Figure S1. **Functional characterization of Pld.** (A) The generation of phosphatidyl butanol (P-butanol) by PLD (via the enzyme's transphosphatidylation activity) was used to measure the enzyme's activity. Thin layer chromatography showed that the formation of phosphatidyl butanol was seen in extracts of cells expressing PLD (A; after induction in S2 cells [+]) and after transfection [+ in COS-7 cells] but not in control cells (− in both cell lines). Monoacylglycerol (MAG), phosphatidylethanolamine (PE), PA, PC, and PI were also seen. (B) The formation of the phosphatidyl butanol product required the presence of both PLD protein and 1% butanol. The formation of the phosphatidyl butanol product was not enhanced by the addition of 200 nM PMA. (C) Finally, the formation of the phosphatidyl butanol product required the presence of overexpressed catalytically active wild-type PLD. No product was seen with the Pld carrying the K/R mutation, demonstrating that in Pld, this mutation renders the enzyme catalytically dead. In all assays, mammalian PLD1 was used as a positive control. (D) Pld was inducibly expressed in Drosophila S2 as well as COS-7 cells, and the protein was detected using the anti-PLD antibody described in this study. Both wild-type and the catalytically dead K/R protein were expressed at equivalent levels. (E) We tested the requirement for Arf in activating Drosophila PLD. Arf1 and Arf3 were knocked down together using RNAi in Drosophila S2 cells overexpressing Drosophila PLD. Knockdown was achieved using double-stranded RNA. Knockdown using GFP oligos was used as a control. After 72 h of treatment, the formation of phosphatidyl butanol was assayed as described previously (Manifava, M., J. Sugars, and N.T. Ktistakis. 1999. *J. Biol. Chem.* 274:1072–1077). (D and E) Unit of measure, M.
Figure S2. Molecular analysis of early secretory transport. (A) Western blots of head extracts from cds' and Pld overexpression. Protein extracts were prepared from dissected retinae of the indicated genotypes. Blots were probed with an antibody to GFP to detect the protein disulfide isomerase [PDI::GFP] fusion protein incubated with an HRP-linked secondary antibody and developed with ECL. Levels of a loading control band are shown. The PDI::GFP signal was equivalent in retinae that had elevated PA levels (cds'; PDI::GFP and +PLD;PDI::GFP) compared with controls (PDI-GFP, cds'/+, PDI::GFP, and GMR-GAL4;PDI::GFP). Wild-type flies (ROR) are shown as a negative control for the GFP antibody. (B) Analysis of CD8 glycosylation in wild-type retinae. Expression of GFP-tagged CD8 (CD8::GFP) was initiated by heat shock induction (60 min at 37°C) of heat shock GAL4* UAS-CD8::GFP flies. Protein samples were prepared from head extracts of animals 1 and 2 h after the end of the heat shock. After 1 h, a single band of newly synthesized CD8::GFP protein can be detected. After 2 h, a band of higher Mr is also seen, representing the glycosylated form of this protein. (C) Analysis of CD8 glycosylation in photoreceptors overexpressing Pld. In both control (CD8::GFP) and experimental (PLD;CD8::GFP) flies, the development of the upper band representing glycosylated CD8::GFP is equivalent. (D) Western blots of head extracts to detect Rh1 protein. Rh1 is a glycosylated protein, and a monoclonal antibody to Rh1 detects multiple bands, representing the mobility of various glycosylation intermediates. The levels of Rh1 and the maturation of these glycosylation intermediates are lost in the ninaA mutant. ninaA encodes an Rh1-specific peptidylprolyl cis-trans isomerase, an ER resident chaperone for this protein. Samples are shown from ninaA', a temperature-sensitive allele at the permissive temperature (19°C) and restrictive temperature (29°C). We have used this mutant to demonstrate a setting in which Rh1 is retained in the ER and does not undergo glycosylation because it does not arrive at the Golgi. Syntaxin is used as a loading control. (E) In cds' and Pld overexpression genotypes, although the levels of Rh1 are reduced, its mobility relative to controls is unaffected. Syntaxin is used as a loading control. (A, C, and E) Unit of measure, Mr, glycosylated; NG, non-glycosylated; WT, wild type.
Figure S3. **Analysis of sec mutants.** (A–C) TEM analysis of photoreceptors from sec152 and sec6 hypomorphs wild type (A), sec152 (B), and sec6pr (C). Rhabdomeres (r) and vesicles (v) are marked. Bar, 2 µm. Rhabdomere size is decreased in both sec152 and sec6pr. (D) Western blot analysis of Rh1 levels in sec152 and sec6pr hypomorphs. Levels of syntaxin are shown as a control. Unit of measure, M. WT, wild type.
Figure S4. **Generation and characterization of the Arf6 mutant.** Arf51F (CG8156) was identified as the Drosophila orthologue of human Arf6 with 97% amino acid identity. A P-element insertion, P[EP]2612, was identified in the 5' region of Drosophila Arf6 in the first intron of the 5' untranslated region of several annotated Arf6 transcripts (Arf6 RA, RB, RC, and RD) and upstream of the predicted start site of a shorter transcript (Arf6 RE). Deletions flanking this insertion were generated by transposase-mediated mutagenesis. One deletion removed most of the insertion and the genomic sequence between the insertion site and the first intron in the coding sequence (nucleotides 11,210,283–11,211,224 inclusive on chromosome arm 2R, genome sequence release 5.8; red horizontal line shown on genome browser image generated on www.flybase.org). This deletion lacked the first coding intron, including the start codon and the rest of the first 28 highly conserved codons.
Figure S5. **Subcellular localization of PLD and *Drosophila* Arf1-GAP.** Subcellular localization of PLD when overexpressed was detected using a rabbit polyclonal antibody to a fragment of the *Drosophila* Pld gene (see Materials and methods). (A) PLD appears to be localized in a compartment within the cell body, which is located at the base of the rhabdomere. Rhabdomeres are marked using a monoclonal antibody to Rh1, whose localization is restricted to the rhabdomere (B). (C) An analysis of the overlay shows that there is no significant overlap of the expression of PLD and Rh1, showing that PLD is not localized to the rhabdomere. Immunofluorescence analysis of retinae from wild-type flies overexpressing *Drosophila* Arf1-GAP is shown. (D) *Drosophila* Arf1-GAP expression in the cell body was detected using a monoclonal antibody to HA. E shows Rh1 detection using a rabbit polyclonal to this protein, and F displays an overlay of both, showing no overlapping staining for these two proteins. Bars, 5 µm.