retinal structure. In sections of wild-type animals (Figure 4A), ommatidia are arranged in a stereotyped, lattice pattern and aligned such that rows of ommatidia share the same polarity (Figure 4A–C'). However, sections of ey-flp; spase12dd4 (Figure 4B–B'), ey-flp; spase12dd10774 (Figure 4C–C'), and ey-flp; spase12dd24 (Figure 4D–D') animals reveal widely disorganized ommatidia with polarity defects. Furthermore, multiple ommatidia have varying numbers of rhabdomeres – the light-sensing organelles of photoreceptor cells. Within a single wild-type ommatidium, rhabdomeres appear as dark circles arranged in a trapezoidal pattern and indicate the presence of photoreceptors. Each wild-type ommatidium includes eight photoreceptors, but only seven rhabdomeres are observed (Figure 4A). In ey-flp; spase12 mutants, both ectopic and missing rhabdomeres are observed (Figure 4B, C). Variations in rhabdomere number suggest that there are incorrect numbers of photoreceptor cells. Within a single wild-type ommatidium, rhabdomeres appear as dark circles arranged in a trapezoidal pattern and indicate the presence of photoreceptors. Each wild-type ommatidium includes eight photoreceptors, but only seven rhabdomeres are observed (Figure 4A). In ey-flp; spase12 mutants, both ectopic and missing rhabdomeres are observed (Figure 4B, C). Variations in rhabdomere number suggest that there are incorrect numbers of photoreceptor cells. Within a single wild-type ommatidium, rhabdomeres appear as dark circles arranged in a trapezoidal pattern and indicate the presence of photoreceptors. Each wild-type ommatidium includes eight photoreceptors, but only seven rhabdomeres are observed (Figure 4A). In ey-flp; spase12 mutants, both ectopic and missing rhabdomeres are observed (Figure 4B, C).
Loss of spase12 causes melanotic mass formation and apoptosis

One of the most striking phenotypes observed in spase12 mutants are black lesions called melanotic masses that occur in approximately 15% of spase12 mosaic eyes (2B'-D'). ey-flp; spase12 mutants typically enclose without lesions, which then appear within one to three days. They may appear and remain static or they may expand to cover the entire eye. spase12 mosaic tissue in the wings (Figure 3A') and in the head region (Figure 3C') may also result in melanizations. Melanotic masses represent an inflammatory response that occurs when the immune system recognizes a foreign body or abnormal or dying tissue that is too large to be phagocytosed [25,26]. Specialized hemocytes converge upon and encapsulate the threat forging an indestructible barrier that is subsequently melanized, preventing further damage to the surrounding tissues [27,28]. Melanizations in ey-flp; spase12C24 animals are restricted to mosaic tissue suggesting that lesions are directly associated with abnormal tissue induced by spase12 LOF rather than a defect in hemocyte function. Sections through melanotic masses in ey-flp; spase12C24 animals (Figure 4D''') reveal degenerating tissue with large black inclusions that are consistent with dying cells.

Notably, melanotic masses have been observed in apoptosis mutants [25] and in LOF PINK-1 (PTEN-induced protein kinase 1) mutants with degenerative eye phenotypes [29]. To test whether spase12 LOF results in increased cell death, spase12 mosaic third instar discs were stained with Caspase antibody. Increased expression of Caspase was observed in all ey-flp; spase12C24 mutant discs (Figure 5B-B'') that also exhibit severe defects in retinal differentiation as judged by expression of Elav, a pan-neuronal marker. Such disruption and cell death is observed in approximately 10% of larval eye discs examined. Together these data suggest that spase12 LOF results in disrupted differentiation, increased apoptosis, and melanotic mass formation.

spase12 LOF results in cell differentiation errors

The extensive disorganization of the usually stereotypic retina as well as aberrant rhabdomere numbers observed in spase12 mosaic eyes suggests defects in tissue structure and cell differentiation. Therefore, immunohistochemistry (IHC) was used to examine the structure and appearance of retinal support cells (pigment cells) during pupal development. Using the hs-flp (M) method, we generated spase12C24 homozygous mutant tissue in a heterozygous background in which clones are marked by the absence of GFP.

We examined the eye at 48 hours after pupal formation, at which time the pupal eye structure is consistent with the organization of the adult retina. Staining for Armadillo, an adherens junction protein expressed at cell boundaries, marks support cells and cone cells, revealing the precise lattice pattern of the developing eye in control tissue (Figure 6A). Each individual ommatidium forms a hexagonal shape framed by secondary pigment cells intersected with tertiary pigment cells and inter-ommatidial bristles (IOBs) positioned at every other vertex (Figure 6A''). At the center of each ommatidium is a cluster of four cone cells enclosed by two primary pigment cells. This arrangement is severely disrupted in spase12C24 mutant tissue (Figure 6B'', C''''). In spase12 clonal tissue, many ommatidia lack the normal hexagonal pattern, instead having only four or five sides or a rounded shape, and some have lost one or both primary pigment cell(s). Additionally, many IOBs and tertiary pigment cells are improperly placed at neighboring vertices, and multiple IOBs may be present at a single vertex. There is also evidence of ommatidial fusions where support cells do not fully surround and enclose each ommatidium (Figure 6B', C'). In the example presented in Figure 6B'', the ommatidium is misshapen, IOBs and tertiary cells are present at only two vertices, three IOBs are located at a single vertex, and the identity of several support cells cannot be determined based on their shape and placement. The ommatidium in Figure 6C'''' retains its hexagonal shape, yet IOBs and tertiary cells are not properly positioned in relation to one another, a secondary pigment cell fails to fully extend and enclose the ommatidium resulting in an ommatidial fusion, and an extra primary pigment cell is present. Unlike larval clones where severe defects are observed in only about 10% of discs examined, strong disruption of development in pupal clones is fully penetrant.

Scanning electron microscopy (SEM) was used to image the external surface of the eye to determine whether these defects are maintained in adults and the results are consistent with the phenotypes observed in the pupal eye. The wild-type eye is well organized, with properly placed IOBs (Figure 7A-A''). ey-flp; spase12C24 eyes, however, are highly disrupted (Figure 7B-B''). Overall, the eye appears misshapen and the surface lens material

Figure 1. spase12 mutant alleles. (A) spase12D6 is a 4 kb deletion (purple shaded region). (B) spase12EY10774 contains a transposon inserted in the second exon. (B) spase12C24 is a 303 bp deletion (purple shaded region). spase12 GR (A) is a 29 kb genomic construct.

doi:10.1371/journal.pone.0060908.g001

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of all ommatidia is highly irregular. Consistent with the pupal eye phenotype, bristles are not properly placed at every other vertex, and malformed ommatidia and ommatidial fusions are observed. There are multiple examples of ectopic IOBs in clusters of two and three bristles.

**Figure 2.** *spase12* LOF results in a disorganized eye, loss of pigmentation, and melanotic mass formation. (A) *yw ey-flp/++; FRT 82B P[w+] cl/FRT 82B* where *w+* marks control tissue and *w−* marks the clone. *yw ey-flp/++; FRT 82B P[w+] cl/FRT 82B spase12<sup>EY10774</sup> P[w+] (ey-flp; spase12<sup>EY10774</sup>) (B), *yw ey-flp/++; FRT 82B P[w+] cl/FRT 82B spase12<sup>224</sup> P[w+] (ey-flp; spase12<sup>224</sup>) (ey-flp; spase12<sup>224</sup>) (D) eyes are disrupted compared to the control (A). Clonal tissue in *ey-flp; spase12<sup>24</sup>* eyes (B) appears lighter in color than *spase12<sup>24</sup>/; which exhibits a strong *P[w+]* eye color (B”). Clonal tissue in *ey-flp; spase12<sup>24</sup>/; spase12<sup>224</sup>* (B”), *spase12<sup>224</sup>/; (C”), and *spase12<sup>224</sup>/; mosaic eyes. A single copy of *spase12* GR rescues *spase12<sup>d4</sup>* in *yw ey-flp/++; FRT 82B P[w+] cl/FRT 82B spase12<sup>224</sup> P[w+] (E). *spase12<sup>d4</sup>* does not rescue *spase12<sup>224</sup>* phenotypes in *w; ubi-gal4/UAS-spase12; spase12<sup>d4</sup>* flies (G).

doi:10.1371/journal.pone.0060908.g002

**spase12 fails to genetically interact with Notch**

The Notch (N) pathway is a predicted SPC target as N and its ligands, Delta and Serrate, are SP-bearing transmembrane proteins [30–32]. Interestingly, *spase12* LOF mosaics exhibit Notch-like phenotypes, in particular, defects in cell differentiation
cell differentiation, disrupted alignment of ommatidia, and melanotic mass formation. From these data we conclude that spase12 is required for viability and development in Drosophila. Although it does not appear to be essential for SPC function, the data show that spase12 mediates cell differentiation, possibly through regulation of SPC activity on specific substrates or through contributing to SPC efficiency. In addition, this study reveals that loss of spase12 function causes melanotic mass formation, suggesting that spase12 LOF may lead to activation of the immune response pathway.

Deducing the role of spase12 in the SPC

In yeast, spc1p mutants are viable, but accumulate uncleaved pre-proteins in the ER. However, over-expression of spc1p rescues lethality of temperature-sensitive sec11 mutants shifted to the restrictive temperature suggesting that Spc1p contributes to the efficiency of the SPC complex in yeast [5]. Although Drosophila spase12 LOF is lethal to the animal as a whole, the fact that many spase12 null mutant cells are viable and can develop and differentiate normally suggests that Spase12, like its yeast homolog Spc1p, is expendable for SPC function. If spase12 were essential for catalytic function of the SPC, we would expect spase12 LOF to result in a fully penetrant cell lethal phenotype.

Spase12 may promote SPC cleavage by facilitating the translocation of SPC substrates into the ER. Human SPC12 is predicted to interact with nearly 100 proteins that are directly involved in this process, including more than 70 ribosomal subunits, as well as the translocon, signal sequence binding proteins, and signal recognition particle components [36,37]. This is consistent with topographical data indicating that mammalian SPC12 is primarily localized to the cytosol [11] where it could interact with ribosomes and translocation machinery on the ER surface. Such interactions may function to stabilize the ribosome-translocon interaction, facilitating the entry of newly translated proteins into the ER.

Loss of spase12 function causes highly variable phenotypes and does not appear to affect any one specific step in differentiation with full penetrance. For example, in the developing eye we observe both loss and gain of photoreceptors as well as supporting pigment and bristle cells. Furthermore, melanotic masses were observed in only 15% of mosaic animals with varying severity. Cleavage of each of the many SPC substrates may be disproportionate from cell to cell and animal to animal resulting in mutable phenotypes and incomplete penetrance. Although strong defects are observed in only 10% of mosaic larval eye discs, disruption of pupal differentiation is fully penetrant, perhaps reflecting a strong maternal contribution of spase12 transcript that rescues most zygotic null phenotypes into larval stages.

Lethality, however, is a fully penetrant phenotype. The SPC is required for the translocation and subsequent localization of transmembrane and secretory factors, many of which play a role in cell signaling. Even a potentially mild disruption of cell signaling during embryogenesis through loss of spase12 expression may be an insurmountable obstacle compared to the induction of spase12 LOF clones at later developmental stages using ey-flp or hs-flp clonal analysis.

**SPC targets in Drosophila**

In eukaryotes, all secretory and transmembrane proteins are expected to be translocated to the ER and cleaved by the SPC before they can be properly localized; however, few putative SPC targets have been confirmed in Drosophila. In vitro experiments provide strong evidence that vitellogenins and Crumbs are cleaved by the SPC. In vitro translation of vitellogenins, which are secreted

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**Figure 3. spase12 LOF disrupts development in multiple tissues.**<br>
yw ubx-flp/+; FRT 82B (M) P[w+] ubi-GFP/FRT control (A), yw ubx-flp/+; FRT 82B (M) P[w+] ubi-GFP/FRT 82B spase1224 wings are crumpled and melanized (A'). Clones in the distal portion of yw hs-flp/+; FRT 82B (M) P[w+] ubi-GFP/FRT 82B spase1224 legs (B) are twisted and stunted compared to yw hs-flp/+; FRT 82B (M) P[w+] ubi-GFP/FRT 82B control (B'). yw hs-flp/+; FRT 82B (M) P[w+] ubi-GFP/FRT 82B control (C). Clones in the head region of yw hs-flp/+; FRT 82B (M) P[w+] ubi-GFP/FRT 82B spase1224 result in eye, bristle, and antennal defects as well as melanotic mass formation (C').

doi:10.1371/journal.pone.0060908.g003

[33–35]. However, IHC failed to detect changes in the expression of N or Delta in spase12 LOF clones. If loss of spase12 function compromises SPC capacity, it would presumably reduce but not abolish expression of a wide range of proteins – though IHC may be insufficiently sensitive to distinguish changes in expression. To overcome this possibility, we tested whether spase12 and N genetically interact. We generated A40/FM7 P[w+] ubi-GFP; FRT 92B spase1224/TM6B flies and crossed virgins to yw ey-flp/Y; FRT 92B P[w+] cl/TM6B males. Progeny were screened for yw ey-flp/Y;N45, FRT 92B cl/FRT 82B spase1224 and yw ey-flp/FM7 P[w+] ubi-GFP; FRT 92B cl/FRT 82B spase1224 mosaic flies to determine whether removing a single copy of N could exacerbate spase12 mutant phenotypes. We observed no effect in response to reduced expression of N; however, these results do not rule out the possibility of a spase12;N interaction (data not shown).

**Discussion**

In the current study, we investigated the developmental role of Drosophila Spase12, a signal peptidease complex member. Thus far, the SPC has been studied primarily in S. cerevisiae. This work represents an effort to expand our understanding of this critical complex in a multicellular model system that is physiologically relevant for the study of human disease. We generated two spase12 LOF alleles, spase1224 and spase1224, that are recessive and embryonic lethal. Clonal analysis using both of these alleles, and the P-element mutant spase1210774, results in defective development of all tissues tested, including the eye, head, antenna, leg and wing. Further investigation into the retinal defects in spase12 mosaics reveals increased apoptosis in the developing eye, errors in...
from the fat body, yields preproteins that are significantly larger than endogenously synthesized vitellogenins, suggesting that they are likely to possess an SP that is cleaved by the SPC [38]. In the presence of microsomes derived from either canine pancreas or Drosophila embryos, in vitro translation of vitellogenins results in polypeptides that are the same size as what is produced in vivo [21]. Crumbs, a transmembrane protein with an exceptionally long SP, is co-translationally cleaved in an in vitro system to which canine pancreas or Drosophila S2 cell-derived microsomes are added [39].

Our investigation in Drosophila reveals that pathways populated by SP-bearing proteins may be affected by spase12 LOF. Loss-of-pigmentation phenotypes in ey-flp; spase12 mutants suggest that spase12 may affect the expression of proteins involved in eye pigmentation, such as the pigment cell membrane localized ABC transporter [40]. Additionally, defects in cell differentiation observed in ey-flp; spase12 mutants suggest that cell signaling pathways that function in retinal development, such as Notch, Hedgehog, Dpp, and EGFR [41], may be sensitive to spase12 expression.

In an effort to identify specific proteins that are disrupted by spase12 LOF and understand the mechanisms resulting in spase12 phenotypes, we conducted IHC in third instar and pupal spase12 mosaic eye discs. Expression of Crumbs, a known SPC substrate, was unaltered in spase12 mosaic tissue. Additionally, IHC failed to detect any changes in the expression of DE-Cadherin, Fasciclin 2, and Notch pathway members Notch and Delta – all of which are

Figure 4. spase12 adult eyes have ectopic and missing rhabdomeres and polarity defects. Thin plastic sections of yw ey-flp/+; FRT 82B P[w+]/FRT 82B control (A), yw ey-flp/+; FRT 82B P[w+]/FRT 82B spase12/+ (ey-flp; spase12) (B), yw ey-flp/+; FRT 82B P[w+]/FRT 82B spase12 (ey-flp; spase12) (C) and yw ey-flp/+; FRT 82B P[w+]/FRT 82B spase12 (ey-flp; spase12) (D) mosaic eyes are disorganized and exhibit spacing defects between ommatidia (red asterisks) and varying rhabdomere numbers. Red circles mark ommatidia with an ectopic inner rhabdomere while yellow circles mark ommatidia with a missing inner rhabdomeres. Polarity of individual ommatidia within ey-flp; 82B (A', A''), ey-flp; spase12 (B', B''), ey-flp; spase12 (C', C''), and ey-flp; spase12 (D', D'') is represented by red arrows. Section through ey-flp; spase12 melanotic mass (D'') reveals degenerating tissue with large black dots consistent with dying cells (red arrows).

doi:10.1371/journal.pone.0060908.g004
Caspase is upregulated and Elav expression strongly disrupted. In contrast, melanotic mass formation is likely to result in a systemic phenotype [25]. In this report we have shown that Spase12 is required for melanotic mass formation and cell death may shed light on the functional role of Spase12 in the SPC.

**Methods**

**Drosophila stocks**

Flies were cultured at 25°C on standard media. The following stocks were used: *ubi-gal4, spase12^EY10774* P[w+] cl/FRT 82B (ey-flp; spase12C24) control (A–A’’) has limited Caspase expression and normal Elav expression compared to *yw ey-flp/+; FRT 82B P[w+] cl/FRT 82B spase12^C24* (ey-flp; spase12^C24) (B–B’’) mosaic discs in which Caspase is upregulated and Elav expression strongly disrupted. doi:10.1371/journal.pone.0060908.g005

SP-bearing, transmembrane proteins. These results, however, do not rule out the possibility that the expression and function of these potential targets are impacted by *spase12* LOF. If Spase12 promotes the overall activity of the SPC but is not absolutely required for SPC function, there may not be a sufficient reduction in the expression or localization of any one protein to be detected with IHC. Conversely, Spase12 may not be necessary for SPC cleavage and subsequent localization of the putative SPC substrates tested. Because *spase12* LOF in the entire animal is lethal, S2 cells may present a viable alternative to identify SPC substrates that require *spase12* expression for localization and function. Partial RNAi knockdown of *spase12* in S2 cells, coupled with quantitative approaches to assay the expression levels of putative SPC targets may aid the identification of proteins affected by *spase12* LOF.

Melanotic masses in *spase12* mutants

*spase12^C24* mosaic flies are susceptible to melanotic mass formation. Mutated genes that result in melanization can be divided into two groups. Class I genes are not involved with the immune pathway, but may induce an immune reaction when altered in response to abnormal or degenerating tissue that is recognized and attacked by the immune system. Class II genes include those known to function in immune response pathways such as *Toll* and *JAK/STAT* [42,43].

Although *spase12* mosaic animals develop melanotic masses, it is unclear whether Spase12 functions directly in immunity pathways. However, melanotic mass formation is executed by hemocytes which circulate freely in the hemolymph and throughout the organism. Therefore, mutations that promote melanization are likely to result in a systemic phenotype [25]. In contrast, melanotic mass formation in *spase12* mosaic flies is restricted to the mutant tissue, suggesting that *spase12* may be a Class I gene.

Several studies have suggested that melanotic masses correlate with both necrotic and apoptotic cell death. In necrotic (nec) mutants, necrosis was shown to correspond with melanotic mass formation [44]. *PINK-1 (PTEN-induced protein kinase 1)* LOF in the eye results in photoreceptor degeneration, melanizations, and necrosis [29]. Mutations in apoptosis genes *dronc* (*Ned2-like caspase*), *dep-1* (*Decapping protein 1*) and *ark* (*Apaf-1-related-killer*) also result in melanotic mass formation [25]. We observe an increase in apoptotic cell death in developing *spase12* mosaic eyes, and melanotic masses, degeneration, and evidence of cell death in adult eyes suggesting a correlation between cell death and melanization in *spase12* mutant tissue.

It is also interesting to note that Drosophila homologs of five of the proteins that human SPC12 is predicted to interact with are linked with melanotic mass formation and each has a role in protein synthesis and translocation into the ER [36,37,45]. These include Gtb-bp and Srp54k, which target SP-bearing proteins to the ER, and ribosomal subunits RpL26, RpL6, and RpS5b [45]. Further investigation into the link between *spase12* LOF, melanotic mass formation, and cell death may shed light on the functional role of Spase12 in the SPC.

**Figure 5. spase12 LOF leads to increased apoptosis.** *yw ey-flp/+; FRT 82B P[w+] cl/FRT 82B (ey-flp; 82B) control (A–A’’) has limited Caspase expression and normal Elav expression compared to *yw ey-flp/+; FRT 82B P[w+] cl/FRT 82B spase12^C24* (ey-flp; spase12^C24) (B–B’’) mosaic discs in which Caspase is upregulated and Elav expression strongly disrupted. doi:10.1371/journal.pone.0060908.g005
and spase12C24 were generated via precise and imprecise excision, respectively, of spase12EY10774 P[w+]
using h2–3 transposase following standard methods.

Constructs

UAS-spase12 was generated by inserting spase12 cDNA (RE02772, DGRC) into pUAST-attB [48]. spase12 GR was generated by recombineering a 29 kb fragment of BACR28B07 into pACMAN [49]. pACMAN was a gift from Hugo Bellen (Jan and Dan Duncan Neurological Research Institute, Houston, TX, USA). Transgenics were generated by injection into VK1 (UAS-spase12) and P2 (spase12 GR) [49].

Clonal analysis

spase12EY10774 P[w+] and spase12C24 were recombined onto FRT82B. Clonal analysis was conducted using yw; ey-flp/+; FRT 82B P[w+]/TM6B, yw hs-flp/+; FRT 82B (M) P[w+ ubi-GFP]/TM6B, and yw; wbx-flp/+; FRT 82B (M) P[w+ ubi-GFP]/FRT 82B/TM6B stocks. hs-flp clones in pupal eye discs were generated via a 1 hour heat shock at 37°C to induce hs-flp expression at 48 hours after egg lay. Pupal discs were dissected 48 hours after pupation.

Immunohistochemistry and confocal microscopy

Antibody staining of third instar and pupal eye discs was performed as described [50]. The following primary antibodies were used: rabbit anti-GFP (1:1000, Molecular Probes), mouse anti-GFP (1:1000, Molecular Probes), rabbit anti-Caspase (Cell Signaling), mouse anti-Arm (1:500, DSHB) [51], and rat anti-Elav (1:500, Jackson ImmunoResearch), CY5 goat anti-rabbit (1:500, Jackson ImmunoResearch), CY5 goat anti-rabbit (1:500, Jackson ImmunoResearch), and CY3 goat anti-rat (1:500, Jackson ImmunoResearch). Images were captured using a Zeiss LSM 510 confocal microscope (Zeiss, Jena, Germany) and processed with ImageJ (NIH, Bethesda, MD, USA) and Adobe Photoshop software (Adobe Systems Incorporated, San Jose, CA, USA).

Figure 6. Loss of spase12 leads to defects in cell differentiation. yw hs-flp/+; FRT 82B (M) P[w+ ubi-GFP]/FRT 82B spase12C24 ey-flp/+; FRT 82B (M) P[w+ ubi-GFP]/FRT 82B spase12C24 48 APF eye discs stained with Armadillo (Arm) (A, B, C). GFP negatively marks clones (A', B', C'). Red dashed boxes outline representative ommatidia which are highlighted in A", B", C". Support cells are color-coded according to their identity; interommatidial bristles (magenta), secondary pigment cells (cyan) and tertiary pigment cells (green) (A", B", C"). The center of each ommatidium contains four cone cells, which strongly express Arm, surrounded by two primary pigment cells. spase12C24 mutant tissue (B, B' and C, C) exhibit multiple defects: ommatidia missing one or both primary pigment cells (stars), ectopic IOBs (arrowheads), ectopic primary pigment cell (asterisk), and gaps in the support cell structure that allow contact between primary pigment cell of neighboring ommatidia (red arrows). (B") A spase12C24 mutant ommatidium is misshapen and has a cluster of three IOBs. Additionally, support cells are not properly placed and the identity of three support cells (purple) cannot be determined by their shape or placement. (C") A spase12C24 mutant ommatidium fails to maintain the appropriate pattern of cell types at the vertices and possesses an ectopic primary pigment cell (asterisk). A secondary pigment cell (red arrow) does not fully extend to separate one ommatidium from its neighbor and one side of the ommatidium has two secondary pigment cells rather than one (white arrow).

doi:10.1371/journal.pone.0060908.g006

(Exelixis) as described [47]. spase12EY10774 and spase12C24 were generated via precise and imprecise excision, respectively, of spase12EY10774 P[w+] using 2–3 transposase following standard methods.
Figure 7. *spase12* LOF results in ectopic IOBs and ommatidial fusions. Scanning electron microscopy (SEM) of (A) *yw ey-flp/++; FRT 82B P[w+1] c/FRT 82B control and (B) *yw ey-flp/++; FRT 82B P[w+1] c/FRT 82B *spase12* at 200X, (A’, B’) 1000X, and (A”, B”) 2000X. Adult *ey-flp; spase12* eyes (B–B”) are disorganized with ectopic interommatidial bristles (red arrows) and ommatidial fusions (white arrows).

doi:10.1371/journal.pone.0060908.g007

Thin plastic sections and light microscopy

Thin plastic, tangential sections of the adult retina were performed as described [53]. Images of external *Drosophila*

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Electron microscopy

Flies were prepared and fixed in HMDS as described [54]. The samples were then coated under vacuum using a Balzer MED 010 evaporator (Technotrade International, Manchester, NH) with platinum alloy for a thickness of 25 nm, then immediately flash carbon coated under vacuum. The samples were transferred to a desiccator for examination at a later date. Samples were examined in a JSM-5910 scanning electron microscope [JEOL, USA, Inc., Peabody, MA] at an accelerating voltage of 3 kV.

Acknowledgments

We thank Kenneth Dunner of the High Resolution Electron Microscopy Facility at UTMDACC [Institutional Core Grant CA16672] for assistance with scanning electron microscopy; the Bloomberg Stock Center, Exelixis, and VDRC for providing *Drosophila* reagents; and the Baylor College of Medicine Ophthalmology Core (5P30EY002520-32) for confocal access.

Author Contributions

Conceived and designed the experiments: EHG SK RC GM. Performed the experiments: EHG SK. Analyzed the data: EHG. Contributed reagents/materials/analysis tools: EHG SK. Wrote the paper: EHG GM.

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morphology and thin plastic sections were captured using a Zeiss Axioplan 2 microscope, Zeiss AxioCam digital camera and AxioVision software and processed with ImageJ (NIH, Bethesda, MD, USA) and Adobe Photoshop software (Adobe Systems Incorporated, San Jose, CA, USA).


doi:10.1371/journal.pone.0060908.g007

Thin plastic sections and light microscopy

Thin plastic, tangential sections of the adult retina were performed as described [53]. Images of external *Drosophila*
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