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The SOCS box protein STOPS is required for phototransduction through its effects on phospholipase C

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Abstract

Phosphoinositide-specific phospholipase C (PLC) isozymes play roles in a diversity of processes including *Drosophila* phototransduction. In fly photoreceptor cells, the PLCβ encoded by *norpA* is critical for activation of TRP channels. Here we describe a novel PLCβ regulator, STOPS, which encodes a SOCS box protein. Mutation of *stops* resulted in a reduced concentration of NORPA and a defect in stopping signaling following cessation of the light stimulus. NORPA has been proposed to have dual roles as a PLC and GTPase activating protein (GAP). We found that the slow termination resulting from expressing low levels of wild-type NORPA was suppressed by addition of normal amounts of an altered NORPA, which had wild-type GAP activity, but no PLC activity. STOPS is the first protein identified that specifically regulates PLCβ protein concentration. Moreover, this work demonstrates that a PLCβ derivative that does not promote TRP channel activation, still contributes to signaling *in vivo*.

Introduction

Phosphoinositide-specific phospholipase C (PLC) isozymes play vital roles in signal transduction by cleaving the polar head group of phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (reviewed in Berridge, 1993). The most intensively studied PLC subtype, PLCβ, functions in signaling cascades initiated by stimulation of seven transmembrane domain G-protein coupling receptors (GPCRs). These GPCRs engage G_q, a member of the heterotrimeric GTP-binding protein family resulting in dissociation of the α and βγ subunits. One of these subunits then binds to and activates PLCβ, increasing its catalytic activity and thereby amplifying signaling.

In vitro analyses of mammalian PLCβ indicate that they also serve as GTPase activating proteins (GAPs), in addition to the more classical role in catalyzing the hydrolysis of PIP_2. PLCβ1 increases the steady-state GTPase activity up to 20 fold (Berstein et al., 1992; Biddlecome et al., 1996; Paulssen et al., 1996; Mukhopadhyay and Ross, 1999), resulting in fast deactivation of G_q. Thus, the regulation of G_q through the GAP activity of PLCβs potentially forms a short negative feedback loop that contributes to high signal resolution. An open question is whether PLCβs function as GAPs *in vivo*.

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The PLCβ required for *Drosophila* phototransduction has been suggested to be a dual functional protein, serving as a both a GAP and phospholipase C (Cook et al., 2000). Fly visual transduction is initiated by light activation of rhodopsin and engagement of the $G\alpha_q$ effector, which leads to stimulation of the PLCβ encoded by the *norpA* (*no receptor potential A*) locus (Bloomquist et al., 1988). The cascade culminates with the opening of the TRP and TRPL cation channels (reviewed in Wang and Montell, 2007).

Null mutations in *norpA* abolish the light response, while weak alleles display reduced light sensitivity, slow activation and decreased rates in the termination of the photoresponse (Paj et al., 1976). Weak *norpA* alleles express reduced levels of the NORPA protein, leading to the suggestion that the defect in termination results from a reduction in GAP activity (Cook et al., 2000). However, an alternative possibility is that the slow termination is a consequence of decreased PLC activity, since the activities of TRP and TRPL are dependent on hydrolysis of PIP_2, and Ca^{2+} influx via the channels is required for rapid termination (Hardie et al., 1993; Henderson et al., 2000; Wang et al., 2005). Thus the basis for the slow termination associated with expression of low quantities of NORPA remains unresolved. Defects in termination of G protein signaling can lead to many deleterious consequences in mammals, such as cardiac dysfunction, decreased fertility, deficits in the immune system, altered sensory responses, including problems in adapting to abrupt changes in light levels, and cell death (Gainetdinov et al., 2004; Nishiguchi et al., 2004). Given the diverse expression patterns and roles of PLCβ, understanding the basis through which PLCβ expression impacts on response termination has broad implications.

The expression of different PLCβ isoforms at appropriate levels and in distinct cell types is potentially a mechanism of control that applies to both mammalian and *Drosophila* PLCβs. For example, the NORPA PLCβ is expressed primarily in the *Drosophila* retina, and the mammalian PLCβ2 which functions in leukocyte signaling, is highly expressed in cells of the immune system (Bloomquist et al., 1988; Lee et al., 1996; Jiang et al., 1997). However, the proteins that regulate the cell-type specific expression of PLC proteins are unknown.

In the current study, we identified a mutation in an eye-enriched SOCS box protein, which we named Slow Termination of Phototransduction (STOPS). Known SOCS box proteins bind to the elongin B/C complex and promote the ubiquitination and proteasomal degradation of target proteins (reviewed in Kile et al., 2002). In contrast to these other proteins, STOPS functions independently of elongin B and C and is essential for expression of maximal levels of NORPA. STOPS is also required for stopping signaling upon cessation of the light stimulus as a consequence of the reduced NORPA expression. The defective termination in the *stops* mutant was due to decreased GAP activity of NORPA rather than a reduction in PLC activity, thereby providing strong evidence that NORPA functions as a GAP *in vivo*. These data demonstrate a novel mode for controlling PLCβ expression in photoreceptor cells.

Results

A mutation causing defective termination of phototransduction

To identify new genes that function in *Drosophila* phototransduction, we screened for mutations on the 3rd chromosome that disrupted the electroretinogram (ERG) (Wang et al., 2005). ERGs are extracellular recordings that measure the summed retinal responses to light (reviewed in Wang and Montell, 2007). Upon initiation of a light stimulus, the wild-type ERG displays a rapid corneal negative deflection, which quickly returns to the baseline after cessation of the light stimulation (Figure 1A). One mutant line, referred to as $stops^1$, displayed slower termination of the light response relative to wild-type (Figure 1B), but exhibited normal ERG amplitudes (Figure 1B; wild-type, 8.96 ±1.79 mV, n=12; 8.79 ±1.20 mV, n=12). The $stops^1$ mutation complemented all known 3rd chromosome mutations that disrupted the ERG,

including *arr2*, *rdgC*, *ninaE*, *trp*, *calx*, *ninaB* and *pinta* (data not shown), and therefore appeared to represent a new locus required for the normal light response.

Most genes required for phototransduction are expressed and function in the retina. However, some genes, such as *ninaB*, *ninaD* and *santa maria* are required outside the retina for a normal visual response (Gu et al., 2004; Wang et al., 2007; Yang and O'Tousa, 2007). To test whether *stops* functions autonomously in the retina, we used a mitotic recombination approach that leads to the generation of homozygous *stops*^{1} mutant eyes in animals that are heterozygous in all other tissues (Stowers and Schwarz, 1999). These mosaic flies displayed a slow termination phenotype similar to the original *stops*^{1} mutant (Figure 1C), indicating that *stops* is required in the compound eye. The *stops*^{1} phenotype was due to a defect in the photoreceptor cells as the slow termination of the light response was evident in intracellular recordings which measure the light response of single photoreceptor cells (Figures 1D and E).

Identification of the *stops* gene

To identify the gene responsible for the *stops*^{1} phenotype, we used meiotic recombination to map the mutation to cytogenetic region 100B (see Experimental Procedures). No deficiencies were available to more precisely localize the site of the *stops*^{1} mutation. Therefore, to identify a candidate *stops* gene, we considered whether expression of any gene near the 100B region was eye-enriched, since most genes that function in phototransduction are expressed primarily in the compound eye (Xu et al., 2004). We have previously performed a genome-wide screen for eye-enriched genes by comparing the expression of genes in wild-type heads and in the heads of an eyeless mutant (*sine oculis; so*) (Xu et al., 2004). In the chromosomal region encompassing *stops*, only one gene was enriched in the compound eye (*CG31006*; 100B4-100B5; wild-type head/*sine oculis* head ratio was ~22.6).

To confirm and extend the data indicating that *CG31006* was eye-enriched, we performed *in situ* hybridizations and Northern blots. Based on *in situ* hybridizations, *CG31006* RNA was spatially restricted to the retina (Figure 1F). The Northern blots supported these results, as the *CG31006* mRNA was present in wild-type but not in *sine oculis* heads or in wild-type bodies (Figure 1G). In addition, the *CG31006* signal was absent from the *glass* mutant (Figure 1G), which eliminated photoreceptor cells (Moses et al., 1989). These results indicated that *CG31006* was expressed primarily in photoreceptor cells and was therefore a good candidate for encoding STOPS.

To test whether mutation of *CG31006* was responsible for the *stops*^{1} phenotype, we sequenced the *CG31006* genomic region from *stops*^{1} flies and compared the sequence with DNA isolated from the original isogenized stock used to conduct the mutagenesis. In the *stops*^{1} mutant, there was a nonsense mutation in place of residue 92 (Figure 2A TGG to TAG), which truncated the 445 amino acid protein encoded by wild-type *CG31006* (Supplemental Figure S1A).

To provide additional evidence that *CG31006* was the *stops* gene, we took advantage of a fly line in which a P-element (KG09937) was inserted into the *CG31006* gene. The P-element resulted in lethality, which was possibly due to the disruption of the *gycβ 100B* gene encoding a guanylyl cyclase, since *CG31006* was located in an intron of *gycβ100B* (Figure 2A). We generated mosaic KG09937 animals containing mutant eyes and found that KG09937 exhibited a slow termination phenotype similar to *stops*^{1} (Figures 2B–D, H). KG09937 failed to complement the *stops*^{1} ERG phenotype (Figures 2E and H), indicating the same gene was disrupted in both mutants.

To confirm that *CG31006* was the *stops* gene, we tested for rescue of the *stops*^{1} phenotype using a transgene containing the wild-type *CG31006* genomic region (*gstops*). Normal termination was restored in the *gstops;stops*^{1} flies (Figure 2F and H). To test whether

CG31006 was required in photoreceptor cells, we expressed *CG31006* in photoreceptor cells using the major rhodopsin promoter (*ninaE*; *neither inactivation nor after potential E*). The *ninaE-stops;stops*^{1} flies showed a normal visual response, demonstrating that *CG31006* was the *stops* gene and was required in photoreceptor cells (Figure 2G and H).

NORPA protein level is down-regulated in the *stops*^{1} mutant

The *stops* gene encodes a 445 amino acid protein including a 40 amino acid SOCS box (Suppressor Of Cytokine Signaling) (Figure 3A; Supplemental Figure S1; residues 294–333). Although only ~20% of the residues in SOCS boxes are widely conserved, a common feature of these domains is that they bind to elongin B/C, which is part of the E3 ubiquitin ligase complex (Kile et al., 2002). SOCS box containing proteins typically include one or more additional motifs. STOPS is most similar to ASB (ankyrin repeat and SOCS box) proteins that include multiple ankyrin repeats (Supplemental Figure S1; nearly 30% identical to residues 191–334). However, STOPS does not appear to include a *bona fide* ankyrin repeat.

Most SOCS box proteins promote the proteasomal degradation of signaling proteins. The mechanism seems to involve ubiquitination of target proteins by linking the substrates with components of the E3 ubiquitin ligase complex. Therefore, disruption of the *stops* locus might lead to a defect in response termination as a consequence of overexpression of one or more signaling proteins. Termination of the light response is achieved at multiple levels to ensure that each component in the signaling cascade is properly inactivated. For example, activated rhodopsin is quenched by arrestin binding (Dolph et al., 1993), termination of the $G\alpha_q$ activity is proposed to be facilitated by the GAP (GTPase activation protein) activity of NORPA (Cook et al., 2000) and closing of the TRP/TRPL channels is regulated by calmodulin (CaM) (Scott et al., 1997) and protein kinase C (INAC) (Popescu et al., 2006). In principle a defect in termination could result if the levels of rhodopsin, $G\alpha_q$ or the TRP channels were increased relative to the signaling proteins that terminate their activities. Therefore, we checked the relative amounts of these and other proteins by performing Western blots. We found that *stops*^{1} expressed normal levels of Arr2, CalX, CaM, $G\alpha_q$, $G\beta_e$, INAC (PKC), INAD, NINAC (myosin III), Rh1 and TRP (Figure 3B).

In contrast to the expectation that the concentration of one or multiple signaling proteins would be increased, we found that the quantity of NORPA was decreased significantly in the *stops*^{1} mutant as well as in KG09937 mosaic flies and in KG09937/*stops*^{1} trans-heterozygous animals (Figure 3C; ~9% wild-type levels; see below). The level of NORPA reverted to wild-type in *gstops;stops*^{1} and *ninaE-stops;stops*^{1} flies (Figure 3C). These data indicate that STOPS is required specifically for normal expression of NORPA.

STOPS is required for expression of NORPA in photoreceptor cells but not in extra-retinal tissues

Although NORPA is expressed predominately in photoreceptor cells, it is also expressed and functions in other head regions including the brain (Zhu et al., 1993; Kim et al., 1995). To address whether STOPS controls the expression of NORPA in non-retinal tissues, we assessed the relative amounts of NORPA in retinas and in heads without retinas. To conduct the comparison, the extracts were prepared in a constant volume consisting of the same actual numbers of retinas, and heads without retinas, rather than including more retinas to adjust for the lower amounts of total proteins in the retinas only samples. The relative quantity of NORPA was greatly reduced (10%) in the *stops*^{1} retina; however, in the head tissue missing the retina, the concentration of NORPA was similar in wild-type and *stops*^{1} (Figure 3D and Supplemental Figure S2). Furthermore, upon elimination of the photoreceptor cells by expression of the proapoptotic gene *hid* (Grether et al., 1995), under the control of an eye-specific promoter (*GMR*) (Hay et al., 1994), the concentration of NORPA was unchanged in *stops*$^{+}$ and *stops*^{1}

flies (Supplemental Figure S3). These results indicate that STOPS is required for NORPA expression specifically in the retina.

The SOCS box is indispensable for STOPS function

The observation that loss of *stops* caused a decrease rather an increase in NORPA indicates that the SOCS box might not function to promote elongin B and elongin C-dependent proteolysis. To test this proposal, we analyzed lethal mutations in *elongin-B* and *elongin-C* in mosaic animals. We found that in *elongin-B* or *elongin-C* mutant eyes, NORPA was produced at normal levels (Figure 4A) and the ERG responses were indistinguishable from wild-type (Figures 4B-E). Moreover, the $stops^{1}$ phenotype was not suppressed in an *elongin-B* background (Figure 4F).

The observation that the $stops^{1}$ phenotype was independent of elongin B/C raised the question as to whether the SOCS box in STOPS was crucial for function, since elongin B/C proteins bind to SOCS boxes in other proteins. Therefore, we generated a Myc-tagged form of STOPS lacking the entire SOCS box and expressed it using the *ninaE* promoter (*ninaE-*$stops^{dsocs}$;$stops^{1}$; Figure 4G, middle panel). As a control, we generated $stops^{1}$ flies expressing full length STOPS fused to a Myc tag (*ninaE-stops*; Figure 4G, middle panel). Introduction of *ninaE-*$stops^{dsocs}$ in $stops^{1}$ flies failed to restore normal amounts of NORPA or wild-type termination (Figures 4G and I). Both wild-type NORPA protein concentrations and ERG responses were restored in *ninaE-stop;*$stops^{1}$ flies (Figures 4G and J).

To confirm the importance of the SOCS box, we mutated three consecutive amino acids in the SOCS box (LRH to PGG, residues 297–299), two of which are highly conserved in SOCS boxes (Figure 3A). We expressed the Myc-tagged derivative of STOPS in flies under the control of the *ninaE* promoter (*ninaE-*$stops^{m3}$;$stops^{1}$) and found that Myc-$STOPS^{m3}$ and wild-type Myc-STOPS were produced at similar levels (Figure 4G). However, expression of $STOPS^{m3}$ did not rescue the $stops^{1}$ phenotype (Figures 4G and K). Taken together, these data indicated that the SOCS box in STOPS was indispensable, even though STOPS did not appear to function through an elongin B/C dependent mechanism.

STOPS controls NORPA protein concentration post-transcriptionally

The STOPS protein could be required for NORPA expression by controlling the amount of *norpA* mRNA. To address this question, we performed Northern blots and found that the quantities of *norpA* mRNAs were indistinguishable in wild-type and $stops^{1}$ flies (Figure 5A). This result suggested that transcription of *norpA* was not controlled by STOPS.

To provide additional evidence that STOPS regulates NORPA protein concentration post-transcriptionally, we generated transgenic flies that expressed the *norpA* cDNA under the control of the *ninaE* promoter. Therefore, production of the *norpA* mRNA was dependent on the *ninaE* promoter instead of its own promoter, and was independent of mRNA splicing since a cDNA was used to express *norpA*. Thus, a requirement for STOPS for transcription or mRNA splicing of *norpA* should be unnecessary in $norpA^{P24}$;*ninaE-norpA* flies. However, the quantity of NORPA protein in *ninaE-norpA;*$stops^{1}$ flies was as low as in $stops^{1}$ flies (Figure 5B). This reduced NORPA concentration was not due to a problem in expression of the *ninaE-norpA* construct since NORPA was detected at levels similar to wild-type in a $norpA^{P24}$;$stops^{+}$ background (Figure 5B; $norpA^{P24}$;*ninaE-norpA*). These data indicate that STOPS regulates NORPA expression post-transcriptionally.

To determine whether STOPS controls the spatial localization of the NORPA protein, we performed immunostaining. However, the concentration of NORPA was too low to be detected in sections of the $stops^{1}$ compound eye. Therefore, we stained whole isolated ommatidia with

anti-NORPA antibodies. *Drosophila* photoreceptor cells contain a specialized microvillar region, the rhabdomere, which contains the major rhodopsin (Rh1), NORPA and other proteins that participate in phototransduction. We found that the residual NORPA in *stops*^{1} co-localized with the major rhodopsin Rh1, indicating that it was in the rhabdomeres (Figure 5C). Thus, STOPS did not affect rhabdomere localization of NORPA, but specifically regulated the quantity of the NORPA protein.

Reduced NORPA concentration was responsible for the slow termination

To test whether the reduction in the amount of the NORPA protein alone was responsible for the *stops*^{1} phenotype, we examined whether decreased expression of wild-type NORPA mimicked the slow termination phenotype in *stops*^{1} flies. We generated transgenic flies that expressed varying levels of wild-type NORPA under the control of the *heat-shock* promoter (*hs-norpA*) and placed the transgene in a null *norpA* background (*norpA*^{P24}). The *heat-shock* promoter results in expression of *norpA* mRNAs in both heads and bodies (Supplemental Figure S4A). However, the NORPA protein expressed under the control of the *heat-shock* promoter was detected predominately in the retina (Supplemental Figure S4B).

We found that the speed of termination of the photoresponse was proportional to NORPA protein expression. After a 15 minute heat shock, *norpA*^{P24};;*hs-norpA* flies expressed about ~3.8% of wild-type NORPA, and displayed a severe slow termination phenotype (Figures 6A, B and F). As the duration of the heat-shock increased, the expression of NORPA rose gradually and the termination of the photoresponse became faster. Exposing the *norpA*^{P24};;*hs-norpA* flies to 30, 60 and 120 minute heat-shock treatments resulted in the production of ~7%, ~15% and ~20% of wild-type NORPA protein respectively, and expression of 20% the normal concentration of NORPA restored a wild-type ERG (Figure 6). Thus, there was a strong correlation between the termination time and amount of NORPA. Moreover, *stops*^{1} flies, which expressed NORPA at ~9% of wild-type levels, displayed a similar termination time as *norpA*^{P24};;*hs-norpA* flies, which expressed similar quantities of NORPA (30 minute heat shock; Figures 6A, B, D and G). These results indicated that the reduction of NORPA protein in *stops*^{1} was the basis underlying the slow termination phenotype.

PLC activity of NORPA is dispensable for termination of the light response

NORPA has been suggested to function as a GAP to promote hydrolysis of the GTP bound to the $G\alpha_q$ (Cook et al., 2000), in addition to its established role as a phospholipase C, which is necessary for activation of the TRP and TRPL channels. Therefore, the slow termination of the light response caused by reduced NORPA expression could result from either of two distinct mechanisms. Decreased Ca^{2+} influx due to low PLC activity might underlie the phenotype, since termination of the photoresponse is dependent on light-dependent Ca^{2+} influx. Alternatively, lower GAP activity might be responsible for the *stops* phenotype due to slow turn-off of the $G\alpha_q$. To differentiate between these two possibilities we used genetic approaches.

If the basis of the termination defect in *stops*^{1} was diminished Ca^{2+} influx during light stimulation, then the termination phenotype might be suppressed by a genetic manipulation that increased intracellular Ca^{2+} during light stimulation. We have shown previously that elimination of the Na^{+}/Ca^{2+} exchanger, CalX, results in an increased Ca^{2+} concentration during light stimulation (Wang et al., 2005). Therefore, to test whether an increase in intracellular Ca^{2+} can suppress the *stops*^{1} ERG phenotype, we examined the light response in *calx*^{B}, *stops*^{1} flies. Although the *calx*^{B} photoresponse terminates rapidly, *calx*^{B}, *stops*^{1} flies display the slow termination characteristic of *stops*^{1} flies (Figure 7). These results suggest that diminished Ca^{2+} due to low PLC activity is not the major factor affecting termination of the light response in *stops*^{1} flies.

To address whether the diminished NORPA GAP activity underlies the *stops*^{1} phenotype, we tested whether we could restore normal termination in *stops*^{1} flies by expressing a NORPA derivative that retained only GAP activity. Currently, mutations that disrupt the GAP activity of PLCβs have not been described. However, specific residues have been identified that are critical for the phospholipase C activity. We reasoned that if the *stops*^{1} phenotype was due to lower GAP activity, then we might suppress the slow termination by expressing normal amounts of a NORPA derivative with a mutation in a critical residue in the PLC catalytic domain, but which does not affect GAP activity. To test this possibility, we introduced a point mutation in the Y domain of the PLC catalytic site of NORPA (serine 559 to alanine; Figure 8A), which has been shown in other PLCs to reduce PLC activity >10,000-fold (Essen et al., 1996; Ellis et al., 1998). The *norpA*^{S559A} was expressed in *norpA*^{P24} flies under the control of the *ninaE* promoter (*ninaE-norpA*^{S559A}), resulting in a normal rhabdomeral distribution of NORPA^{S559A} (Figure 5C), and expression at a level comparable to NORPA in wild-type flies (Figure 8B).

Expression of NORPA^{S559A} did not restore a light response in *norpA*^{P24} null mutant flies (Figure 8F) consistent with previous studies that mutation of serine 559 has a profound effect on PLC activity. Although the *norpA*^{P24};*ninaE-norpA*^{S559A} flies were devoid of an ERG response, they displayed normal light-induced GAP activity (Figure 8C).

Expression of NORPA^{S559A} under the control of the *ninaE* promoter was low, if the *ninaE-norpA*^{S559A} transgene was placed in a *stops*^{1} background (Figure 5B). Therefore, to test whether the slow termination due to a reduction in wild-type NORPA protein could be suppressed by NORPA^{S559A}, we used an alternative approach. We expressed NORPA^{S559A} in a *norpA*^{P24};;*hs-norpA* genetic background (*norpA*^{P24};*ninaE-norpA*^{S559A};*hs-norpA*) and induced low amounts of wild-type NORPA protein using a 30 minute heat shock. After expressing low amounts of wild-type NORPA alone in a *norpA*^{P24} background (30 minute heat shock of *norpA*^{P24};;*hs-norpA*), the termination of the photoresponse was slow relative to wild-type (Figures 8D, G and I). Of significance here, production of normal levels of NORPA^{S559A} restored wild-type response termination kinetics in flies expressing low quantities of wild-type NORPA (30 minute heat shock of *norpA*^{P24};*ninaE-norpA*^{S559A};*hs-norpA*) (Figure 8G and H). The suppression of the termination phenotype could be due to the increase in GAP activity. Alternatively, expression of NORPA^{S559A} might increase the concentration of wild-type NORPA and raise the PLC activity. However, we found that the NORPA PLC activities were not raised in *norpA*^{P24};*ninaE-norpA*^{S559A};*hs-norpA* relative to *norpA*^{P24};;*hs-norpA* flies, indicating that the level of wild-type NORPA was not increased by expression of NORPA^{S559A} (Supplemental Figure S5). These results indicate that the slow termination phenotype resulting from a low amount of NORPA protein is due to the reduced GAP activity of NORPA.

Discussion

Low levels of PLC cause slow termination of phototransduction

Many PLCβ isoforms, such as the NORPA protein, which functions in fly phototransduction, are expressed in distinct subsets of cells. In the current work, we demonstrate that NORPA expression in photoreceptor cells depends on the STOPS protein. Mutations in *stops* caused a decrease in NORPA protein concentration, resulting in slow termination of the photoresponse.

A correlation between low NORPA expression and slow response termination has been proposed previously since multiple *norpA* alleles as well as *inaD*^{2} express reduced concentrations of NORPA and display termination defects (Pearn et al., 1996; Shieh et al., 1997; Tsunoda et al., 1997). However, these mutants have additional alterations, precluding the conclusion that low levels of NORPA prevent normal termination of the photoresponse.

For example, hypomophic *norpA* mutants such as $norpA^{P57}$ and $norpA^{P16}$ express mutant rather than wild-type NORPA (Pearn et al., 1996). Moreover, the interaction of NORPA with the INAD scaffold protein is disrupted in $inaD^{2}$ and $norpA^{C1094S}$, suggesting that the INAD-NORPA interaction contributes to the termination phenotype in these mutant flies (Shieh et al., 1997; Tsunoda et al., 1997).

We provide two lines of evidence that a normal concentration of NORPA is essential for stopping signaling after cessation of the light stimulus. First, the *stops* mutant expresses decreased quantities of wild-type NORPA but not other proteins in photoreceptor cells, resulting in slow termination of the light response. Second, we manipulated the amount of wild-type NORPA using the *heat shock* promoter. Our results showed a strong relationship between the termination rate and the concentration of NORPA protein.

Low GAP activity underlies the termination defect due to reduced NORPA levels

A question concerns the mechanism through which reduced amounts of NORPA cause a termination defect. In principle, the slow termination could result from a combination of low NORPA levels, and a decrease in intrinsic enzyme activity, due to a requirement for STOPS as a cofactor. However, this does not appear to be the case as the termination defect is indistinguishable between $stops^{+}$ flies that express the same low levels of NORPA (expressed under the control of the *hsp70* promoter in a *norpA* mutant background) as is produced in $stops^{1}$ flies. STOPS does not co-immunoprecipitate with the TRP channels, suggesting that it does not cause defects in termination through direct interactions with the TRP channel (Supplemental Figure S6). Moreover, the ERG response is the same in trp^{P343} and trp^{P343}, $stops^{1}$ flies (Supplemental Figure S7), suggesting that STOPS does not alter the activity of the TRPL channel. Furthermore, STOPS does not co-immunoprecipitate with the scaffold protein, INAD (Supplemental Figure S6), or alter the concentration of any known member of the signalplex other than NORPA, indicating that the termination phenotype in $stops^{1}$ does not result from destabilization of the signalplex.

In addition to serving as a phospholipase C, mammalian PLCβs and NORPA have been proposed to be GAPs (Berstein et al., 1992; Biddlecome et al., 1996; Cook et al., 2000). Therefore, according to one model, low NORPA levels cause a termination defect by decreasing GAP activity, thereby leading to sustained activity of the $G\alpha_q$ following light stimulation (Cook et al., 2000). However, previous work did not exclude that the slow termination resulting from a reduced NORPA concentration was due to a requirement for PLC activity. Thus, an alternative possibility is that the slow termination is a consequence of decreased light-dependent Ca^{2+} influx, since the NORPA phospholipase C activity leads to opening of the TRP and TRPL channels. A rise in Ca^{2+} is important for termination, as the rate is decreased in flies overexpressing the Na^{+}/Ca^{2+} exchanger, CalX, or if the photoreceptor cells are illuminated in a Ca^{2+}-free bath (Henderson et al., 2000; Wang et al., 2005).

Our results indicate that the slow termination due to a low concentration of NORPA is caused by reduced GAP activity rather than diminished intracellular Ca^{2+}. In support of this conclusion, the delayed termination was not suppressed by a mutation in *calx*, which eliminates Ca^{2+} extrusion. Rather, we found that slow termination of the photoresponse was fully reversed by expression of a PLC-derivative, $NORPA^{S559A}$, which contains a mutation in a residue critical for activation of the photoresponse. Since $NORPA^{S559A}$ fully retained GAP activity, these data indicate that the GAP activity of NORPA is essential for arresting signaling following cessation of the light stimulus. The observation that introduction of normal levels of $NORPA^{S559A}$, suppresses the termination defect resulting from expression of a low concentration of wild-type NORPA, indicates that more than one NORPA molecule can interact successively with the same activated $G\alpha_q$. This proposal is consistent with the evidence that NORPA forms a homodimer (Kimple et al., 2001). In addition, it appears that a single

activated $G\alpha_q$-GTP is capable of interacting sequentially with multiple independent NORPA molecules (Hardie et al., 2002).

Following activation, rapid termination of the light response is essential for high temporal resolution to ensure appropriate responses to subsequent stimuli. Since G-protein deactivation appears to be the rate-limiting step for termination in mammalian phototransduction cascade (Sagoo and Lagnado, 1997), timely deactivation of G-protein may be equally important for regulation of *Drosophila* phototransduction. Given that NORPA is the direct target for the G-protein, stimulation of the GTPase activity of the $G\alpha_q$ represents a highly efficient and rapid mode of negative feedback regulation.

A novel role for a SOCS box protein

In the current work, we demonstrate that the SOCS box protein, STOPS, is required specifically for expression of NORPA in photoreceptor cells. SOCS proteins were initially identified as suppressors of cytokine signaling and contain a common C-terminal 40 amino acid SOCS box (Starr et al., 1997). Genomic analyses led to the identification of many additional SOCS box-containing proteins, which are subdivided into groups based on their diverse N-terminal domains. Examples include an SH2 domain (SOCS), WD40-repeats (WSB), a SPRY-domain (SSBs), a RAB domain (RAR), a domain conserved in the Neuralized family of proteins (Neuralized-like) and ankyrin-repeats (ASBs) (Supplemental Figure S1B) (Hilton et al., 1998; Hilton, 1999; Kile et al., 2002). Among the mammalian proteins, STOPS is most similar to the human CRA-b isoform of ASB15, since STOPS and CRA-b share additional homology (STOPS domain) that extends N-terminal to the SOCS box.

Current understanding about SOCS box containing proteins is that they function as adaptor molecules for the E3 ubiquitin ligase complex to target signaling proteins to the protein degradation pathway (Kile et al., 2002). The variable N-terminal domain interacts with target proteins thereby defining the substrate specificity for the E3 ubiquitin ligase complex. The SOCS box binds to a heterodimer composed of ubiquitin-like elongin B and Skp1-like elongin C, and forms an E3 ubiquitin ligase complex with Cullin-2 and Rbx-1 (Kamura et al., 1998; Zhang et al., 1999; Wilcox et al., 2004). In *C. elegans*, the ZIF SOCS box protein functions in the exclusion of germ-line proteins from somatic lineages by interacting with the elongin B/C complex and targeting the somatic CCCH finger protein for degradation in a cullin and Rbx-dependent manner (DeRenzo et al., 2003).

In contrast to all other characterized SOCS box proteins, which function in protein degradation, STOPS has an opposite role in promoting expression of NORPA in photoreceptor cells. Consistent with this observation, the elongin B/C pathway was not required for expression of NORPA. The *elongin-B* and *elongin-C* mutants expressed normal amounts of NORPA and displayed wild-type light responses. Thus, as expected the elongin B/C complex does not function as a positive regulator of NORPA. Furthermore, a double *elongin-B*, $stops^1$ mutant displayed the same decreased NORPA concentration and termination defect as the single $stops^1$ mutant, indicating that the elongin B/C complex does not function in a pathway opposing STOPS. Nevertheless, mutations in the SOCS box disrupt STOPS function. Taken together, these data indicate that although STOPS is dependent on the SOCS box, it acts independently of the elongin B/C complex *in vivo*. Thus, STOPS is distinct from previously characterized SOCS box proteins.

Control of NORPA expression exclusively in photoreceptor cells

The expression patterns of many signaling proteins are restricted to ensure that they function in a cell-type specific manner. In wild-type flies, NORPA is expressed predominately in photoreceptor cells, but is still detected in other tissues. We found that the STOPS protein is

required for expression of NORPA only in photoreceptor cells since in the *stops*^{1} retina, NORPA is reduced by >90% without affecting NORPA in other tissues. In *hs-norpA* flies, which expressed NORPA under the control of *heat-shock* promoter, the NORPA protein was expressed primarily in the head but not in the body, although the mRNAs were present in both the head and body (Supplemental Figure S4C). Moreover, in the head of *hs-norpA* flies, NORPA protein was enriched in the retina (Supplemental Figure S4D).

Although STOPS is required for relatively high expression of NORPA, it is not sufficient. In heat-shocked *norpA*^{P24}*;hs-stops;hs-norpA* flies, which expressed both the *stops* and *norpA* RNAs broadly, NORPA was still expressed principally in the retina (Supplemental Figure S4). In HEK293T cells, coexpression of STOPS with NORPA did not result in an elevation of NORPA levels beyond that obtained in cells expressing NORPA alone (unpublished observations). Moreover, NORPA and STOPS did not co-immunoprecipitate either in fly heads or after expressing the two proteins in HEK293T cells (Supplemental Figure S6 and unpublished observations). Thus, there appear to be one or more additional factors that remain to be identified that function in concert with STOPS to promote NORPA expression. Finally, the current work describes a novel mode for controlling the expression of a PLCβ and raises the possibility that there exist mammalian SOCS box proteins that function independently of the elongin B/C complex to promote the expression of PLCβ and other signaling proteins in distinct subsets of cells.

Experimental Procedures

Fly stocks

The *stops*^{1} flies were isolated by performing EMS (ethylmethylsulfonate) mutagenesis and by screening for 3rd chromosome mutations affecting the ERG (Wang and Montell, 2005; Wang et al., 2005). The Bloomington Stock Center was the source for the "3rd chromosome deficiency kit" and the following stocks: 1) KG09937, 2) P{*lacW*}*Idh*^{L3852}, 3) *y*^{1}*w**;P{*neoFRT*}*42D* P{*Car20y*}*44B* P{*GMR-hid*}*SS2* l(2)*/CyO;P{GAL4-ey.H}SS5 P{*UAS-FLP1.D*}*JD2*, 4) *y*^{1}*w**;P{*GAL4-ey.H*}DH1 P{*UAS-FLP1.D*}JD1;P{*neoFRT*}82B P{*GMR-hid*}SS4 l(3)*/TM2, 5) P{*EPgy2*}*elongin-B*^{EY04022} (*elongin-B*), 6) P{*lacW*}*elongin-C*^{SH1299} (*elongin-C*), 7) *so*^{1} (*sine oculis*), and 8) *gl*^{1} (*glass*). *EP3575* and *EP3089* were from the Szeged *Drosophila* Stock Center. Dr. W. Pak provided the *norpA*^{P24} stock. The "wild-type" control described in this work is *w*^{1118}. The *stops*^{1}, *norpA*^{P24} and *calx*^{B} mutants were in a *w*^{1118} background. The transgenic flies were red-eyed since the marker used for screening for the transgenic flies was *w*$^{+}$.

Mapping the *stops* mutation by genetic recombination

We performed deficiency mapping by crossing the *stops*^{1} mutant to the fly stocks that comprised the "3rd chromosome deficiency kit". However, *stops*^{1} was not uncovered by any deficiency. Therefore, we used recombination mapping in combination with *ninaE* (92B), *trp* (99C) and three P-element lines: P{*lacW*}*Idh*^{L3852} (66C), *EP3575* (94A) and *EP3089* (97E). The following recombination rates were observed: *trp* (1.1%), *ninaE* (37.5%), P{lacW}*Idh*^{L3852} (45.5%), *EP3575* (25%), *EP3089* (11.2%). Based on these findings, we localized the *stops* gene to genomic region 100B.

Generation of transgenic flies

The *stops* (*CG31006*) genomic DNA was subcloned from BAC11C03 between the *SmaI* and *SacII* sites of the pCaspeR4 vector (Thummel and Pirrotta, 1992). To express the *stops* cDNA under the control of the *ninaE* promoter, the cDNA (EST clone GH07253, DGRC) was subcloned between the *NotI* and *XbaI* sites of pCNX (Wang and Montell, 2006). To express Myc-tagged wild-type or mutated STOPS in flies, the sequence encoding a Myc tag was fused

to the 3′ end of the wild-type *stops* cDNA. Mutations were introduced using the QuickChange method (Stratagene). The wild-type and mutant *stops* cDNAs were subsequently subcloned between the *NotI* and *XbaI* sites of pCNX.

To express *norpA* under control of the *hsp70* and *ninaE* promoters, the full length *norpA* cDNA was excised from pHKX-*norpA* and subcloned into pCaspeRhs (Thummel and Pirrotta, 1992) and between the *NotI* and *XbaI* sites of pCNX. To express the mutated *norpA* under control of the *ninaE* promoter, the mutations were first introduced into the *norpA* cDNA using the QuickChange method, before subcloning into pCNX. The constructs were injected into of w^{1118} embryos and transformants were identified by w^{+} pigmentation.

Electroretinogram and intracellular recordings

ERG recordings were performed as previously described (Wes et al., 1999). Briefly, two glass microelectrodes filled with Ringer's solution were inserted into small drops of electrode cream placed on the surfaces of the compound eye and the thorax. A Newport light projector (model 765) was used for stimulation. The ERGs were amplified with a Warner electrometer IE-210 and recorded with a MacLab/4s A/D converter and the Chart v3.4/s program. Intracellular recordings were performed by placing a low-resistance microelectrode filled with 3 M KCl into a small hole on the compound eye and a reference electrode on the thorax as described previously (Xiong et al., 1994).

Western blots

To perform the Western blots, fly heads were homogenized in SDS-sample buffer, the proteins were fractionated by SDS-PAGE and transferred to Immobilon-P transfer membranes (Millipore) in Tris-glycine buffer. The blots were probed with mouse anti-Tubulin primary antibodies (1:3000 dilution, Developmental Studies Hybridoma Bank), mouse anti-Rh1 antibodies (1:2000 dilution, Developmental Studies Hybridoma Bank), mouse anti-$G\beta_e$ antibodies (1:1000 dilution), rabbit anti-INAC antibodies (1:2000 dilution), rabbit anti-NINAC antibodies (1:2000 dilution), rabbit anti-Arr2 antibodies (1:2000 dilution), rabbit anti-$G\alpha_q$ antibodies (1:2000 dilution), rabbit anti-INAD antibodies (1:2000 dilution), rabbit anti-TRP antibodies (1:2000 dilution), rabbit anti-CalX antibodies (1:2000 dilution), rabbit anti-NORPA antibodies (1:2000 dilution) and rabbit anti-Myc antibodies (1:1000, Santa Cruz). The blots were subsequently probed with either anti-rabbit, mouse IgG peroxidase conjugate (Sigma), ^{125}I-labeled protein A (NEN), ^{125}I-labeled anti-mouse IgG (NEN), IRDye 680 goat anti-Rabbit IgG (LI-COR) or IRDye 800 Donkey anti-mouse IgG (LI-COR), and the signals were detected using ECL reagents (Amersham), a phosphoimager or the Odyssey infrared imaging system (LI-COR).

Northern blots

Total RNAs were prepared using Trizol (Invitrogen) and the samples were fractionated on 3% formaldehyde, 1.0% agarose gels. The RNAs were transferred to nitrocellulose membranes and allowed to hybridize with ^{32}P-labeled probes, which were prepared using random primers in combination with the following DNA templates as described by Roche: *norpA* (nucleotides 1288–2211 from the *norpA* cDNA), *stops* PCR product (nucleotides 675–1500 obtained using the *stops* cDNA), *rh1* PCR product (nucleotides 300–900 of *ninaE* cDNA) or an *rp49* PCR product (nucleotides 24–585 of *rp49* cDNA). The hybridizations were performed at 65 °C in 7% SDS, 2 mM EDTA, 0.5 M Na_2HPO_4 (pH 7.2) and the membranes were washed at 65 °C in 0.5X SSC, 0.1% SDS.

In situ hybridizations

Frozen 8 μm sections of adult fly heads were prepared and hybridized to DIG labeled sense and anti-sense RNA probes prepared from a full-length *stops* cDNA clone (pcDNA3-*stops*) using either SP6 polymerase or T7 polymerase. The hybridizations were at 70 °C, 50% formamide, 5X SSC 0.02% SDS, 1X Blocking Buffer (Roche). The hybridized sections were incubated with anti-DIG alkaline phosphatase conjugated antibodies (Roche) and the signals were detected using NBT and BCIP.

GTPase Assay

Total membrane proteins were prepared from fly heads that were dark-adapted overnight. The GTPase assays were performed essentially as described (Cook et al., 2000) in 100 μl containing 50 mM KH_2PO_4/K_2HPO_4 buffer pH 6.8, 5 mM $MgCl_2$, 2.5 mM NaF, 0.25 μ;M [γ-P^{32}]GTP (300–500 cpm/nmol), 0.5 mM NaATP, 2 mM DTT and 10 mg total membrane proteins. After a 5 min incubation at room temperature under bright blue light or in the dark, the reactions were terminated by addition of 0.6 ml 5% charcoal suspension (in 20 mM phosphoric acid). After centrifugation at 12,000 RPM for 10 min, the $^{32}P_i$ generated by hydrolysis of GTP in the supernates were assayed using a scintillation counter. Light-dependent GTPase activities were determined by calculating the differences between the samples kept in the dark with those exposed to blue light.

Immunofluorescence staining of isolated ommatidia

Ommatidia from adult flies were dissected as described (Hardie, 1991) except that they were performed in glass bottom culture dishes (MatTek, MA) filled with Schneider's *Drosophila* medium. The dissociated ommatidia were attached to poly-D-lysine coated glass surfaces by placing in an incubator for at least 6 hr at room temperature. The stainings were performed in the same culture dishes. The ommatidia were washed once in PBS, fixed with 3.5% formaldehyde in PBS for 20–30 min, washed 2–3 times in PBS and then treated with 1% Triton X-100 in PBS for 5 min. The samples were then washed 3 times in PBS, blocked with PBSG (0.2% BSA, 1% goat serum, 0.01% saponin) and then incubated with the following primary antibodies in PBSG for 1 hr: mouse anti-Rh1 antibodies (1:500 dilution) and rabbit anti-NORPA antibodies (1:100 dilution). After washing 3–5 times in PBS with 0.01% saponin, the samples were incubated with the following secondary antibodies in PBSG for 1 hr: Alexa Fluor 568 anti-mouse IgG and Alexa Fluor 488 anti-rabbit IgG (Invitrogen). After washing in PBS with 0.01% saponin 3–5 times, the samples were mounted by adding Vectashield (Vector Laboratories, CA) to the cover glasses in the culture dishes. Confocal images were acquired using an UltraView LCI System (Perkin-Elmer).

Phospholipase C activity assay

Phospholipase C activity in fly heads was assayed according to procedures similar to those described (Zhu et. al, 1993; Pearn et. al, 1996). Briefly, we homogenized 20 compound eyes in 50 mM Tris-Cl, pH 7.5, 250 mM KCl, 0.05% (w/v) sodium deoxycholate, 0.1 mM dithiothreitol, and 1x protease inhibitor (Sigma). We combined 10 μl head extracts and 90 μl of a buffer containing 50 mM Tris-HCl, pH 7.5, 1.5 mM $CaCl_2$, 1 mM EDTA, 0.1 mg/ml BSA, 0.2 mM PI (L-α-phosphatidylinositol Na^+ salt, Sigma), 44,000 dpm [^{3}H]PIP_2 (phosphatidylinositol-4,5-biphospate, [Inositol-2-^{3}H(N)], PerkinElmer), which were incubating for 5 min at room temperature. The reactions were stopped by addition of 100 μl 5% trichloroacetic acid followed by 50 μl 10 mg/ml BSA. After 15 min incubation on ice, the unhydrolyzed [^{3}H]PIP_2 was separated from [^{3}H]IP_3 by centrifugation at 5000 g for 4 min. The [^{3}H]IP_3 in the supernatants were assayed using a scintillation counter.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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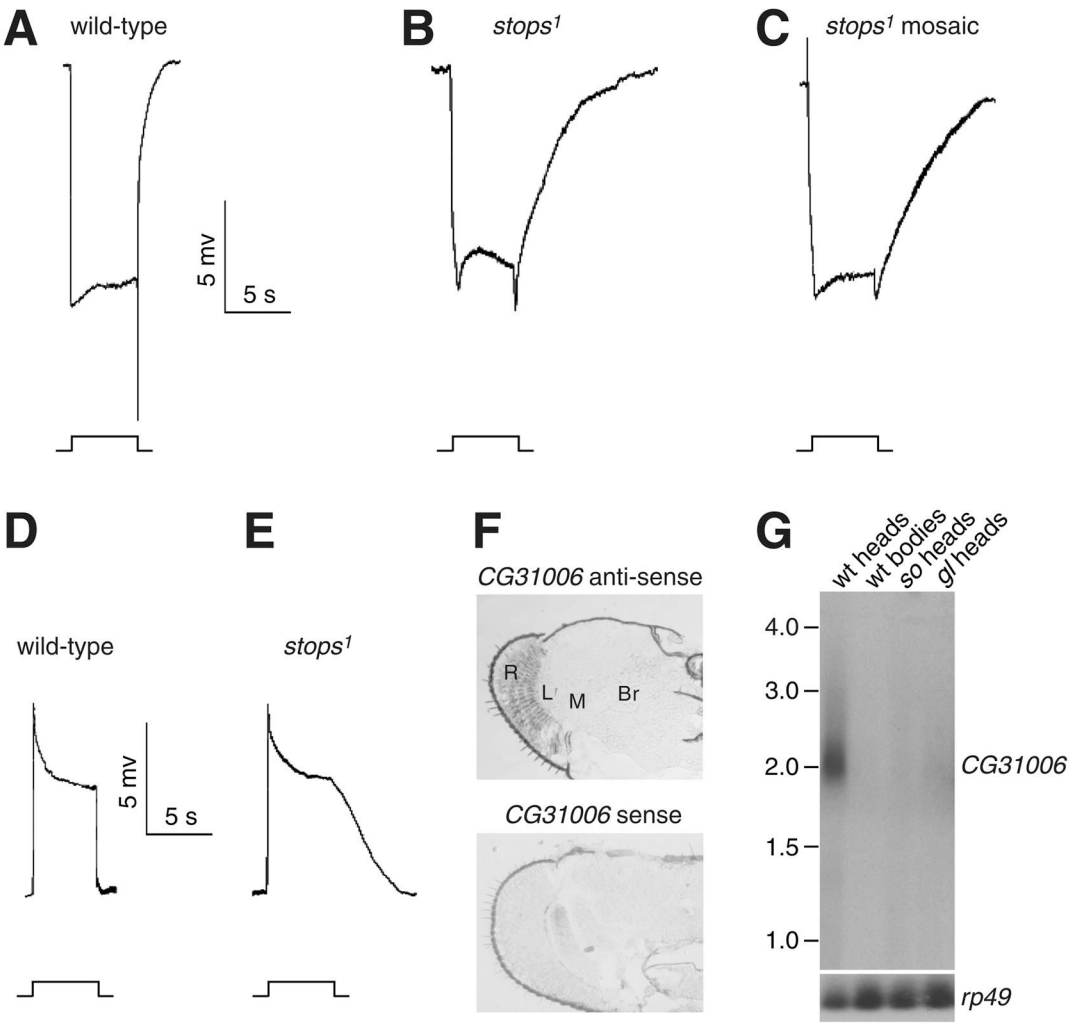

Figure 1.

Slow termination of the visual response in *stops*^{1}. (A–C) Light responses assayed by ERG recordings. Flies ~1 day post-eclosion were dark-adapted for 1 min prior to exposure to 5 sec pulses of orange light indicated by the event markers below the ERGs. The "wild-type" stock used throughout this work was *w*^{1118} and the *stops*^{1} mutant was in a *w*^{1118} background (*w*^{1118};;*stop*^{1}). (A) wild-type. (B) *stops*^{1}. (C) *stops*^{1} mosaic flies (*EGUF;FRT82B GMR-hid/ FRT82B stops*^{1}) with mutant eyes. (D and E) Light responses of photoreceptor cells assayed by intracellular recordings. 1 day-old flies were dark-adapted for 2 min before being exposed to 5 sec pulses of orange light. (D) wild-type. (E) *stops*^{1}. (F) *In situ* hybridizations to 8 μm cryostat sections of adult heads hybridized with *CG31006* anti-sense and sense RNA probes. Br, brain; L, lamina; M, medulla; R, retina. (G) *CG31006* mRNA levels in wild-type heads, bodies, *sine oculis* heads (*so*; flies missing eyes) and *glass* heads (*gl*; missing photoreceptor cells). Total RNAs were isolated from <3 day-old dark-reared flies. 20 μg total RNAs were used in each lane. Single-stranded RNA markers are indicated to the left. The same blot was reprobed with an *rp49* (*ribosomal protein 49*) probe (bottom).

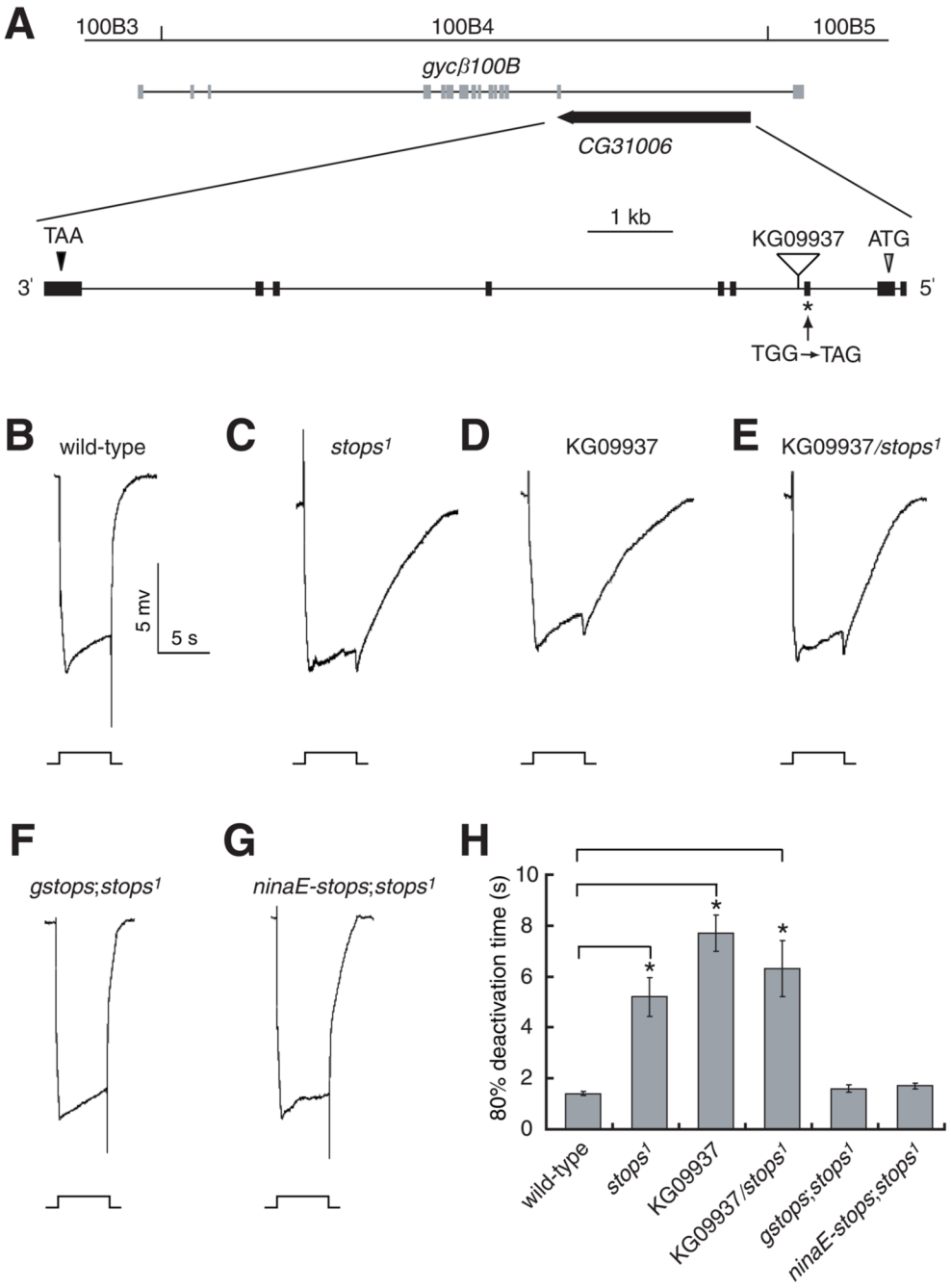

Figure 2.

Identification of the *stops* gene. (A) *stops* (*CG31006*) locus. The *stops* gene was localized to the 100B region using recombination mapping. *CG31006*, which was situated in the intron of the *gycβ100B* gene, was the only eye-enriched gene (Xu et al., 2004) in this region. The insertion site of a P-element (KG09937), which presumably disrupted both *stops* and *gycβ100B* genes, is indicated. The nonsense mutation in *stops*^{1} (TGG to TAG) is indicated. (B–E) ERG recordings showing that *stops*^{1} is not complemented by KG09937. (B) wild-type. (C) *stops*^{1}. (D) KG09937 mosaic flies (*EGUF;FRT82B GMR-hid*/*FRT82B* KG09937) with mutant eyes. (E) KG09937/*stops*^{1}. (F) Slow termination was rescued in *gstops;stops*^{1} flies. (G) Rescue of the slow termination phenotype in *stops*^{1} by expression of a *stops* cDNA transgene

under control of the *ninaE* promoter (*ninaE-stops;stops*^{1}). ERG recordings were performed on flies ~1 day post-eclosion. The flies were exposed to a 5 sec pulse of orange light after a 2 min dark adaptation. (H) Quantification of the time required for 80% recovery after termination of the light stimuli in (B–G). The standard errors of the mean (SEMs) and number of flies examined (n) were as follows: wild-type (1.4 ±0.1, n=12), *stops*^{1} (5.2 ±0.8, n=12), KG09937 (7.7 ±0.7, n=9), KG09937/*stops*^{1} (6.3 ±1.1, n=7), *gstops;stops*^{1} (1.6 ±0.2, n=7), *ninaE-stops;stops*^{1} (1.7 ±0.1, n=6). Asterisks indicate statistically significant differences (Student's unpaired t test; $p < 0.05$) from wild-type.

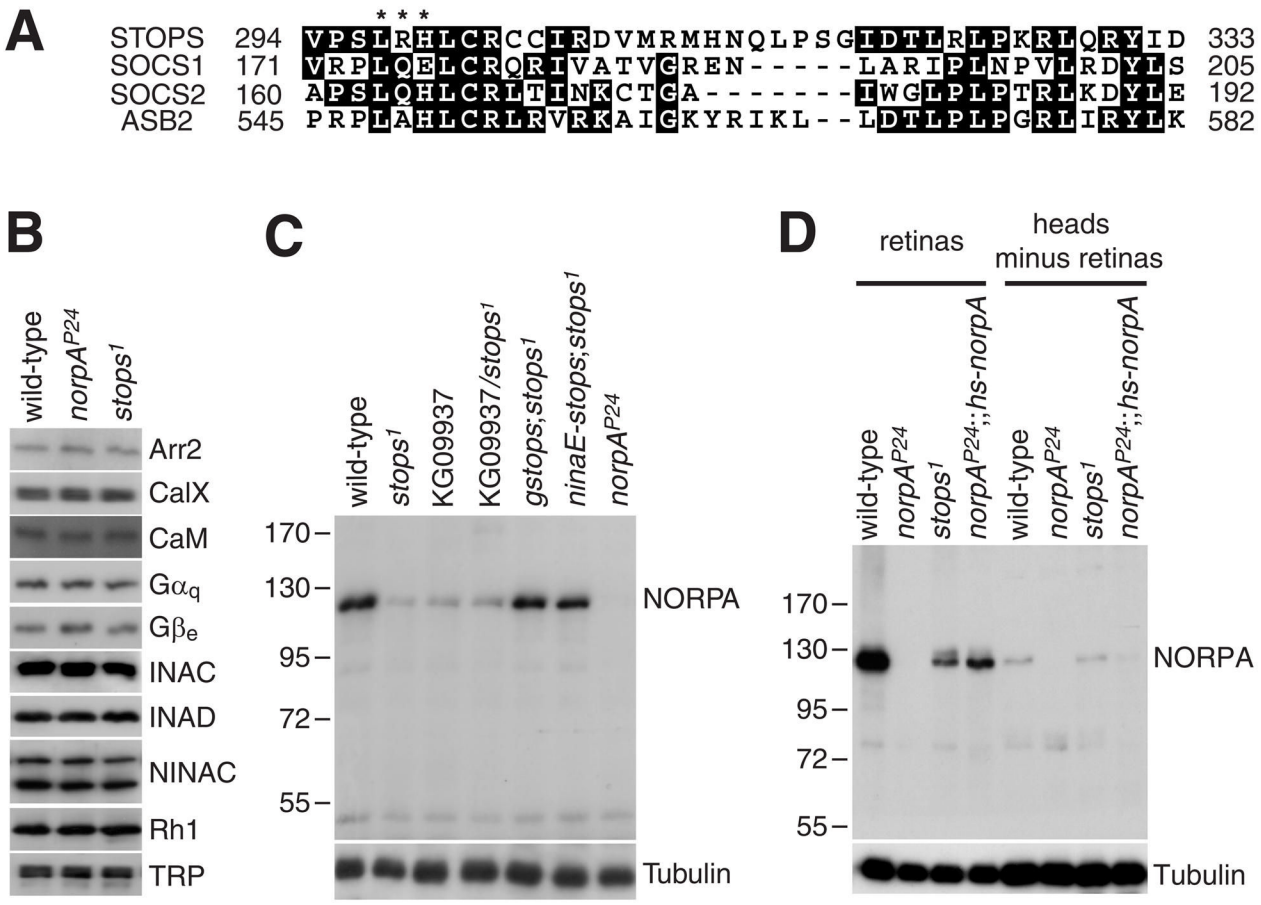

Figure 3.

STOPS contains a SOCS domain and is required for expression of NORPA. (A) Alignment of the SOCS box amino acid sequences from STOPS, human SOCS1, human SOCS2 and human ASB2. The asterisks indicate three amino acids in STOPS mutated in STOPS^{m3}. (B) Western blots showing that expression of most phototransduction proteins were the same in wild-type, *norpA*^{P24} (*w*^{1118},*norpA*^{P24} throughout this study) and *stops*^{1}. Extracts were prepared from fly heads (~1 day post-eclosion), fractionated by SDS-PAGE and the Western blots were probed with antibodies to the indicated proteins. NINAC is expressed as two proteins, p174 and p132 (Montell and Rubin, 1988). (C) Expression of NORPA was reduced in *stops*^{1}. A Western blot containing head extracts prepared from 1 day-old flies of the indicated genotypes was probed with anti-NORPA antibodies and reprobed with anti-Tubulin antibodies. Molecular weight markers (kDa) are indicated to the left. (D) STOPS controls NORPA expression in photoreceptor cells but not in extra-retina tissues. Expression of *hs-norpA* results in expression primarily in the retina. Retinas were dissected from flies ~1 day post-eclosion. Newly enclosed *norpA*^{P24};;*hs-norpA* flies were heat-shocked for 5 hrs at 37 °C, and retinas were dissected one day later. The Western blot was probed with anti-NORPA antibodies and re-probed with anti-Tubulin antibodies.

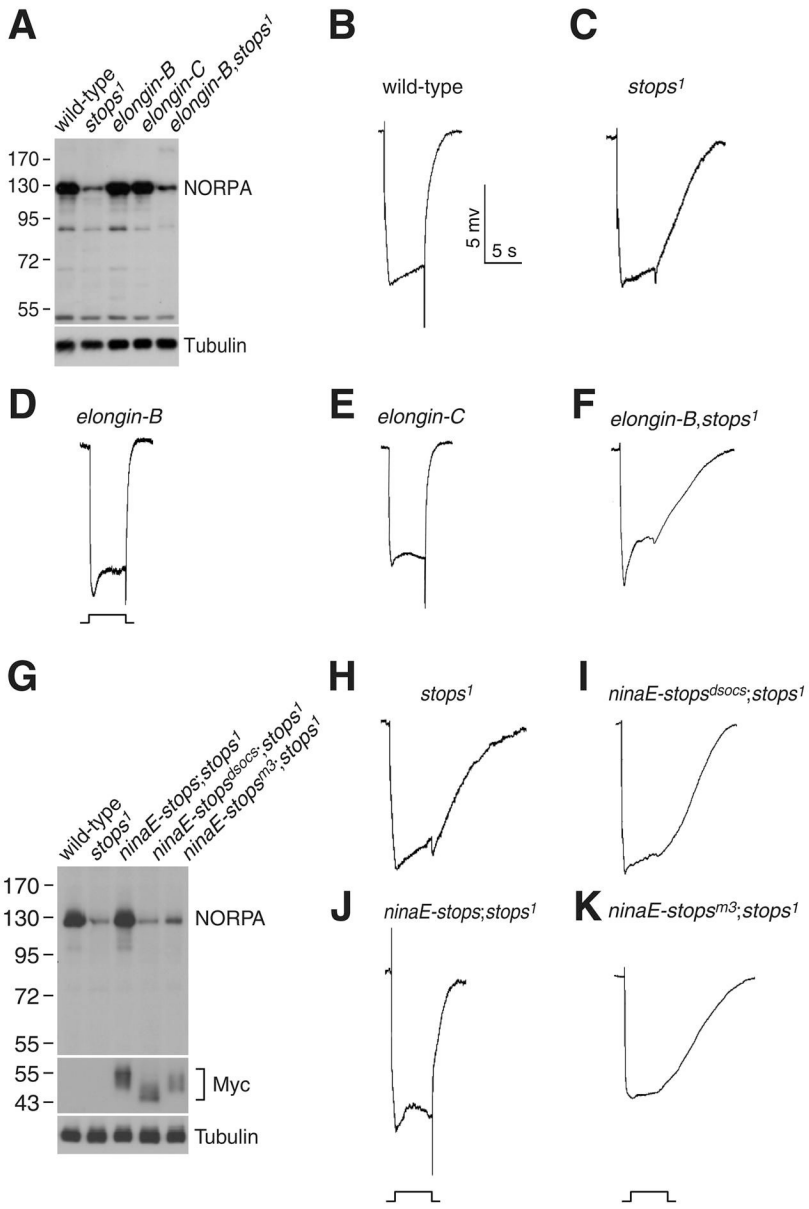

Figure 4.

The SOCS domain is critical for STOPS function independent of the elongin B/C pathway. (A–F) Mutations in *elongin-B* and *elongin-C* do not phenocopy *stops*^{1} or suppress the *stops*^{1} phenotype. (A) Mutations in *elongin-B* and *elongin-C* did not decrease NORPA levels and mutation of *elongin-B* did not restore NORPA levels in *stops*^{1} flies. The Western blot contained head extracts prepared from flies of the indicated genotypes ~1 day post-eclosion. The blot was probed with anti-NORPA and reprobed with anti-Tubulin antibodies. (B–F) ERG recordings. (B) wild-type. (C) *stops*^{1}. (D) *elongin-B* mosaic flies (*EGUF;FRT82B GMR-hid*/*FRT82B elongin-B*). (E) *elongin-C* mosaic flies (*EGUF;FRT42B GMR-hid*/*FRT42B elongin-C*). (F) *elongin-B, stops*^{1} mosaic flies (*EGUF;FRT82B GMR-hid*/*FRT82B elongin-B, stops*^{1}). (G–K) The SOCS domain is required for STOPS function. (G) Normal NORPA levels were restored in *stops*^{1} flies by introducing a Myc-tagged wild-type *stops* gene but not by expression of *stops* genes with mutated SOCS domains. The head extracts used for the Western blot were

prepared from 1 day-old flies. The blot was probed with anti-NORPA and reprobed with anti-Tubulin and anti-Myc antibodies. (H–K) ERG recordings performed on flies ~1 day post-eclosion. (H) *stops*^{1}. (I) *ninaE-stops*^{dsocs};*stops*^{1}. (J) *ninaE-stops;stops*$^{1.}$ (K) *ninaE-stops*^{m3};*stops*^{1}.

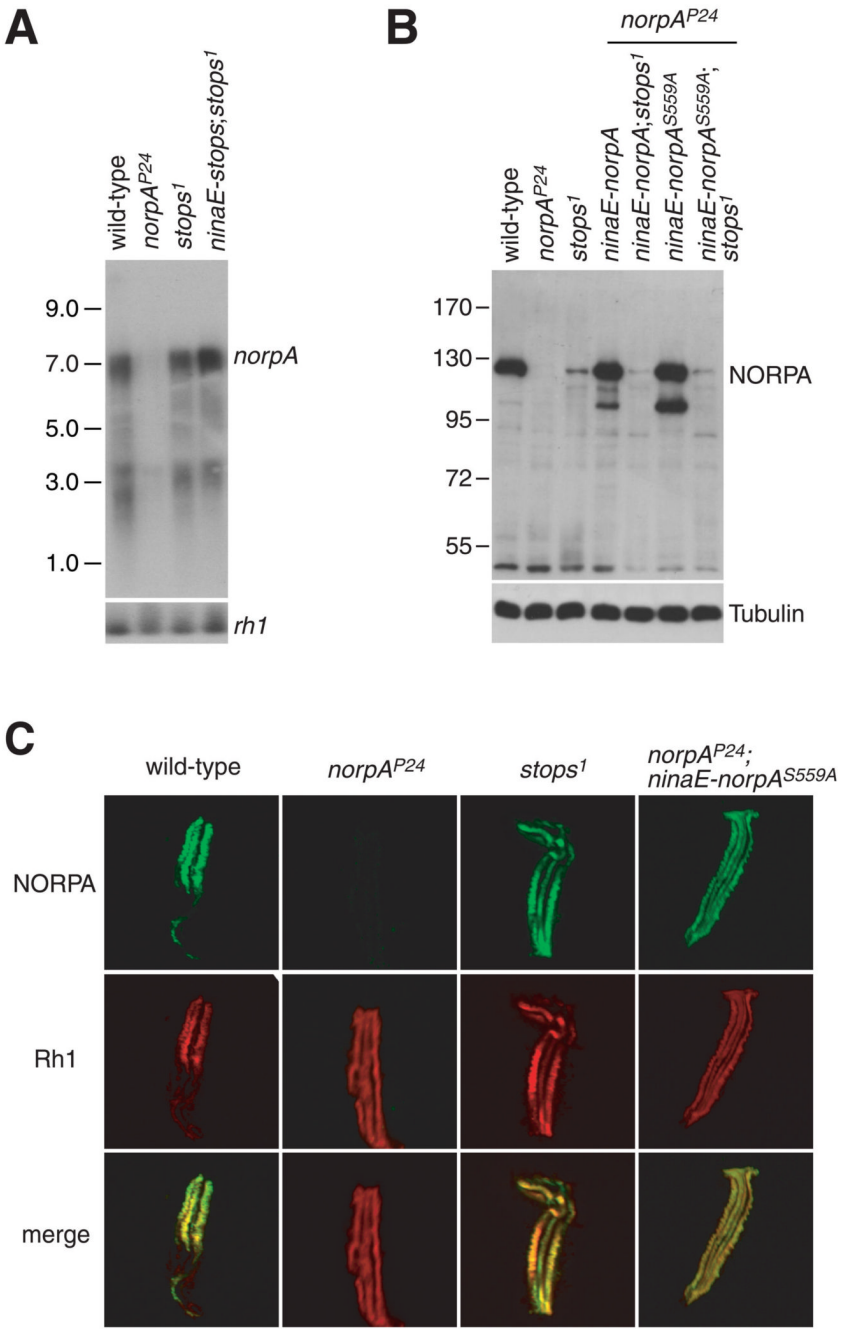

Figure 5.

STOPS controls NORPA expression post-transcriptionally. (A) *norpA* mRNA expression was not affected in *stops*^{1}. The Northern blot contained samples (~10 μg total RNA each) isolated from the heads of the indicated dark-reared flies (<3 days post-eclosion). The blot was probed with a *norpA* DNA probe and reprobed with an *rh1* probe (bottom). Single-stranded RNA markers are indicated to the left. (B) Reduction of NORPA protein concentration in *stops*^{1} flies was not restored by expression of *norpA* under control of the *ninaE* promoter. The head extracts used for the Western blot were prepared from the following fly stocks (~1 day post-eclosion): 1) wild-type, 2) *norpA*^{P24}, 3) *stops*^{1}, 4) *norpA*^{P24};*ninaE-norpA*, 5) *norpA*^{P24};*ninaE-norpA;stops*^{1}, 6) *norpA*^{P24};*ninaE-norpA*^{S559A}, and 7) *norpA*^{P24};*ninaE-norpA*^{S559A};*stops*^{1}. The

blot was probed with anti-NORPA and reprobed with anti-Tubulin antibodies. (C) NORPA localization was not disrupted in the *stops*^{1} flies. Ommatidia were dissected from wild-type, *norpA*^{P24}, *stops*^{1} and *norpA*^{P24};*ninaE-norpA*^{S559A} flies, and stained with rabbit anti-NORPA (green) antibodies and mouse anti-Rh1 (red) antibodies.

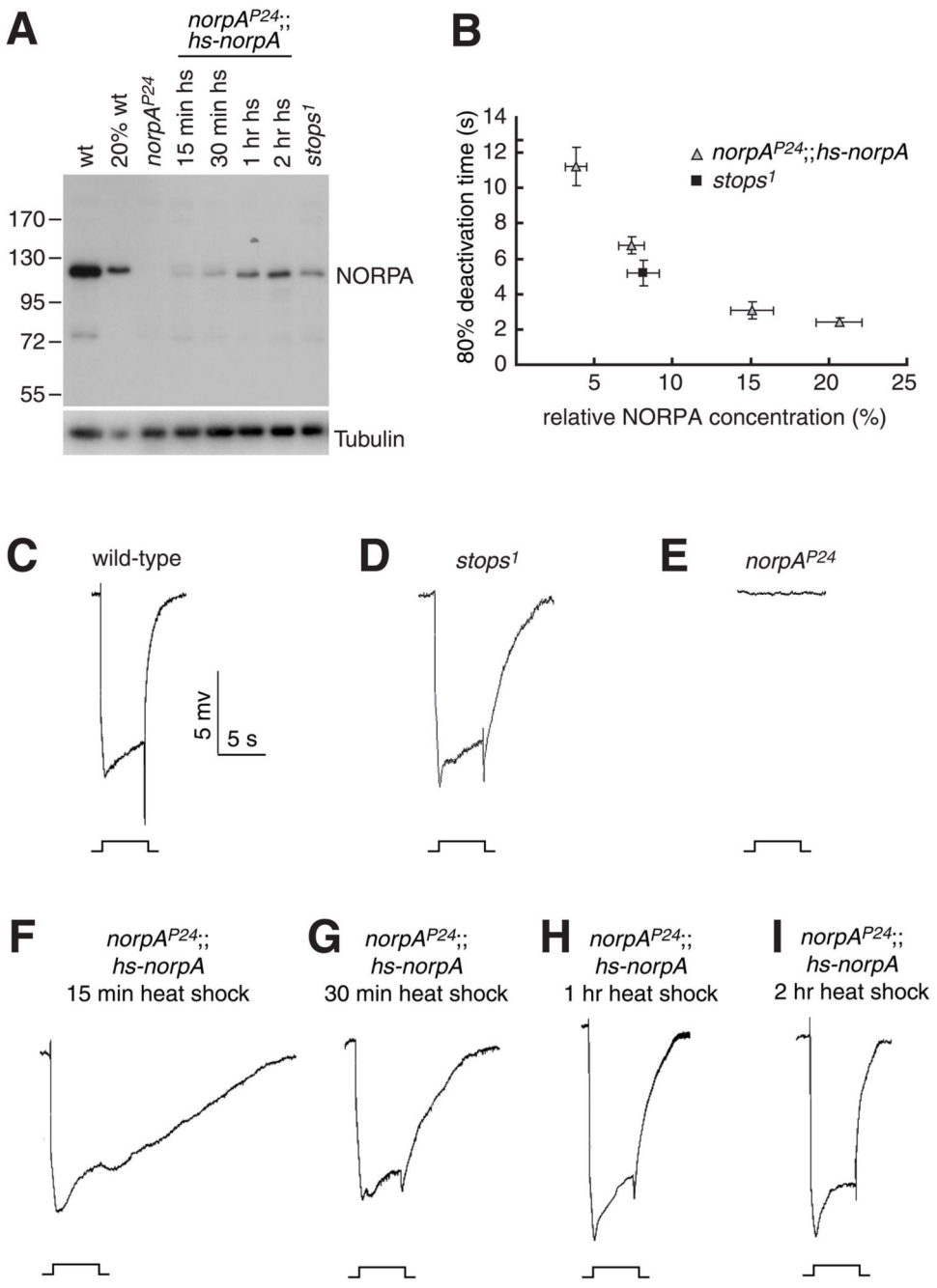

Figure 6.

Reduced NORPA expression underlies the slow termination phenotype. (A) Relative levels of NORPA expressed in *norpA*^{P24};;*hs-norpA* flies exposed to heat-shock treatments of different durations. The flies were heat-shocked for either 15 min, 30 min, 1 hr or 2 hrs at 37 °C immediately post-eclosion, and the head extracts for the Western blot was prepared one day later. The blot was probed with anti-NORPA antibodies and reprobed with anti-Tubulin antibodies. (B) Termination times plotted against the relative levels of NORPA. The times necessary for an 80% return to the baseline after cessation of the light stimuli were determined by performing ERGs. The quantification of NORPA protein levels was performed using a phosphoimager. The error bars represent the SEMs. (C–E) ERG recordings. (C) wild-type. (D)

stops^{1}. (E) *norpA*^{P24}. (F–I) ERG recordings obtained in *norpA*^{P24};;*hs-norpA* flies exposed to heat-shock (hs) treatments. Flies were heat shocked for either 15 min, 30 min, 1 hr or 2 hrs at 37 °C immediately post-eclosion and the ERGs were performed 1 day later.

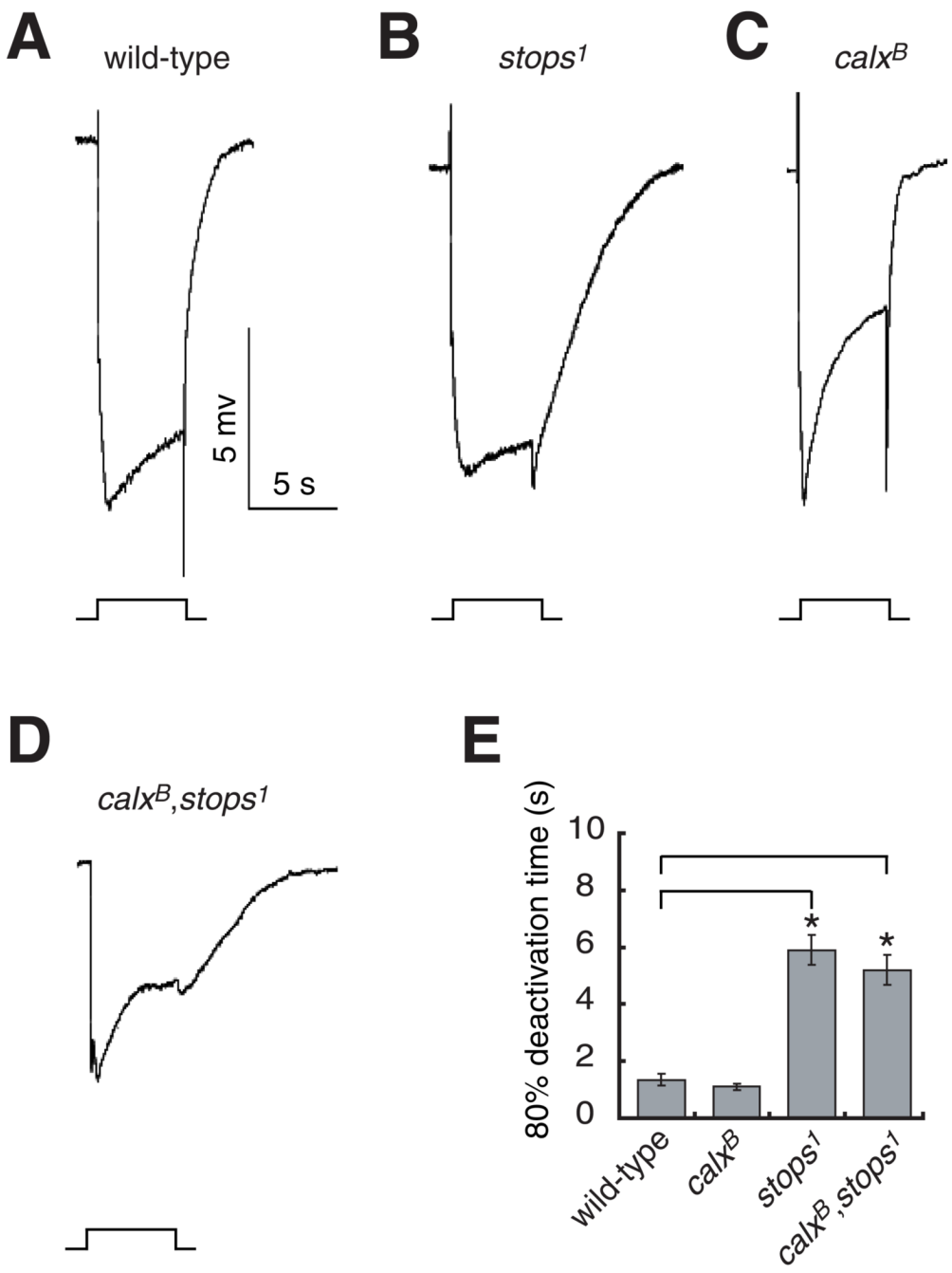

Figure 7.

Slow termination caused by reduced amounts of NORPA is not suppressed by mutations in *calx*. (A-D) ERG recordings. (A) wild-type. (B) *stops*^{1}. (C) *calx*^{B}. (D) *calx*^{B}, *stops*^{1}. The flies (~1 day old) were dark-adapted for 2 min before they were exposed to a 5 sec pulse of orange light. (E) Average times required for 80% recovery after termination of the light stimuli (±SEMs): wild-type (1.4 ±0.2, n=6), *calx*^{B} (1.1 ±0.1, n=6), *stops*^{1} (5.9 ±0.7, n=9), *calx*^{B},*stops*^{1} (5.3 ±0.5, n=6). Asterisks indicate statistically significant differences relative to wild-type (Student's unpaired t test; $p < 0.05$).

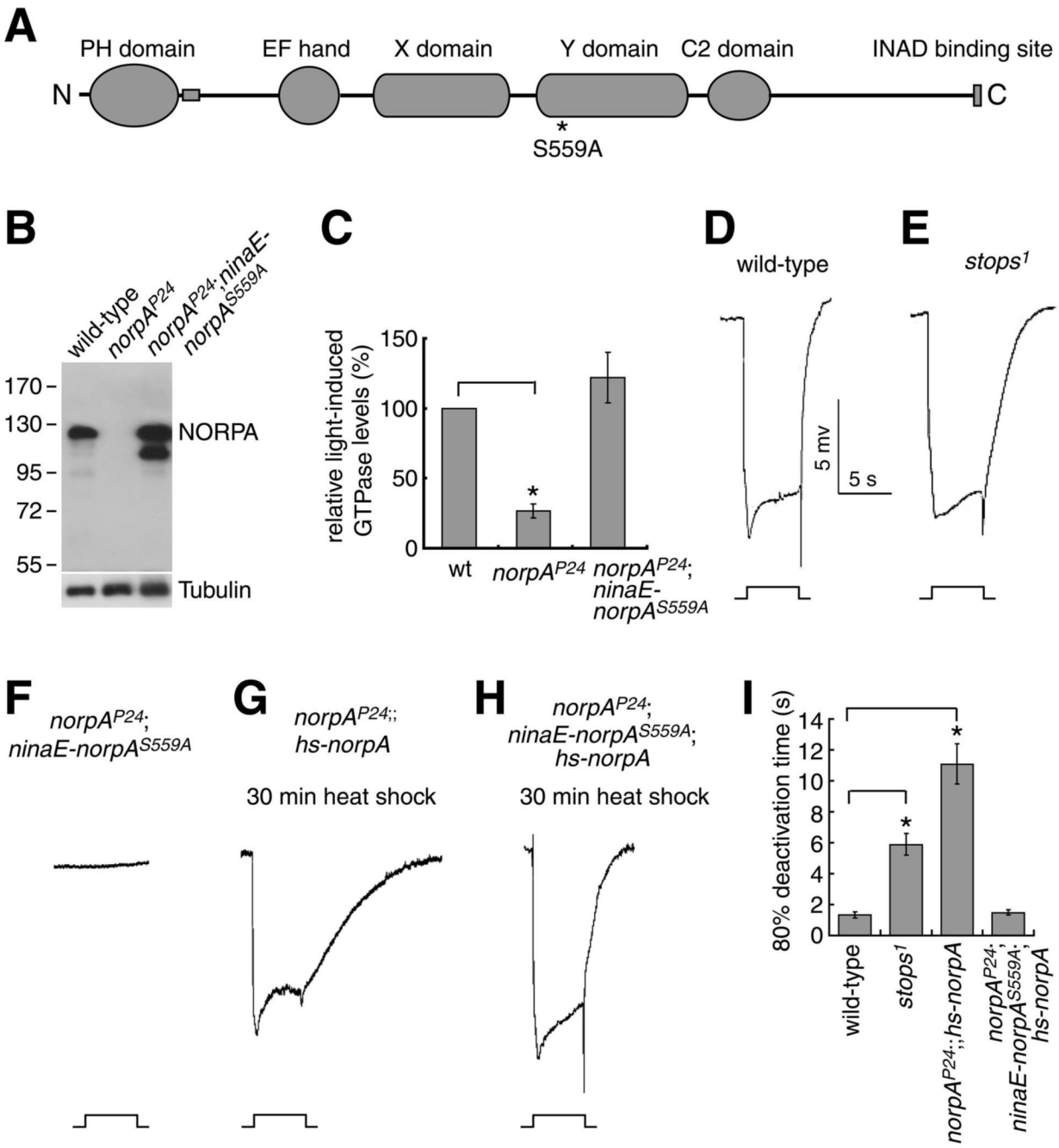

Figure 8.

Slow termination of the light response caused by reduced NORPA GAP activity. (A) Domain organization of NORPA. Indicated are a possible membrane binding PH domain, an EF-hand, a split phospholipase catalytic region (X and Y core domains), a putative $G\alpha_q$ binding C2 domain and the INAD binding site. The small oval adjacent to the C-terminal end of the PH domain represents a region containing 14 amino acid differences between NORPA subtypes I and II. The asterisk indicates a point mutation (S559A) in the Y domain that eliminates the ERG response. (B) Expression of $NORPA^{S559A}$. The Western blot containing extracts prepared from wild-type, $norpA^{P24}$ and $norpA^{P24}$;$ninaE\text{-}norpA^{S559A}$ fly heads (~1 day post-eclosion) was probed with anti-NORPA antibodies and reprobed with anti-Tubulin antibodies. (C)

Normal light-induced GTPase activity in *norpA*^{P24};*ninaE-norpA*^{S559A} flies. The GTPase assays were performed using head extracts prepared from dark-raised wild-type, *norpA*^{P24} and *norpA*^{P24};*ninaE-norpA*^{S559A} flies (< 3 days post-eclosion). The asterisk indicates a statistically significant difference between *norpA*^{P24} and wild-type (Student's unpaired t test; $p < 0.05$; n=3). (D–H) ERG recordings. The flies (~1 day post-eclosion) were dark-adapted for 2 min before being exposed to a 5 sec pulse of orange light. (D) wild-type. (E) *stops*^{1}. (F) *norpA*^{P24};*ninaE-norpA*^{S559A}. (G) *norpA*^{P24};;*hs-norpA*/+. (H) *norpA*^{P24};*ninaE-norpA*^{S559A}/+;*hs-norpA*/+. (I) Quantification of time required for 80% recovery after termination of the light stimuli (±SEMs): 1) wild-type (1.4 ±0.2, n=6), 2) *stops*^{1} (5.9 ±0.7, n=9), 3) *norpA*^{P24};;*hs-norpA* (11.1 ±1.3, n=6), and 4) *norpA*^{P24};*ninaE-norpA*^{S559A};*hs-norpA* (1.5 ±0.2, n=7). The asterisks indicate statistically significant differences compared to wild-type (Student's unpaired t test; $p < 0.05$).