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Functional Interactions between the erupted/tsg101 Growth Suppressor Gene and the DaPKC and rbf1 Genes in Drosophila Imaginal Disc Tumors

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Abstract

Background: The Drosophila gene erupted (ept) encodes the fly homolog of human Tumor Susceptibility Gene-101 (TSG101), which functions as part of the conserved ESCRT-1 complex to facilitate the movement of cargoes through the endolysosomal pathway. Loss of ept or other genes that encode components of the endocytic machinery (e.g. syntaxin7/avalanche, rab5, and vps25) produces disorganized overgrowth of imaginal disc tissue. Excess cell division is postulated to be a primary cause of these ‘neoplastic’ phenotypes, but the autonomous effect of these mutations on cell cycle control has not been examined.

Principal Findings: Here we show that disc cells lacking ept function display an altered cell cycle profile indicative of deregulated progression through the G1-to-S phase transition and express reduced levels of the tumor suppressor ortholog and G1/S inhibitor Rbf1. Genetic reductions of the Drosophila aPKC kinase (DaPKC), which has been shown to promote tumor growth in other fly tumor models, prevent both the ept neoplastic phenotype and the reduction in Rbf1 levels that otherwise occurs in clones of ept mutant cells; this effect is coincident with changes in localization of Notch and Crumbs, two proteins whose sorting is altered in ept mutant cells. The effect on Rbf1 can also be blocked by removal of the γ-secretase component presenilin, suggesting that cleavage of a γ-secretase target influences Rbf1 levels in ept mutant cells. Expression of exogenous rbf1 completely ablates ept mutant eye tissues but only mildly affects the development of discs composed of cells with wild type ept.

Conclusions: Together, these data show that loss of ept alters nuclear cell cycle control in developing imaginal discs and identify the DaPKC, presenilin, and rbf1 genes as modifiers of molecular and cellular phenotypes that result from loss of ept.

Introduction

Genetic screens in Drosophila have identified a relatively small group of mutations that disrupt normal epithelial architecture and lead to neoplastic overgrowth of developing larval imaginal discs, a set of polarized epithelial tissues that grow during larval stages and develop into the majority of adult structures [reviewed in 1]. The genes affected by these mutations encode proteins with conserved human homologs and fall generally into two functional classes: those involved in the establishment and maintenance of apicobasal polarity [reviewed in 2], and those involved in vesicular trafficking of transmembrane proteins [3–8]. Genes in this latter group have been termed ‘endocytic tumor suppressor genes’ and include rab5, syntaxin-7/avalanche (syx7/avl), erupted/tumor susceptibility gene-101 (ept/tsg101) and referred to hereafter as ept, and vps25. Each of these genes is required at distinct steps in the trafficking proteins from the apical membrane to the lysosome. The latter two genes, ept and vps25, respectively encode components of the ESCRT [endosomal sorting complex required for transport]-I and ESCRT-II complexes which promote maturation of late-endosomes into multi-vesicular bodies (MVBs) prior to subsequent fusion with the lysosome [9,10].

Though it is assumed that mutations in these vesicular trafficking factors promote tissue growth in part by removing developmental blocks to excess cell division, there is little direct evidence that links ept, vps25, or syx7/avl mutations to specific cell cycle transitions or to core components of the nuclear cell cycle machinery. Mutations in ept are known to block the trafficking and degradation of certain apically localized trans-membrane proteins, including the apical membrane determinant Crumbs and the transmembrane receptor Notch [3,5,6,8], but the effects these molecules have on the cell division process in ept mutant cells is not known. Notch has many context-specific links to the cell cycle including controlling levels of the mitotic regulator Cyclin A [11], activity of the dE2f1 transcription factor [11,12], and expression of the daxapo, string, and fizzy related genes in ovarian follicle cells [13,14]. Notch has also been reported to collaborate with chromatin modifying factors to silence expression of the rbf1 gene in eye imaginal disc tumors [15]. Thus, there are many pathways through which Notch could potentially affect either the G2/M or
G1/S cell cycle transitions in ept mutant cells. The ability of cbh overexpression to drive imaginal disc neoplasia [4] argues that cbh can also directly or indirectly affect the cell division process. Yet the potential links between CDr-an integral membrane scaffolding molecule with no known intrinsic signaling activity – and the cell division process are not well understood. cbh indirectly regulates Notch in the larval wing by modulating activity of the γ-secretase complex [16]. However, since mechanisms that deregulate cell division in endocytic tumor suppressor mutants are poorly understood, it is difficult to discern specific pathways through which Notch, CDr, and the myriad of other receptors that are candidate targets of the ESCRT pathway (for example those shown to be affected by loss of the hsm gene [17]) might exert pro-proliferative effects in these mutant backgrounds.

We have taken a dual approach to examine cell division control in ept mutant eye-antennal tumors: we have sought to identify genetic manipulations that suppress ept tumor growth, and in parallel we have characterized the effect of ept loss on cell cycle phasing and expression of core cell cycle regulatory factors. We have found that genetic reduction of the DaPKC apical-membrane kinase effectively suppress the growth of ept mutant eye-antennal tumors. In parallel, we have found that ept mutant eye and wing imaginal discs are enriched for cells in the G2/M phase and depleted for those G1 phase, and this correlates with reduced expression of the nuclear S-phase inhibitor and tumor suppressor homolog Rbf1. These two phenotypes are linked by the observation that expression of a dominant-negative DaPKC transgene (DN-DaPKC) in ept eye cells is sufficient to prevent the reduction in Rbf1 levels. It is also sufficient to prevent high-level expression of the Upd protein, which is induced in a Notch-dependent manner in ept2 mutant cells [3]. A similar rescue of Rbf1 levels is observed following removal of the presenilin gene (psn) from ept mutant clones, indicating that cleavage of a Psn substrate(s) contributes to the effect of the ept genotype on Rbf1 levels. To test the physiologic significance of the Rbf1 reduction in ept cells, we have re-expressed exogenous rbf1 in either wild type or ept mutant eye-antennal tissue and found that excess rbf1 is able to completely ablate mutant tissue while having little effect on normal tissue. These data indicate that DaPKC- and psn-dependent loss of Rbf1 from ept cells may be a significant factor in their overgrowth.

**Results**

**DaPKC is required for ept tumor growth**

Under normal circumstances, ept mutant eye-antennal tumors created using the cell-lethal Minute (M) technique (the genotype cyFLP;epiF,RFT30B/P[m-c Armenian];Rpl14,FRT30B) is hereafter referred to as ept/M(3) grow into large unstructured masses (Fig. 1A) that fail to differentiate into recognizable eye tissue and kill the animal bearing them during the late larval and pupal phases [6]. To test the genetic requirements of this tumor-like phenotype, we screened a small collection of alleles of signaling, polarity, and growth regulatory genes (stat92E, cbh, lgI, Drosophila aPKC, yki, cyclinD, dMyc, d6k and others) for their ability to suppress size and/or architectural phenotypes associated with loss of ept/psn101. Alleles of two of these genes had significant effects on the morphology of ept/M(3) tumors: the DaPKC gene, which encodes an apical-membrane kinase that controls epithelial polarity and endocytosis [18–21], and the stat92E gene, which encodes the soluble homolog of the Stat family of mammalian transcription factors (Fig. 2B). The expression of DaPKC transgene alone (Ept/M(3)) mutant discs caused these tissues to develop as enlarged eye/antennal structures (Fig. 1B) that are morphologically similar to normal eye discs (Fig. 1C) and contain differentiated photoreceptor neurons (data not shown). Most animals bearing these DN-DaPKC/ept/M(3) discs survive to late-pupal and pharate adult stages well beyond the point where animals bearing ept/M(3) tumors normally die; a few survive to eclosion and emerge with irregular heads and eyes (Fig. 1F) that are enlarged relative to those expressing the DN-DaPKC transgene alone (Fig. 1G). To confirm that this genetic interaction is not an artifact of the DN-DaPKC transgene, ept/M(3) tumors were also generated in a background heterogeneous for the genomic DaPKC loss-of-function allele [25]. This DaPKC D046010+/+ genotype also shrank the size of ept/M(3) tumors (Figs. 1D,E), confirming that DaPKC is required for the ept tumor phenotype.

**ept mutant cells exhibit G1/S cell cycle deregulation**

Because excess cell proliferation is a key factor in tissue hyperplasia, we examined the effect of ept loss on the cell cycling properties of cells in eye-antennal discs and whether DaPKC might influence this effect. Fluorescence-activated cell sorting (FACS) analysis of ept/M(3) eye-antennal discs shows that the population of cells within them is under-represented for 2N G1-phase cells and enriched for S- and G2/M-phase cells relative to control FRT/M(3) discs (Fig. 2A). Cell size is also increased in ept/M(3) tumors relative to control cells (Fig. 2A, see inset), ept/M(3) eye-antennal discs show widespread BrdU incorporation relative to control discs (Figs. 2C–D) and lack cells that express the neuronal marker Elav (Figs. 2E). FACS analysis of ept mutant wing disc tumors [genotype UbxFlp;M(3),FRT30B/epiF,RFT30B] show similar cell cycle and cell size shifts as ept/M(3) eye-antennal disc cells (Fig. 2B), although the G2/M-shift is less pronounced in the wing. Thus cell cycling changes associated with loss of ept function are not solely due to a block in progression of the eye-specific morphogenetic furrow [26], indicating that loss of ept affects G1/S progression in multiple larval discs.

Expression of DN-DaPKC in the background of ept/M(3) eye-antennal discs led to a reproducible shift of a fraction of cells back into the G1-phase (dotted line, Fig. 2C). The cell cycle shift induced by the DN-DaPKC transgene partially restored cell cycle phasing and had no discernable effect on the enlarged size of ept cells, suggesting that additional factors contribute to each of these phenotypes. Consistent with this hypothesis, a reduction in the dose of the stat92E gene affects both cell cycle phasing and cell size in ept/M(3) tumors (see accompanying paper by Gilbert et al.).

We next examined the levels of G1/S regulatory proteins in clones of eye disc cells doubly mutant for ept and the H99 chromosomal deletion, which removes the genes bup, gin, and hid [27] and prevents activation of the pro-death caspase enzymes [28–33] that are otherwise detected at high levels in ept mutant clones of cells (Figure S1); their removal thus allows recovery of larger ept mutant clones and permits molecular analysis of protein epitopes that might otherwise be degraded. The expression of two key regulators of G1/S, the pro-divison protein Cyclin E (CycE) and Rbf1, the Drosophila homolog of the retinoblastoma (Rb) tumor suppressor protein [34], were found to be affected by the ept genotype. Compared to H99 control clones (Fig. 3A–A’), some ept/H99 mutant clones express elevated levels of CycE protein (Fig. 3B–B’, see arrows). CycE is also slightly elevated in some normal cells that surround mutant clones, which is likely a reflection of the previously described non-autonomous mitogenic effect of ept mutant cells [6]. Neither of these effects on CycE are not apparent in all clones, and are thus not a strongly penetrant part of the ept/H99 phenotype. By contrast, immunostaining with a monoclonal antibody specific to Rbf1 [34] shows that levels of this
protein are clearly reduced in ept,H99 disc cells compared to surrounding control cells (Fig. 3C–C0). Clones of H99 mutant cells do not show the same effect (Figure S2), indicating that ept is required to maintain normal levels of Rbf1 protein in eye-antennal disc cells.

**DaPKC and psn are required for the effect on Rbf1 levels in ept mutant cells**

Given the effect of the DaPKC alleles on ept/M(3) tumor growth, we next examined whether DaPKC activity might affect Rbf1 levels in ept mutant cells. To do this, the DN-DaPKC transgene was expressed specifically in ept,H99 mutant cells using the MARCM technique [35]. Although this led to a significant reduction in the size of ept,H99 clones (paralleling the effect of the DN-DaPKC transgene on ept tumor size), close examination of DN-DaPKC+ept,H99 clones stained with the anti-Rbf1 antibody revealed no obvious difference in Rbf1 levels relative to surrounding normal cells (Fig 3D–D0). By this measure, DaPKC activity is required for the effect of the ept,H99 genotype on Rbf1 levels. Because of the somewhat variable effect of the ept,H99 genotype on tumor size, we used the MARCM technique to express DN-DaPKC specifically in ept/H99 mutant cells. This led to a significant reduction in the size of ept,H99 clones (Fig 3D–D0), indicating that DaPKC activity is required for the effect of ept,H99 on tumor growth.

**Figure 1. DaPKC is required for ept tumor growth.** Bright-field images of ept/M(3) mutant (A), ept/M(3) mutant expressing DN-DaPKC (B), or FRT80B/M(3) (C) eye-antennal discs dissected from wandering 3rd instar larvae. (D,E) Side-by-side image of an ept/M(3) mutant eye antennal disc and an ept/M(3) disc that is heterozygous for the DaPKC06403 allele. (F,G) Heads from surviving ept/M(3)+DN-DaPKC adults or DN-aPKC control animals. Grouped images are to scale.

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genotype on CycE, CycE levels were not examined in this MARCM DN-DaPKC background. DaPKC is known to regulate a number of cellular processes and pathways ([18] and reviewed in [19]), including the Notch pathway in larval brain neuroblasts [36]. To test whether an allele of a Notch pathway component might also alter the effect of the ept,H99 genotype on Rbf1, a strong loss-of-function allele of the presenilin gene (psn227;[37]) was recombined onto the ept,H99 chromosome. psn encodes a required component of the γ-secretase that cleaves and releases the Notch intracellular domain and its activity is needed for Notch activation in vivo [38,39]. Because all three loci are on the left arm of chromosome 3, this ept,H99,psn mutant chromosome allows for the production of somatic clones of triple mutant cells. These cells are deficient in ept function and Psn activity, but give rise to easily detectable clones due to their inability to die. Immunostaining with the anti-Rbf1 antibody indicates that the level of Rbf1 in these ept,H99,psn cells (arrows in Fig. 3E–E0) is similar to that in surrounding normal cells. Thus, loss of psn has a similar effect on levels of the anti-Rbf1 epitope in the ept,H99 genotype as does expression of the DN-DaPKC allele.
Figure 3. ept mutations reduce levels of the Drosophila Rb ortholog Rbf1. Confocal images of larval eye discs containing clones of H99 mutant cells (A–A’), ept,H99 double-mutant cells (B–C’) or ept,H99,psn triple mutant cells (E–E’) marked by the absence of GFP (green) co-stained for CycE (red in A–B’), or Rbf1 (red in C–C’ and E–E’). Tracing in A’ and B’ outlines H99 and ept,H99 mutant clones respectively. The anti-CycE signal was recorded at the same optical settings in panels A’ and B’. Arrowheads in panels B and B’ denote ept,H99 double mutant clones that express CycE. (D–D’) MARCM-mediated expression of DN-DaPKC (blue) in ept,H99 double-mutant cells marked by GFP (green) restores levels of Rbf1 (red). Arrows in panel E denote clones of ept,H99,psn cells that express normal levels of Rbf1 relative to adjacent control cells.

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Effect of DN-DaPKC on Notch and Upd in ept cells

The similar effect of the psn227 allele and the DN-DaPKC transgene on the Rbf1 epitope in ept mutant cells led us to examine Notch protein in ept mutant cells in backgrounds in which DaPKC activity is reduced. ept/M(3) tumors grow as disorganized masses that lack ‘landmarks’ normally associated with the eye disc (e.g. morphogenetic furrow, optic stalk, eye-antennal boundary, etc) thus depriving the tissue of any A/P or D/V reference points. We have therefore used the sole remaining disc feature, the overlying peripodial membrane, to orient each image. As shown in a prior study [6], loss of ept causes the eye disc proper (DP) to grow as disorganized groups of cells surrounded by a layer of peripodial cells (PP) in which the Notch protein shows increased co-staining with the endosomal protein Hrs (Fig. 4A–A'). This co-localization of Notch and Hrs is most apparent in PP cells and in more cortical regions of the DP (upper portion of image in Fig. 4A), suggesting that Notch/Hrs-positive endosomes may accumulate in more apical regions of DP cells. Notch-Hrs co-localization has also been observed in cells lacking the ESCRT-II subunit gene vps25 [3,5] and has been interpreted as an indication that Notch normally traffics through ESCRT-I and -II vesicular compartments on its way to the lysosome. Interestingly reducing DaPKC activity, either by expression of the DN-DaPKC transgene (Fig. 4B–B') or heterozygosity for the DaPKCk06403 loss-of-function allele (Fig. 4C–C'), alters the pattern of Notch protein localization in ept/M(3); a larger proportion of the anti-Notch signal is detected on outer surface of the DP and on the apical face of PP cells (arrows in Fig. 4B), with a corresponding drop in the proportion detected in Hrs-positive structures in cytoplasm of cells in the DP. DaPKC alleles have a similar effect DP and PP populations of Crumbs (Fig. 4D–F), a protein that is normally trapped in cytoplasmic puncta in ept mutant cells [6] and that is known to be regulated by DaPKC [24]. As recent studies have suggested that Notch cleavage and activation requires internalization of Notch protein into specific endosomal compartments [40], we sought to determine if changes in Notch localization following expression DN-DaPKC might correlate with changes in expression of a validated Notch target. It has previously been shown that expression of a Notch RNA interference ‘knock-down’ construct in vps25 mutant eye disc cells is sufficient to block overproduction of the Unpaired protein (Upd), which is otherwise expressed at very high levels in vps25 mutant cells [3]. The overexpression of Upd in ept mutant cells has also been shown to occur by a Notch-dependent mechanism [6]. We find that expression of the DN-
DaPKC is sufficient to substantially blunt the elevation in Upd levels as measured by immunoblotting for total Upd protein present in ept/M(3) eye-antennal discs (Fig. 4G). Thus, in addition to its effect on ept/M(3) tumor growth, Rbf1 levels, and Notch localization, DaPKC is also required for Notch-dependent hyper-accumulation of Upd in ept/M(3) tumors.

Transgenic expression of rbf1 in ept/M(3) tumors

In light of the well-known role Rb family proteins play in regulating the G1-to-S phase cell cycle transition [reviewed in 41], reduced Rbf1 expression in ept mutant cells might be predicted to impair the Rbf1-mediated block to unregulated S-phase entry. To test what effect re-expression of rbf1 might then have on the ept tumor phenotype, a UAS-rbf1 transgene was driven in the background of either normal eye-antennal discs or ept mutant eye/antennal tumors. rbf1 expression in control FRT80B/M(3) eyes/heads leads to a moderate reduction in eye and head size (Fig. 5A). In contrast, re-expression of rbf1 in ept eye/antennal tumors completely blocks the growth of the mutant tissue and results in headless pharate adults (Fig. 5B–C). A residual lump of cuticle (arrow in Fig. 5C) is all that remains of tumors that would otherwise overgrow and kill the animal as an enlarged larva (e.g. Fig. 5B–B). Expression of rbf1 thus has a significant and specific effect on the terminal organismal phenotype resulting from ept eye-antennal tumors. To test if the effect of rbf1 on ept/M(3) tumor growth is due to enhanced rates of apoptosis, the previous experiment was repeated in ept,H99/M(3) tumors that express UAS-GFP under control of the eyFLP and Actin>CD2>Gal4 transgenes [42], such that each ‘flip-out’ event creates a clone of GFP-positive mutant cells. In the absence of the UAS-rbf1 transgene, these ept,H99 mutant tumors are uniformly green (Fig. 5D–D’), indicating that clones of GFP-expressing ept,H99 mutant cells take over the majority of the organ. However, in the presence of the UAS-rbf1 transgene, GFP-positive clones of ept,H99 mutant cells remain quite small (Fig. 5E’), indicating that exogenously produced Rbf1 retains the ability to retard the overgrowth of ept,H99 mutant cells. At an organ level, re-expression of rbf1 in the apoptosis-compromised background of ept,H99/M(3) tumors results in pharate adults with small heads and eyes (inset, Fig. 5E). Thus, the ability of rbf1 to block ept tumor growth is only partly reduced in a background in which cell death is compromised. These observations demonstrate that an eye-antennal disc composed of ept mutant cells responds differently to over-expression of rbf1 than a disc composed of normal cells eye-antennal cells, and suggests that re-introducing rbf1 either slows proliferation of ept,H99 mutant cells or kills them by an H99-
independent mechanism; alternatively, specification of early progenitors of the head/eye fate may be defective in cells that simultaneously overexpress *rbf* and lack ept.

**Discussion**

The data presented here indicate that the DaPKC kinase plays a significant role in the growth of ept mutant imaginal disc tumors. DaPKC is also required for tumor growth in other Drosophila mutants [36,43–45], suggesting that DaPKC may be generally required for excess proliferation in many different backgrounds. The molecular mechanism through which reduced DaPKC activity exerts these effects in ept/*M(3)* cells is not known, although it correlates with increased apical membrane localization of two membrane-bound proteins, Crumbs and Notch, that otherwise aggregate in vesicle-associated structures in the cytoplasm of ept mutant cells [6]. DaPKC directly phosphorylates Crumbs and the endocytic adaptor Numb [36,44,46], which can in turn inhibit Notch [reviewed in 47]. Thus the effect of reduced DaPKC activity on ept tumor growth could be mediated exclusively through effects on Notch and Crb. However two considerations suggest this is unlikely: first, mutations in the *hs* gene affect the vesicular trafficking of many different receptors [17], suggesting that signals transduced through many receptors may contribute to the overall ept tumor phenotype; second, aPKC kinases appear to play a general role in promoting endocytic internalization [18], again suggesting that reduced DaPKC function may affect many different pathways. In light of these considerations, it seems more likely that DaPKC alleles partially rescue ept phenotypes not because they specifically affect the endocytic uptake of one or two apical-membrace proteins (e.g. Crb and Notch), but because they generally lessen the endocytic uptake of a spectrum of proteins that are otherwise trapped in the late-endosome of ept mutant cells. Thus DaPKC might act as a ‘permissive factor’ in ept tumor growth via a positive role in endosomal trafficking upstream of ESCRT-1; in its absence a set of proteins that normally enter the growth via a positive role in endosomal trafficking upstream of Thus DaPKC might act as a ‘permissive factor’ in ept tumor growth via a positive role in endosomal trafficking upstream of ESCRT-1; in its absence a set of proteins that normally enter the growth via a positive role in endosomal trafficking upstream of Notch activity [36,43,44]. Though these observations suggest that the effect of DaPKC and *psn* on Rbf1 levels in ept mutant cells may reflect a Notch-regulatory role for both genes, it remains possible that *psn* and DaPKC are also involved in a Notch-independent signaling pathway that is responsible for the drop in Rbf1 levels in ept mutant disc cells. As certain endocytic tumor suppressors activate Notch (e.g. ept and *ips*25) and others do so to a much lesser extent (e.g. *ayx*/*avl*), one way to begin to address this question may be to examine the pattern of Rbf1 expression in each of these mutant backgrounds.

As a component of the ESCRT-1 complex, ept developmental phenotypes are expected to reflect the combined deregulation of the myriad proteins that traffic through ESCRT-1 dependent compartments of the endolysosomal pathway. Indeed certain elements of the ept mutant phenotype (e.g. increased cell size) do not appear to respond to reduced DaPKC activity. Many other prominent signaling molecules are mislocalized in *Drosophila* wing disc cells mutant for the endocytic regulator *hs* [17], which acts at a sorting step prior to the ESCRT-1 complex [49]. The pathways in which these molecules act should thus be considered as additional candidate effectors of ept mutations. This expanding array of potential ESCRT targets raises the possibility that inactivation of ept will have tissue- and stage-specific phenotypes that reflect the changing pattern of proteins targeted for endolysosomal degradation in various cell types. If targeted endocytosis regulates a similar array of proteins in certain mammalian epithelial cells as it does in *Drosophila* imaginal discs, it may be that defects in this process will act via an aPKC- and *psn*-dependent pathway to produce neoplastic phenotypes similar to those observed with alleles of *Drosophila ept*.

**Materials and Methods**

**Genetics**

Crosses were carried out at 25°C unless otherwise indicated. *DN-DaPKC*-mediated rescue of ept tumor phenotypes was optimal at 20°C. ept mutant eye clones were generated as described previously [4,6]. *ept*, *H99* and *ept*, *H99*, *psn* mutant eye clones were generated by crossing either *w;*+/*Df[3L]H99,FRT080/TM6B or *w;*UAS-*Df[3L]H99*, *FRT280/TM6B males to *y*;CyO:twi-GFP;*FRT080B females. ept mutant eye-antennal tumors were generated by crossing *w;*UAS-*Df[3L]H99*, *FRT280/TM6B; P[w-m'-ubiGFP]*, *FRT080B females. ept mutant eye-antennal tumors were generated by crossing *w;*UAS-*Df[3L]H99*, *FRT280/TM6B; P[w-m'-ubiGFP]*, *FRT080B females. ept mutant eye-tumor clones were generated as described previously [4,6]. *ept*, *H99* and *ept*, *H99*, *psn* mutant eye clones were generated by crossing either *w;*+/*Df[3L]H99,FRT080/TM6B or *w;*UAS-*Df[3L]H99*, *FRT280/TM6B males to *y*;CyO:twi-GFP;*FRT080B; P[w-m'-ubiGFP]*, *FRT080B females. ept mutant eye-antennal tumors were generated by crossing *w;*UAS-*Df[3L]H99*, *FRT280/TM6B; P[w-m'-ubiGFP]*, *FRT080B females. ept mutant eye-tumor clones were generated as described previously [4,6]. *ept*, *H99* and *ept*, *H99*, *psn* mutant eye clones were generated by crossing either *w;*+/*Df[3L]H99,FRT080/TM6B or *w;*UAS-*Df[3L]H99*, *FRT280/TM6B males to *y*;CyO:twi-GFP;*FRT080B; P[w-m'-ubiGFP]*, *FRT080B females. ept mutant eye-antennal tumors were generated by crossing *w;*UAS-*Df[3L]H99*, *FRT280/TM6B; P[w-m'-ubiGFP]*, *FRT080B females. ept mutant eye-tumor clones were generated as described previously [4,6]. *ept*, *H99* and *ept*, *H99*, *psn* mutant eye clones were generated by crossing either *w;*+/*Df[3L]H99,FRT080/TM6B or *w;*UAS-*Df[3L]H99*, *FRT280/TM6B males to *y*;CyO:twi-GFP;*FRT080B; P[w-m'-ubiGFP]*, *FRT080B females. ept mutant eye-tumor clones were generated as described previously [4,6].
The UAS-DN-DaPKC chromosome 2 stock was a gift of D. Bilder. DN-DaPKC-expressing ept mutant clones were generated by crossing the eyFLP; tub-Gal4/CyO; UAS-DaPKC/FRT80B stock (gift of J. Treisman) to ey; UAS-DN-DaPKC/CyO, FRT80B/FRT80B/TM6B.

Flow Cytometry
Eye or wings discs were dissociated in PBS Trypsin-EDTA and stained with 20 μM DRAQ-5 [Biostatus Limited]. Data were acquired on a Becton Dickinson LSR II flow cytometer via a 755 nM Red laser with a 780/60 nM BP collection filter and were analyzed with FACSDiva Software.

Microscopy & Immunohistochemistry
Immunostaining and confocal microscopy was performed as described previously [50]. Antibodies used were: rat anti-Crb-extra (gift of U. Tepass and E. Knust) 1:500; guinea pig anti-Hrs (gift of H. Bellen) 1:1000; mouse anti-BrdU (Becton Dickinson) 1:100; mouse anti-CycE, 1:5 (gift of H. Richardson); mouse anti-C63 anti-Notch (DSHB) 1:50; mouse anti-Rbf1 DX5 (gift of W. Du) 1:50; rat anti-ELAV (DSHB) 1:1000; goat anti-rabbit Cy5, goat anti-mouse Cy3, goat anti-guinea pig Cy3, and goat anti-rat Cy3 (Jackson Laboratories) each at 1:50. BrdU incorporation assays were performed as described previously [51].

References

Supporting Information
Figure S1 Levels of cleaved Caspase-3 are elevated in ept/tsg101 mutant eye clones. Clones of ept/tsg101 mutant cells marked by the absence of GFP (green) contain many dying cells, as indicated by staining for cleaved Caspase-3 (blue).

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Figure S2 Rbf1 levels are unaffected by the H99 deletion. Confostral image of H99 clones lacking GFP, in a mosaic 3rd instar eye imaginal disc stained with the anti-Rbf1 antibody (red).

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Author Contributions
Conceived and designed the experiments: MMG BSR KHM. Performed the experiments: MMG BSR. Analyzed the data: MMG BSR. Contributed reagents/materials/analysis tools: MMG. Wrote the paper: MMG BKM.