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Bap170, a Subunit of the Drosophila PBAP Chromatin Remodeling Complex, Negatively Regulates the EGFR Signaling

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ABSTRACT

BAP and PBAP constitute the two different forms of the *Drosophila melanogaster* Brahma chromatin remodelers. A common multisubunit core, containing the Brahma ATPase, can associate either with Osa to form the BAP complex or with Bap170, Bap180, and Sayp to constitute the PBAP complex. Although required for many biological processes, recent genetic analyses revealed that one role of the BAP complex during Drosophila wing development is the proper regulation of EGFR target genes. Here, we show that Bap170, a distinctive subunit of the PBAP complex, participates instead in the negative regulation of EGFR signaling. In adults, loss of Bap170 generates phenotypes similar to the defects induced by hyperactivation of the EGFR pathway, such as overrecruitment of cone and photoreceptor cells and formation extra veins. In genetic interactions, *bap170* mutations suppress the loss of veins and photoreceptors caused by mutations affecting the activity of the EGFR pathway. Our results suggest a dual requirement of the PBAP complex: for transcriptional repression of *rhomboid* and for efficient expression of *argos*. Interestingly, genetic evidence also indicates that Bap170-mediated repression of *rho* is inhibited by EGFR signaling, suggesting a scenario of mutual antagonism between EGFR signaling and PBAP function.

DURING Drosophila development, the EGFR signaling pathway plays essential roles in multiple processes, such as cell fate specification, proliferation, and cell survival (Shilo 2005). The role of EGFR signaling in cell differentiation has extensively been studied in Drosophila eye and wing development, where EGFR promotes the recruitment of ommatidial cells and the differentiation of veins, respectively (reviewed in Freeman 1997; Schweitzer and Shilo 1997; Kumar and Moses 2001; de Celis 2003; Crozatier *et al.* 2004; Blair 2007). In these processes, the expression of *rhomboid* (*rho*), which encodes a membrane protease with ligand-processing activity, represents the limiting factor regulating the spatial and temporal hyperactivation of the EGFR receptor (Bier *et al.* 1990; Sturtevant *et al.* 1993; Golembo *et al.* 1996). In addition, multiple mechanisms of negative modulation of the EGFR pathway ensure its temporal and spatial restricted activity. Thus, the short-range action of the EGFR pathway requires the inhibitory action of secreted proteins encoded by *argos (aos)*, *kekkon1*, and *sprouty*, which are transcriptionally induced by the pathway and are required to inhibit EGFR signaling in cells that are more distant from the source (Shilo 2005). Additional repressors, acting downstream of EGFR signaling, include E(spl), required to repress *rhomboid* in interveins in response to the Notch signaling (de Celis *et al.* 1997), Capicua (Roch *et al.* 2002; Tseng *et al.* 2007), Atrophin (Charroux *et al.* 2006), and Groucho (Hasson *et al.* 2005). Except for different ligands used in diverse developmental processes, EGFR signaling usually involves a common cytoplasmic transduction cascade, which includes Ras, Raf, Mek, and Mapk (Perrimon and Perkins 1997). During eye development, activated Mapk transmits the Ras signaling cascade into the nucleus by phosphorylating two members of the ETS family of transcription factors, the repressor Yan (Rebay and Rubin 1995) and the transcriptional activator Pointed P2 (Brunner *et al.* 1994; O'Neill *et al.* 1994). This allows EGFR targets to be relieved from Yan-mediated repression and induced by the Pnt-P2 activation function.

Although much is known about the mechanisms that trigger the precise spatial and temporal activation of the EGFR signaling, knowledge is still limited about the mechanisms that, in the nucleus, translate the signaling to the wide network of downstream genes required to execute the differentiation programs (Roberts *et al.* 2000). The global corepressor Groucho seems an ideal target for such widespread nuclear regulation. The finding that Gro-dependent gene silencing can be relieved in response to Mapk activation signal, suggests a mechanism of coordinated derepression of a considerable number of genes in distinct developmental settings (Hasson and Paroush 2006).

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Also, some connections between EGRF signaling and the function of other widely used transcriptional machinery, the Brahma (Brm) chromatin remodeling complex, have been described in Drosophila. Thus, in addition to the documented role played by the Brm complex as positive regulators of *Hox* genes (Kennison and Tamkun 1988; Papoulas *et al.* 1998; Collins *et al.* 1999; Vazquez *et al.* 1999) and repressors of *wingless* targets (Treisman *et al.* 1997; Collins and Treisman 2000), analyses of point mutations affecting key subunits of the complex core, such as Snr1 or Brahma, demonstrated the ability of the Brm complex to have opposite roles in some EGFR-dependent processes of wing development, as well as a differential ability to function as either activator or repressor of *rho* expression (Elfring *et al.* 1998; Zraly *et al.* 2003; Marenda *et al.* 2003, 2004). This dual role of the Brm complex on EGFR signaling might be explained in part by the recent finding that the complex exists in Drosophila, as in human and yeast, in two different forms, which execute distinct and in part antagonistic functions in transcription control. A common core complex, which includes Brahma, Moira, and Snr1, can associate to the distinctive subunit Osa to form the BAP complex or alternatively with Polybromo/Bap180, Bap170, and Sayp, but not Osa, to constitute the PBAP form (Mohrmann *et al.* 2004; Moshkin *et al.* 2007). In the hypothesis that BAP and PBAP have opposite roles on EGFR target gene regulation, any mutation affecting core complex subunits would deplete both BAP and PBAP functions, inducing opposite EGFR phenotypes. Recent analyses on the role of Osa in the control of EGFR target gene regulation in wing discs have better defined the role of BAP in the control of the response to EGFR signaling (Molnar *et al.* 2006; Terriente-Felix and de Celis 2009), suggesting that Osa is required to activate the expression of EGFR target genes in response to EGFR signaling.

Here we show that Bap170, an essential and distinctive subunit of the PBAP complex, is involved in the negative regulation of EGFR signaling. In a screening for metamorphosis mutants in Drosophila, we isolated *half-life* (*hfl*) mutations as alleles of *bap170*. Surprisingly, although *bap170* is specifically expressed in imaginal tissues in third instar larvae and loss-of-function alleles cause early prepupal lethality with severe defects in imaginal disc eversion, Bap170 is dispensable for the transcriptional control of a subset of primary EcR (Ecdysone receptor) responsive genes throughout the larval–prepupal period. Conversely, loss of Bap170 generates phenotypes similar to those induced by hyperactivation of EGFR signaling, such as overrecruitment of cone and photoreceptor cells and formation of extra vein tissues. Genetic and epistatic analyses show that *bap170* interacts with components of EGFR signaling, acting during wing vein development downstream of *knirps* and upstream of *rhomboid*. Interestingly, the lack of *Bap170* function causes upregulation of *rho* and downregulation of *argos*, thus uncoupling the transcriptional response of these genes to EGFR signaling. Our results suggest that Bap170 participates in transcriptional repression of *rhomboid* but is also required to ensure a proper level of *argos* expression. Interestingly, genetic evidence also indicates that Bap170-mediated repression of *rho* is inhibited by EGFR signaling, thus depicting a scenario of a reciprocal regulation between EGFR signaling and PBAP function.

MATERIALS AND METHODS

Drosophila strains: The following GAL4 and *UAS* lines were used: *UAS-Ras*^{V12} and *UAS-Ras*^{N17} (Lee *et al.* 1996), *UAS-net* (Brentrup *et al.* 2000), *UAS-GFP* (Ito *et al.* 1997), *UAS-E(spl)mβ* (de Celis *et al.* 1996), *UAS-bs* (Montagne *et al.* 1996), *elav*-GAL4 (Brand and Perrimon 1993), *Omb*-GAL4, *dll*-GAL4 (Calleja *et al.* 1996), *MS1096*-GAL4 (Milan *et al.* 1998), *sev-Ras*^{N17} (Karim *et al.* 1996), *sev*-GAL4 (Basler *et al.* 1989), and *tub*-GAL4 (Lee and Luo 1999). The following alleles and enhancer trap lines were obtained from the Bloomington Stock Center: *rho*^{ve}, *vn*^{c221}, *vn*^{1}, *Egfr*^{T1}, *Egfr*^{f24}, *bs*^{2}, *kni*$^{ri\text{-}1}$, *pnt*^{1277}, *pnt*$^{\Delta 88}$, *raf*^{HM7}, *ras*^{e1B}, *aos-lacZ*^{05845}, *aos-lacZ*^{W11}, and *Dl-LacZ*^{05151}, while *bap170*$^{\Delta 65}$ and *bap170*$^{\Delta 135}$ mutants were kindly provided by J. Treisman.

Genetic and molecular mapping of *half-life* locus: *hfl*^{1} mutation was isolated in a small-scale *P*-element mutagenesis screen (Giordano *et al.* 1999) performed essentially according to the "reversion jumping" scheme (Tower *et al.* 1993) and aimed to recover metamorphosis lethal mutations. *hlf*^{1} was recovered as a prepupal lethal mutation located on a second chromosome, which also carried a viable PZ-element insertion within the *clot* gene in 25E1 (Giordano *et al.* 2003). Subsequent recombination tests with wild-type chromosome, and *P*-excision experiments aimed at rescuing the prepupal lethality, demonstrated that *hlf*^{1} mutation was not caused by the PZ insertion in 25E1. A clean stock carrying *hlf*^{1} (*CyO/hfl*^{1}*; ry*^{506}), but not the PZ in 25E1, was prepared by recombination with a wild-type second chromosome and used for subsequent analysis. Because Southern blot experiments demonstrated the lack of any sequence related to the PZ element in *CyO/hfl*^{1}*; ry*^{506} flies, we argued the *hlf*^{1} mutation might have originated either spontaneously or more likely by a defect (for example, defective repair of double strand breaks) caused by a secondary insertion of the PZ followed by its excision. *hlf*^{1} was genetically mapped at ~13 cM from *L* (*Lobe*) and 23 cM from *Bc* (*Black cells*) mutations, toward the centromere (supporting information, Figure S1). Complementation tests with deficiencies overlapping the region between *Lobe* and the centromere were used to map *hfl*^{1} mutation to the 42B3-C2 cytological region, between the proximal breakpoint of *Df(2R)Drl*^{rv17} and the distal breakpoint of the *Df(2R)nap1*, within the *Df(2R)ST1* deficiency. To better define the *hlf*^{1} position we thought to mobilize individually single *P*-element insertions available in the 42B3-C2 region with the aim of generating, by imprecise excisions, small *hlf*^{1}-no-complementing deficiencies. Among 40 excisions recovered for each used *P*-element (*P{PZ}Adf1*^{01349}, *P{EP}Vha16*^{EP2372}, *P{lacW}geminin*^{k14019}, *P{PZ}l(2)01289*^{01289}, and *P{PZ}jing*^{01094}), a single event (named *hfl*^{37}), which failed to complement *hfl*^{1}, was obtained using the *P{EP}Vha16*^{EP2372} insertion. Complementation analyses with the *P* lethal insertions mapped in the same region showed that *hfl*^{37} behaves genetically as a deficiency extending at least from the *Vha16* (identified by the

P{EP}Vha16^{EP2372}) and the *Adf1* (identified by the *P{PZ}Adf*^{01349}) genes (see Figure S1). Given that *hfl*^{37} retained both extremities of the PZ elements, as determined by Southern blot hybridizations, inverse PCR was used to map the *hfl*^{37} limits. This showed the proximal breakpoints of the *hfl*^{37} deficiency maps within the *Vha16* gene, whereas the distal breakpoint is located within the first intron of the *CG9422* gene thus extending *hfl*^{37} for ~40 kb (Figure S1). A detailed search at the FlyBase genomic database identified in the *hfl*^{37} deficiency at least 17 potential candidate gene. Since the *hfl*^{1} allele complemented lethal *P* insertions in the *Vha16*, *geminin* and *Adf1*, these genes were excluded as *hfl* candidates. Thus, to identify the *half-life* gene, a series of genomic fragments covering the remaining 14 genes in this region were used for germline transformations and the obtained transgenic flies were each tested for their ability to rescue the lethality of the *hfl*^{1} homozygotes (Figure S1). Only a bap170-containing transgene was able to fully rescue the *hfl*^{1} lethality. The identity between *half-life* and *bap170* was also confirmed by the rescue of *hlf*^{1}/*hfl*^{1} lethality obtained using the full-length *bap170* cDNA (*UAS-bap170)* expressed under the control of the *tub*-GAL4 driver.

Molecular biology: Standard methods were used for PCR, cloning, sequencing, and Southern hybridizations. Northern and Western blot analyses and transgenic line preparation were performed as previously described (Giordano *et al.* 1999). *bap170*-specific RT–PCRs on RNA from larval tissues was performed as follows. Ten third instar larvae per corresponding stage (−18 or −4 hr after pupal formation, APF) were dissected and their wing discs, fat bodies, and salivary glands separately harvested. RNAs from each tissue were extracted and subjected to oligo-dT priming and cDNA synthesis using the RETROscript system (Ambion). Thirty-five cycles of PCR amplification were carried out with primer pairs spanning the third (5′-CAGTTGGCGGTACGGTGG-3′) and the fourth exon (5′-GCGCCAGTGTGCAACGTCGCC-3′) of *bap170* genomic DNA. Primers from the *minifly* gene (Giordano *et al.* 1999) were used as internal controls. For quantitative real time RT–PCR of *rho* and *argos*, three RNA samples of 10 wing discs each (dissected from third instar larvae at −2h APF) were prepared for each genotype (*wild type*, *bap170*^{hfl1}/*bap170*^{hfl1}, *omb-GAL4,UAS-Ras*^{N17}, or *omb-GAL4,UAS-Ras*^{N17}*;UAS-Bap170*). Each RNA sample was first reverse transcribed with oligo-dT primers and subsequently PCR amplified using SYBR green master mix (Applied Biosystems), and the following primer pairs relative to *argos*, *rho*, and, as internal normalizer, to *mfl* gene: aosFor, 5′-TGCGCATCCTCTACCAAGTG-3′; aosRev,5′-CATTGTTGGGCATGCGATTC-3′; rhoFor, 5′-ACTGGCCCT GGTTCATCCTA-3′; rhoRev, 5′-GGAACGGGTAGCCCGAAA T-3′; mflFor, 5′-GCCATGTGGCTGTACGAAAA-3′; mflRev, 5′-GTAATCTTGGCACCATAGCAA-3′). PCR amplification efficiencies were determined for each gene and ΔΔCT relative quantification was done using *mfl* gene expression as internal control to normalize the results.

For full-length *bap170* cDNA cloning, the 3.6-kb cDNA clone GH12174, purchased from the Drosophila Genome Resource Center, was extended by 5′ RACE toward 170 bp of the 5′-UTR. Two classes of cDNAs were isolated and sequenced. One is the full-length 5.1-kb *bap170* cDNA, used in this work, which encodes the 1681-amino-acids-long Bap170. The other is a putative female germline-specific 5.0-kb species since it can be detected by RT–PCR only in early 0- to 2-hr embryos and ovaries of adult female. The C2 cDNA derives from an alternative splicing which, removing a 62-nt segment at the beginning of the second exon, introduces a stop codon just at the end of the AT-rich interaction domain (ARID) (Figure 2). The C2 form is not functional for the somatic function of *bap170* given that it fails to rescue *bap170*^{hfl1} lethality when expressed as *UAS-C2* transgene under the control of the *tub-GAL4* driver. Although we did not investigate the role of this female-specific variant, it likely represents the product of some sort of regulatory mechanism of *bap170* maternal function during oogenesis. The C1 bap170 cDNA was confirmed to be effective in rescuing the *bap170*$^{\Delta 135}$ or *bap170*^{hfl1} lethality when expressed as *UAS-bap170* transgene using the *tub*-GAL4 driver. *UAS-bap170RNAi* transgene was prepared by inserting a spaced inverted repeat of the 3.6-cDNA clone GH12174 into the pUAST vector (Brand and Perrimon 1993). To obtain the *bap170-LacZ* reporter, a 1.3-kb DNA fragment derived from the 5′ end of the *bap170* gene was linked inframe upstream of the *LacZ* coding region in the pCasper-βgal vector (Thummel *et al.* 1988). This fragment, which encompasses the genomic region between exon 1 of the *trap1* gene to the *Bam*HI site in exon 2 of *bap170* (Figure 2D), was selected for its ability to rescue to wild-type conditions *bap170*^{hfl1}/*bap170*^{hfl1} or *bap170*$^{\Delta 135}$/*bap170*$^{\Delta 135}$ homozygous flies, when used to drive the expression of the *bap170* cDNA. The derived Bap170-βGAL fused protein contains the first 194-aa N-terminal residues of Bap170 joined to the complete β-galactosidase amino acid sequence.

Phenotypic analysis: Flies and crosses were maintained at 22° except when differently specified. Lethal phase analysis was performed as previously described (Giordano *et al.* 1999). Time lapse on fluorescently labeled prepupae was performed by capturing images at 20′ intervals starting from white prepupa formation (0 hr APF) of control *dll*-GAL4/*UAS-GFP* and mutant *dll*-GAL4, *bap170*^{hfl1}/*UAS-GFP*, *bap170*^{hfl1} individuals. Scanning electron microscopy was performed as described by Kimmel *et al.* (1990). Fixation, resin embedding, and thin sectioning of adult retina for light microscopy analyses were performed as described by Wolff and Ready (1991). Mutant *bap170*^{hfl1} eyes were obtained from *dll*-GAL4, *bap170*^{hfl1}/*bap170*^{hfl1}*;UAS-bap170*/+ flies, in which the *distalless*-GAL4 driver was used to rescue BAP170 function in the distal region of the leg discs, wing margins, antennal but not eye primordia (Cohen *et al.* 1989). The average number of photoreceptors per ommatidium (ANP) was determined, for each genotype, on ~100 ommatidia derived from three eyes taken from different organisms. Cobalt sulphide staining of pupal retinas was performed as described by Wolff and Ready (1991) on pupae at +60 hr APF. In genetic interaction analyses of wing phenotypes, identical results were obtained using either *bap170*^{hfl1} or *bap170*$^{\Delta 135}$ allele, whereas *bap170*$^{\Delta 65}$ mutation generated weak and sporadic interaction effects. Adult wings were dissected from 4- to 5-day-old females of each genotype and mounted in Permount.

Drosophila immunocytochemistry, *in situ* hybridizations, and X-gal staining: Third instar larvae were staged on the basis of the bromophenol blue method (Andres and Thummel 1994). For immunohistochemical staining, imaginal discs were dissected in PBS, immediately fixed in 4% paraformaldehyde in PBS for 1 hr at 4°, and permeabilized in PBS + 0.3% Triton X-100 for 2 hr at 4°. Incubations of primary and secondary antibodies were all carried out at 4° for 12 hr. Secondary biotinylated antibodies and HRP reagents for ABC detection were from Vector Lab. We used mouse anti-phosphorylated ERK (Sigma), mouse monoclonals anti-bs (Active Motif), guinea pig anti-Bap180, and rabbit polyclonal anti-Bap170 (Carrera *et al.* 2008). *In situ* hybridizations were carried out as described (Giordano *et al.* 1999). *rhomboid*, *net*, and *E(spl)m*β riboprobes were prepared from plasmids carrying gene-specific genomic fragments obtained by PCR amplification using the sequence data available in FlyBase. Detection of the β-gal activity for *lacZ* reporters was carried out according to standard protocols except for *Dl-lacZ*, for which 0.3% Triton X-100 was added to the X-gal staining

solution. Detection of *aos-lacZ* and *Dl-lacZ* expressions was repeated several times and always performed by incubating the sample and control tissues in parallel, under the same conditions (24 hr a 37° for *aos-lacZ* and 2 hr at 25° for *Dl-lacZ*). Images were captured using either a Leica MZ stereomicroscope or a Reichert-Jung Polyvar microscope.

RESULTS AND DISCUSSION

Identification, lethal phase, and morphological analysis of *half-life* mutants: We isolated *half-life* (*hfl*) mutations in a *P*-element mutagenesis screen for second chromosome metamorphosis mutants in Drosophila. hfl^{1} was identified as a prepupal lethal mutation while hfl^{37} was recovered as a *P*-induced deficiency overlapping the *hfl* locus (see MATERIALS AND METHODS). Lethal phase analyses revealed that hfl^{1}/hfl^{1} or hfl^{1}/hfl^{37} individuals display normal development and growth rate throughout larval life but terminate further development within the 12 hr of the prepupal period with no evidence of imaginal discs or head capsule eversion (Figure 1, A and B). *In vivo* time-lapse imaging of fluorescently labeled leg and wing discs was used to compare the behavior of wild-type and hfl^{1} mutant discs during the prepupal period (Figure 1C). This analysis revealed that mutant leg and wing discs normally start the elongation phase (point +4 hr APF) but, as development proceeds (points +7 to +14 hr APF), the eversion outside the imago body fails and the imaginal discs remain with the shape of partially elongated structures. In contrast, proper larval tissues of hfl^{1}/hfl^{1} mutants proceed through all morphological changes characteristic of this stage of metamorphosis, such as the morphological changes of the salivary glands and guts and the disaggregation of fat bodies (not shown).

***hfl* encodes Bap170, a signature subunit of the PBAP complex:** Standard genetic and molecular approaches were used to map *half-life* within a 40-kb genomic region between the *Vha16* and *CG9422* genes on the second chromosome (MATERIALS AND METHODS and Figure S1). Rescue of hfl^{1} lethality with transgenes carrying single candidate genes in this region, revealed that *half-life* corresponded to *bap170* (MATERIALS AND METHODS and Figure S1), a gene encoding a multidomain nuclear protein of 170 kDa, which includes an N-terminal AT-rich interaction domain (ARID), a region with multiple LXXLL motifs, and two C-terminal C_2H_2 Zn-fingers (MOHRMANN *et al.* 2004 and Figure 2A).

Bap170 has recently been identified as one of the three signature subunits characterizing, together with Bap180 and Sayp, the PBAP form of the Brahma chromatin remodeling complex (MOHRMANN *et al.* 2004; CHALKLEY *et al.* 2008). The second member of the Drosophila Brm complex is the BAP form, which contains Osa, but not Bap170, Bap180, or Sayp. A functional comparison between the two complexes in Drosophila S2 cells revealed largely antagonistic functions for the BAP- and PBAP-specific subunits, showing that BAP is mainly involved in cell cycle regulation, whereas PBAP is in part involved in signal transduction cascades (MOSHKIN *et al.* 2007). This analysis demonstrated that the three PBAP signature subunits act as a single functional unit that is essential for PBAP activity (CHALKLEY *et al.* 2008). The role the PBAP complex *in vivo* has recently been investigated by microarray analysis in double *bap170* and *bap180* mutants at puparium formation, finding a PBAP requirement in the expression of genes involved in morphogenesis and the immune response (CARRERA *et al.* 2008). Interestingly, both *in vivo* and in S2 cell, Bap170 is required for stability of Bap180 (MOSHKIN *et al.* 2007; CARRERA *et al.* 2008) and the stability of Bap170, in turn, depends on Sayp (CHALKLEY *et al.* 2008). The crucial role of Bap170 for PBAP function has also been demonstrated by the recent finding that Bap170 is essential to anchor the PBAP complex to the transcriptional initiator factor TFIID, allowing the formation of the transcriptional supercomplex BTfly (VOROBYEVA *et al.* 2009).

***bap170* is specifically expressed in imaginal tissues at the onset of metamorphosis:** The defects of imaginal disc morphogenesis and the prepupal lethality observed in $bap170^{hfl1}$ mutants prompted us to investigate whether these phenotypes might be correlated to a temporally regulated or tissue-restricted expression of *bap170* during the onset of metamorphosis. Northern blot analyses on poly(A)$^+$ RNA extracted at several stages during Drosophila development revealed that *bap170* is constitutively expressed throughout the life cycle (Figure 2B). No quantitative differences in mRNA accumulation or qualitative variations in the splicing pattern, which could potentially suggest a stage-specific regulation of *bap170*, are evident during the late larval–prepupal period. The sole exception is the high level of *bap170* mRNAs detectable in early embryos, which likely represents the maternal contribution. To analyze potential tissue-specific expression of bap170, we made use of a *bap170-lacZ* transgene that we prepared by joining a 1.3-kb genomic fragment, containing the *bap170* transcriptional regulatory sequences, to the *LacZ* gene (Figure 2D and MATERIALS AND METHODS). This fragment was selected for its ability to fully rescue the $bap170^{hfl1}$ lethality when used to drive *in vivo* the expression of a full-length *bap170* cDNA. Independent transgenic lines for the *bap170-lacZ* transgene were then used to monitor the reporter expression throughout development. The *LacZ* expression, although detectable in embryos and adult germline cells (not shown), in third instar larvae is restricted to all imaginal discs, brains, and to other imaginal tissues such as the imaginal rings of salivary glands, the foregut imaginal rings, and the midgut imaginal histoblasts (Figure 2D). In imaginal discs, *bap170-lacZ* is uniformly expressed and not restricted to specific territories, thus confirming previous data, which showed ubiquitous detection of BAP170 in all cells of imaginal discs (CARRERA *et al.*

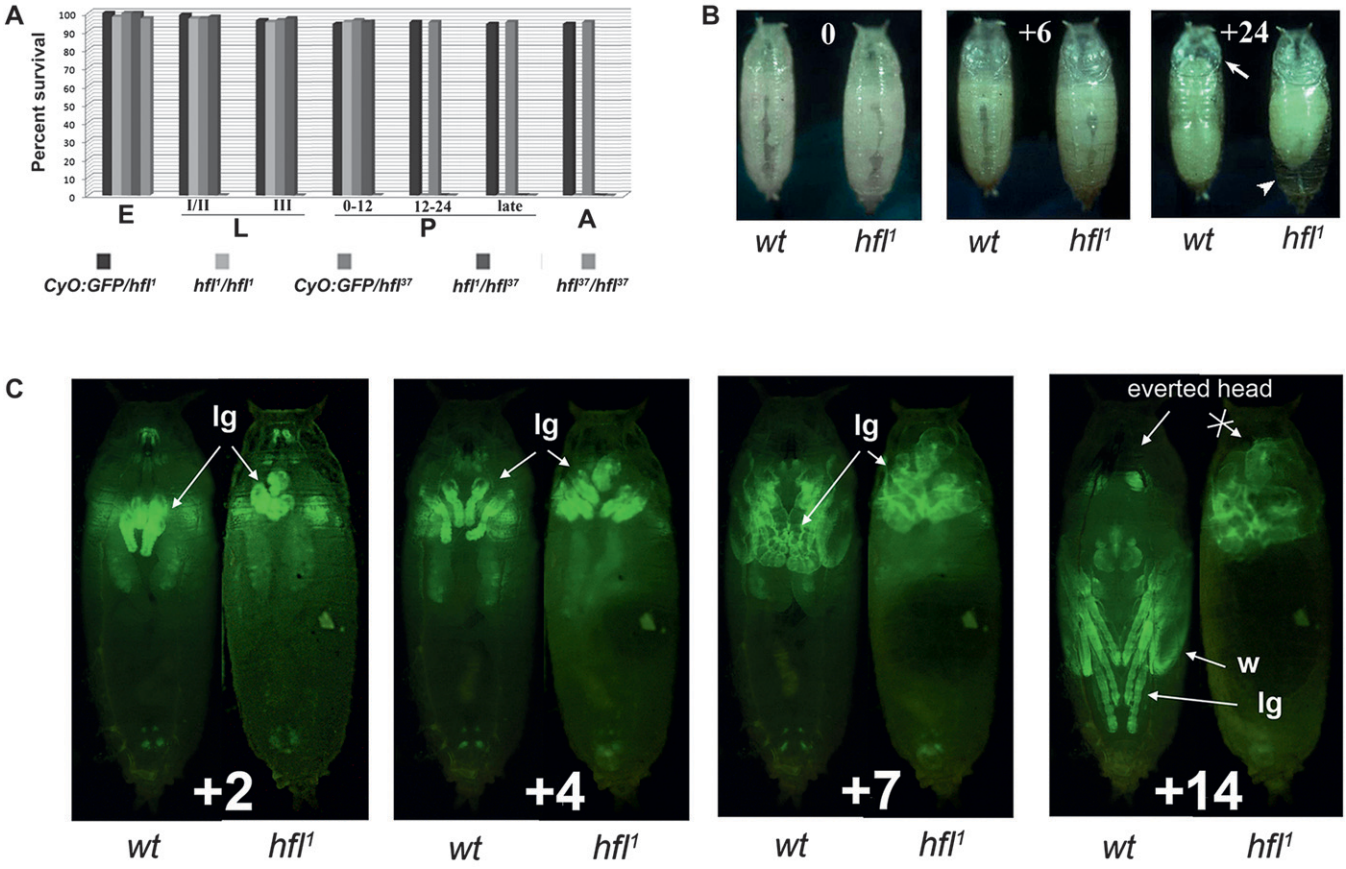

Figure 1.—Lethal phase and discs eversion defects of hfl mutants. (A) Lethal phase analysis of different allelic combination of *half-life* mutants. Homozygous hfl^1/hfl^1 and hfl^{37}/hfl^{37} or heterozygous hfl^1/hfl^{37} organisms were scored for each indicated stage by the lack of GFP fluorescent balancer *CyO:GFP*. The hfl^{37} allele is a deficiency of 40 kb, which removes several genes nearby the *half-life* locus, and hence results in embryonic lethality. (B) Time course of the morphological changes during the 12 hr of the prepupal period (time is relative to puparium formation). hfl^1 homozygous display a normal phenotype until +6 hr APF. At +24 hr APF, gas bubble translocation and head eversion have not correctly occurred. (C) Micrographs from time-lapse experiments of wild type and hfl^1 homozygous mutants carrying both *dll-GAL4* and UAS-GFP transgenes. The photograms at +2, +4, +7, and +14 hr APF show that leg (lg) and wing (w) disc elongation starts normally in hfl^1 homozygous prepupae (+2 and +4). Later in development, discs remain partially elongated (+7) and never evert outside the imago (+14).

2008). The sole exception to the generalized expression in imaginal tissues are the salivary gland cells, in which the β-gal activity is detectable at −18 hr APF, but disappears at −4 hr APF (Figure 2D). RT–PCR analyses of *bap170* transcription, performed on wild-type organs that displayed different expression patterns of the *bap170-LacZ* transgene (*i.e.*, wing discs, fat bodies and salivary glands), confirmed the tissue-specific expression of *bap170* in third instar larvae (Figure 2C).

***bap170^{hfl1}* mutants lack both Bap170 and Bap180 subunit of the PBAP complex:** In a recent study performed to investigate the role of the PBAP complex, deletion mutants of *bap170* and *bap180* genes have been described (Carrera *et al.* 2008). Surprisingly, homozygous animals for the null $bap180^{\Delta 86}$ allele are viable but female sterile. Conversely, two specific mutations have been obtained for *bap170*. The first is the viable hypomorphic $bap170^{\Delta 65}$ mutation, a deletion of the 5′ end of the gene, which reduces adult viability and causes formation of ectopic wing vein material. Homozygous $bap170^{\Delta 65}$ flies still encode a mutated Bap170 protein lacking the ARID domain, which is sufficient to sustain some Bap180 accumulation (Carrera *et al.* 2008). Conversely, the second mutation, $bap170^{\Delta 135}$, a nearly complete deletion of the *bap170* gene, causes total loss of Bap170 and Bap180 and results in a fully penetrant prepupal lethality (Carrera *et al.* 2008). We found that $bap170^{\Delta 135}/bap170^{\Delta 135}$ organisms display an identical lethal phase and the same defects of disc elongation observed in $bap170^{hfl1}/bap170^{hfl1}$ mutants. The similarity between $bap170^{hfl1}$ and $bap170^{\Delta 135}$ mutations was also confirmed at the molecular level. Through sequence analysis of the $bap170^{hfl1}$ allele, we found that a 16-bp deletion, within the fourth exon of the *bap170* gene, introduced a frameshift, which predicts the synthesis of a mutated Bap170 deleted from the last C-terminal 304 aa containing the two Zn fingers (Figure 2A). The transcription of the mutated gene is not affected, as determined by Northern blot analysis (Figure 2E). In contrast, Western blots carried out on extracts of

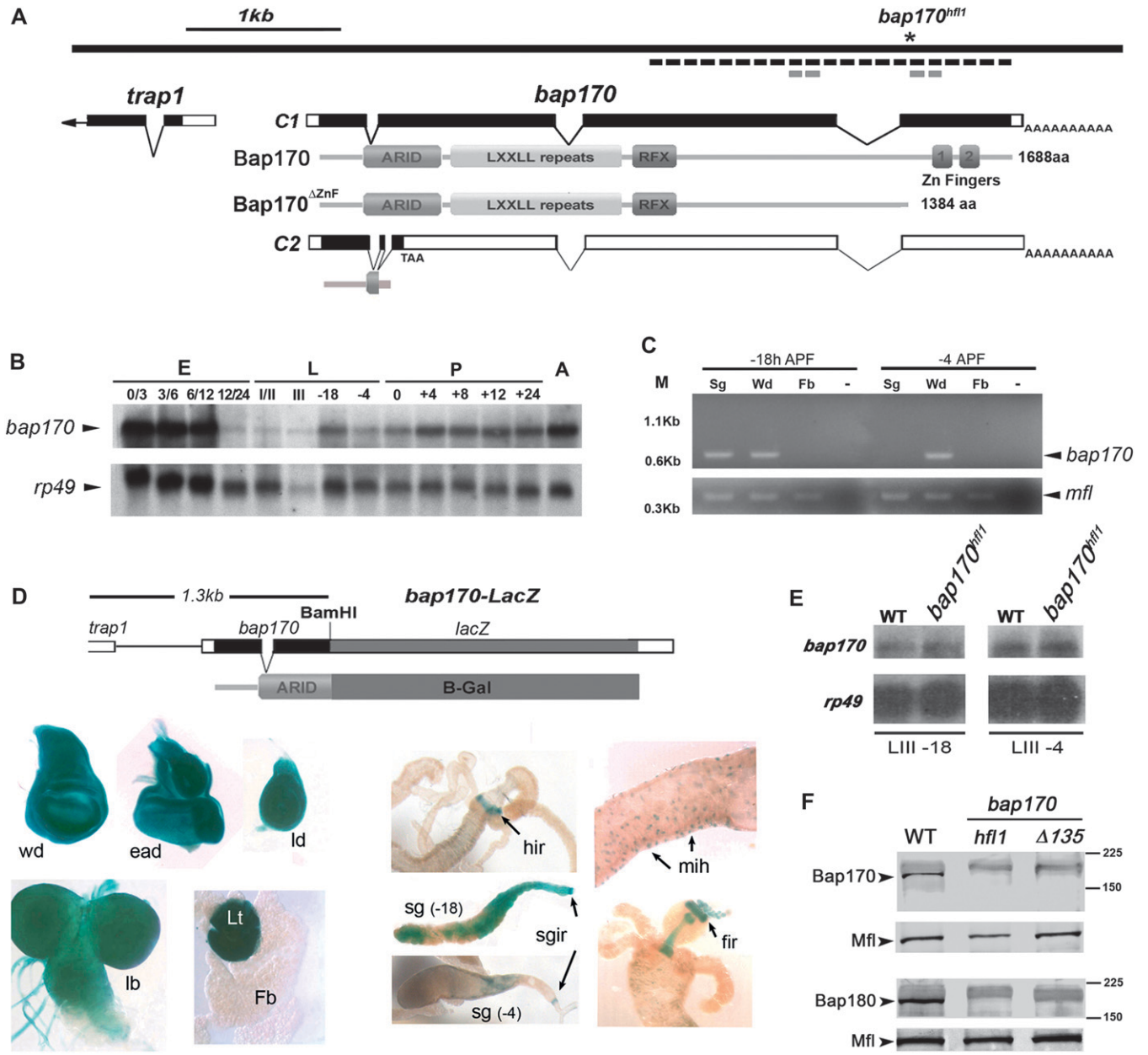

FIGURE 2.—Expression analysis of *bap170* and molecular characterization of *bap170^{hfl1}* allele. (A) Diagram of the *bap170* genomic region with indicated extension of the full-length *bap170* cDNA C1 and the structure of the wild-type Bap170. The structure of the alternative spliced maternal C2 cDNA is indicated with its putative encoded polypeptide.The asterisk on the genomic map indicates the position of the 16-bp deletion within the *bap170^{hfl1}* allele. The black dashed line indicates the genomic fragment used as probe for Northern blot analyses in B and E. The truncated BAP170 protein encoded by *bap170^{hfl1}* (BAP170$^{\Delta ZnF}$) is depicted below the wild-type protein. Gray dashed lines represent the positions of the primers used for RT–PCR analyses shown in C. (B) Developmental Northern blot analysis of *bap170* expression on poly(A)$^+$ RNA samples extracted at all stage of development (E, embryos; L, larvae; P, pupae; A, adults). The high level of *bap170* mRNA accumulating at 0–12 hr embryos represents the maternal contribution. (C) Detection of *bap170* expression by RT–PCR analysis on oligo-dT primed cDNA libraries prepared using RNAs extracted from larval tissues at −18 hr or −4 hr APF. Sg, salivary glands; Wd, wing discs; Fb, fat bodies. As control, RT–PCR was also performed with primers belonging to the ubiquitously expressed *minifly* gene (GIORDANO *et al.* 1999). (D) Expression pattern of *bap170* revealed by the *bap170-lacZ* transgene. Above, diagram of the *bap170-lacZ* transgene (see MATERIALS AND METHODS). Below, dissected tissues from *bap170-lacZ* transgenic larvae after hystochemical staining for β-gal activity. The *LacZ* reporter is expressed in all imaginal disc cells (wd, wing disc; ead, eye-antennal disc; ld, leg disc), in larval testes (Lt), in some cells of larval brains (lb), but not in fat bodies (Fb), malpighian tubules and thacheae (not shown). In the gut, *LacZ* expression is restricted to the hindgut imaginal ring (hir), foregut imaginal ring (fir), and midgut imaginal histoblasts (mih). The weak expression in salivary glands at −18 hr fades at −4 hr APF in the gland cells, but persists in the salivary imaginal rings (sgir, arrows). (E) Northern blot analysis of *bap170* expression in wild type and *bap170^{hfl1}/bap170^{hfl1}* mutant larvae on poly(A)$^+$ RNA samples prepared from larvae at −18 and −4 hr APF. Northerns in B and E were also probed, as control of loaded RNAs, with a *rp49* gene fragment. (F) Bap170 and Bap180 accumulation in extracts of mixed imaginal discs and brain tissues from *wild type*, *bap170$^{\Delta 135}$*, or *bap170^{hfl1}*

bap170^{hfl1} homozygous larvae showed that the predicted 151-kDa Bap170$^{\Delta ZnF}$ form is undetectable (Figure 2F). This indicates a critical role for the two Zn finger domains for Bap170 stability. As expected, the lack of Bap170 is associated with a corresponding loss of Bap180 (Figure 2F), demonstrating that *bap170^{hlf1}*, like *bap170$^{\Delta 135}$*, is a null Bap170 allele. Thus, the prepupal lethality of *bap170^{hlf1}* or *bap170$^{\Delta 135}$* mutants results from a complete deficit of both BAP170 and BAP180 proteins, and therefore each allele represents a *bona fide* loss-of-function condition of the PBAP complex.

Bap170 is required for photoreceptor and cone cell recruitment and development of wing veins: Although the pupal lethality caused by loss of Bap170 and Bap180 suggests a potential role of the PBAP in ecdysone-controlled gene expression, previous studies (Carrera *et al.* 2008) and our observations have excluded a clear role of PBAP on the expression of genes regulated by ecdysone receptor (EcR) during the larval–prepupal period (see Figure S2). With the aim of further investigating the role of the PBAP complex in the development of imaginal discs, we decided to analyze the effects caused by loss of *bap170* activity on the differentiation of two well-characterized adult organs, the eyes and wings. Since *bap170* maps in 42C1, mutant clones by mitotic recombination could not be produced with the available 42D FRT elements. Therefore, to generate adult mutant eyes, we decided to rescue Bap170 function in some tissues of *bap170^{hlf1}/bap170^{hlf1}*mutants with the exception of the eye primordia. When an *UAS-bap170* transgene is expressed using the *dll*-GAL4 driver in antennal discs, wing margins, and distal parts of leg discs, but not in eye primordia (see materials and methods) of *bap170^{hfl1}* mutants, the prepupal lethality can be rescued, allowing the development of adult flies. In these adults, the eyes are slightly larger than wild-type eyes and show an irregular organization of ommatidial surface (compare Figure 3, A and D). In a Drosophila wild-type eye section each ommatidium contains seven photoreceptor (PR) cells (R1–R7 or R1–R6 and R8), four cone cells, and eight accessory cells arranged in a highly structured pattern (Figure 3, B and C). Conversely, in *bap170^{hfl1}*mutant eyes, the ommatidia often contain more than seven PRs cells of comparable size, with an average number of 7.35 PRs per ommatidium ($n = 100$) (Figure 3E). Some ommatidia show supernumerary outer photoreceptors, whereas others have extra putative R7. Since the extra PRs do not have a fixed identity, we assumed that the *bap170* mutation might cause abnormal activation of a general differentiation program common to all PR cells, such as that induced by EGFR signaling. Cone cell recruitment, which also depends by EGFR signaling, is also affected in *bap170^{hfl1}* eyes, given that ommatidia often display more than four cone cells (compare Figure 3, C and F). All the described phenotypes of *bap170^{hfl1}* mutant eyes can be restored to normal condition in control flies in which Bap170 was expressed under the control of the ubiquitous tub-GAL4 driver (Figure 3 G–I), demonstrating that the recruitment of extra cone and photoreceptor cells is due to loss of Bap170. To verify the role of Bap170 on another EGFR-promoted developmental program, the differentiation of wing veins, we analyzed the effect of *bap170* downregulation in adult wings using RNAi-mediated depletion. We observed that the expression of the UAS-*bap170*RNAi transgene in wing discs, using different GAL4 drivers, always causes the formation of adult wings with extra vein tissues (an example in Figure 3K), a phenotype which is again reminiscent of an ectopic EGFR signaling activation. Two doses of the UAS-*bap170*RNAi transgene led to a severe wing phenotype (Figure 3L) similar to that generated by mutations of *bs*, a gene required for intervein cell differentiation and repression of EGFR activation. Together, the over recruitment of cone and photoreceptor cells as well the formation of extra veins caused by Bap170 loss, suggests that the PBAP complex might act as a negative regulator of EGFR signaling during ommatidial and vein differentiation.

Bap170 genetically interacts with component of EGFR signaling: The EGFR signaling pathway is required during eye development to recruit and differentiate the cone cells and all types of PRs, with the exception of the R8 (Freeman 1997; Dominguez *et al.* 1998). To determine whether *bap170* genetically behaves as a negative regulator of the EGFR pathway in retinal differentiation, we tested whether *bap170* mutations could suppress the ommatidial phenotypes caused by viable hypomorphic mutations in components of the EGFR pathway. Downregulation of the Egf receptor, using the viable allelic combination *Egfr^{f24}/Egfr$^{(T1)}$*, generates adult flies with severe rough eyes, which show a reduced number of PRs per ommatidium (Figure 4B). By halving the amount of *bap170* in these flies, the order of ommatidial facets, as well as the average number of PRs, are partially rescued (Figure 4C). *bap170^{hfl1}* can also suppress the eye roughness and the loss of R7 cells caused by the expression of a dominant negative form of Ras1 in R7 cells (*sev-Ras^{N17}*) (Figure 4, D and E). When tested with *Raf^{HM7}*, a temperature-sensitive *Raf* allele, *bap170^{hfl1}* dominantly rescues the severe lack of photoreceptors caused by *Raf^{HM7}* at 18° (compare F and G in Figure 4) and, partially, the lethality of *Raf^{HM7}/Y* males at 25° (not shown). Finally, *bap170^{hfl1}* can also dominantly suppress the loss of PRs caused by a viable combination of *pnt* alleles (compare H

homozygous mutant larvae at ~ −10 hr APF. Western blots were blotted with the previously described anti-Bap170 or anti-Bap180 antibodies (Carrera *et al.* 2008). As control of loaded extracts, the filters were also blotted with anti-*Mfl* antibodies, recognizing the ubiquitous 75-kDa rRNA-pseudouridine-synthase of Drosophila.

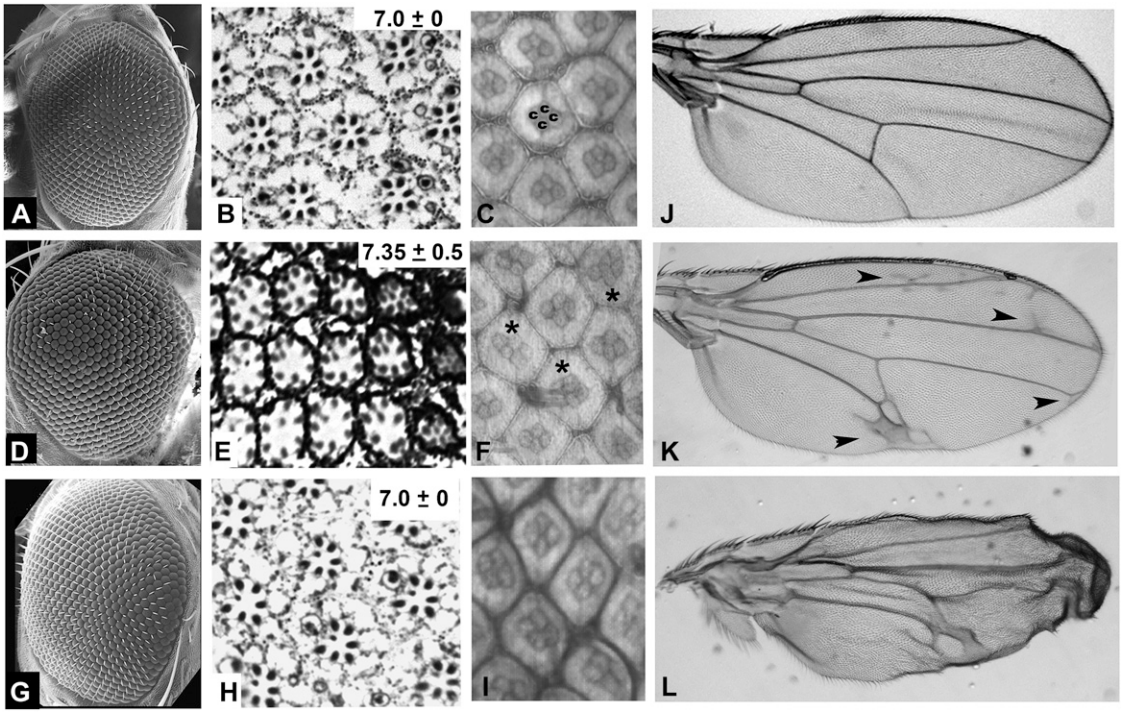

FIGURE 3.—Phenotypes generated by Bap170 depletion in adult eyes and wings. Scanning electron micrographs of adult eyes (A, D, and G); apical sections through adult retinas (B, E, and H); pupal retinas stained with cobalt sulphide (C, F, and I). Wild-type tissues (A, B, and C); mutant *bap170*^{hfl1} tissues (D, E, and F); and eye tissues from *bap170*^{hfl1} mutants rescued by ubiquitous expression of Bap170 (G, H, and I). *bap170* mutant eyes were obtained from *bap170*^{hfl1}*/bap170*^{hfl1}*; dll*-GAL4*/UAS-bap170* flies in which Bap170 function was rescued in some tissues but not in eye primordia (see MATERIALS AND METHODS), whereas rescued control eyes were obtained from *bap170*^{hfl1}*/ bap170*^{hfl1}*; tub-GAL4/UAS-bap170* flies. *bap170*^{hfl1} mutant eyes are rough and larger (D) than wild-type eyes (A). Mutant ommatidia often contain extra photoreceptor cells, with an ANP = 7.35 ($n = 100$) (ANP, average number of PRs/ommatidium) (E), rather than 7.0 PRs/ommatidium as in wild-type eyes (B). Cobalt-sulfide staining of pupal eyes reveals that 30% of *bap170*^{hfl1} ommatidia have five cone cells (asterisks in F), instead of four cone cells as in wild-type eyes (C; c, cone cells).The eye morphology (G), and the number of photoreceptors (H) and cone cells (I) are restored to wild-type condition in *bap170*^{hfl1} mutants rescued by ubiquitous Bap170 expression. Effects of RNAi-mediated depletion of Bap170 using the T80-GAL4 driver on wings phenotype. Adult wings of *T80*-GAL4/+ flies display veins arranged in a normal stereotyped pattern as in wild type (J), while *T80-GAL4;* UAS-*bap170RNAi* animals show extra vein tissues (arrowheads in K). Two doses of *UAS-bap170RNAi*, driven by *T80*-GAL4, increases the extra vein phenotype (L).

and I in Figure 4). Therefore, PBAP appears to antagonize EGFR signaling during PR recruitment. To test whether BAP170 antagonizes the EGFR signaling also in wing discs, we performed genetic interaction analyses between null *bap170* alleles and mutations in genes involved in vein/intervein development (here we report only the results for *bap170*^{hfl1}, but *bap170*$^{\Delta 135}$ gave identical results, not shown). In some hypomorphic mutants for components of EGFR signaling, activation of the Mapk pathway in presumptive vein cells is prevented and veins fail to differentiate. In mutant flies for the hypomorphic allelic combination of the EGFR ligand *vein*, *vn*^{C221}*/vn*^{1}, the L4 vein fails to differentiate (Figure 5C). Conversely, *bap170*^{hfl1}*/+;vn*^{C221}*/vn*^{1} flies develop almost completely the L4 veins (Figure 5D). Similarly, the lack of L4 vein caused by allelic combination of Egf receptor, *Egfr*^{f24}*/Egfr*$^{(T1)}$ (Figure 5K), is fully rescued in *Egfr*^{f24}, *bap170*$^{hfl1)}$*/Egfr*$^{(T1)}$*,+* flies (Figure 5L). This rescue is associated with recovered expression of *rho* (Figure 5, M and N) and Mapk activation in L4 (Figure 5, O and P), indicating that reduced levels of Bap170 can induce a complete restoration of EGFR signaling. This role of BAP170 as inhibitor of vein differentiation is also demonstrated by interaction with *blistered*, a gene required to repress EGFR activity in the interveins. Thus, both the extra veins phenotype and wing size reduction characteristic of *bs*^{2}*/bs*^{2} mutants are enhanced by halving the dose of *bap170* (compare Figure 5, E and F). The interaction with *knirps* supports the view that PBAP might antagonize vein-promoting activity downstream of the A/P patterning. The transcription factors encoded by *knirps* locus, expressed along the L2 provein in response to the antero–posterior subdivision of the wing discs, have as a direct target the activation of *rho* in the L2. The *kni*^{ri1} allele, a deletion of the L2 enhancer element that abolishes *kni* expression in L2, causes loss of *rho* activation in L2 and the lack of the second vein (Figure 5G) (LUNDE *et al.* 2003). Conversely, in *bap170*^{hfl1}*/+;kni*$^{ri\text{-}1}$*/kni*$^{ri\text{-}1}$ flies, the L2 vein is nearly completely restored (Figure 5H), demonstrating that the BAP170-mediated repression of the EGFR activity operates downstream or in parallel to *kni*. Interestingly, when both null alleles of *bap170* were each tested with

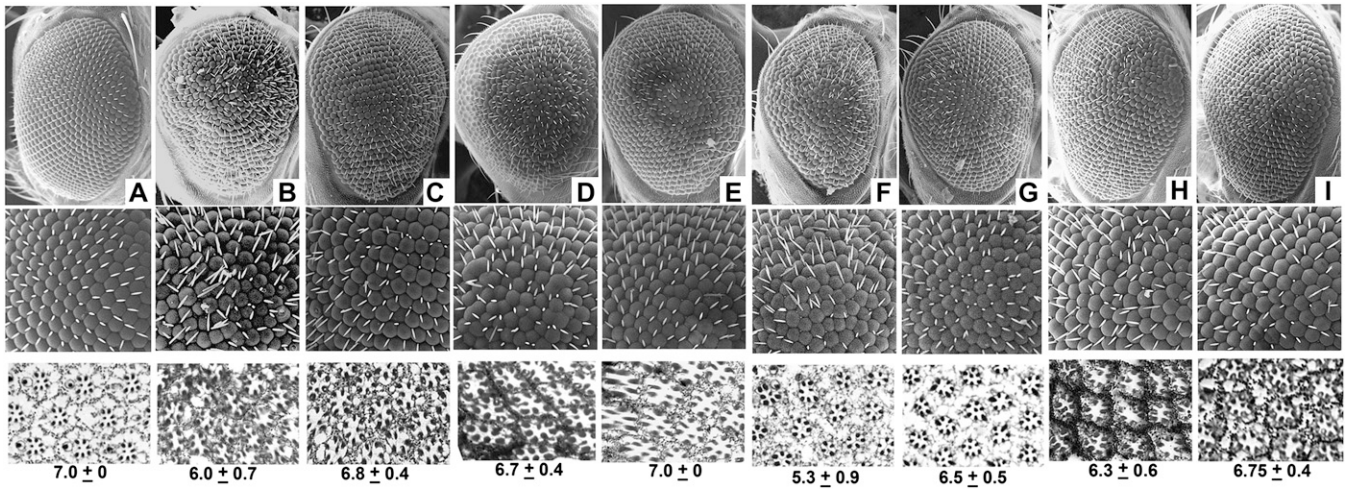

Figure 4.—*bap170* genetically interacts with components of the EGFR signaling during eye development. Scanning electron micrographs of adult eyes (top) and their relative apical sections through retinas (bottom), with indicated the average number of PRs/ommatidium (ANP). A wild-type eye possesses ~750 ommatidia arranged in a highly ordered scheme and a characteristic pattern of seven rhabdomeres within each ommatidium (A). $Egfr^{T1}/Egfr^{f24}$ mutant flies have rough eyes with a reduced number of photoreceptor cells (ANP = 6.0) (B), and both phenotypes are significantly rescued when flies are also heterozygous for $bap170^{hfl1}$ (ANP = 6.8) (C). Overexpression of Ras^{N17} under the control of the *sevenless* enhancer (*sev-*Ras^{N17}/+) generates a weak irregular eye surface and the lack of many R7 cells (D), and both phenotypes are fully suppressed in *sev-*Ras^{N17}/+; $bap170^{hfl1}$ eyes (E). The eye roughness and severe reduction of the photoreceptor cell number of raf^{HM7}/Y flies raised at 18° (ANP = 5.3) (F) are both significantly recovered in raf^{HM7}/Y; $bap170^{hfl1}$/+ adult flies (ANP = 6.5) (G). Ommatidia within adult $pnt^{\Delta 88}/pnt^{1277}$ eyes display an average of 6.3 PRs/ommatidium (H), which is rescued to 6.75 in $bap170^{hfl1}$/+; $pnt^{\Delta 88}/pnt^{1277}$ adult eyes (I).

rho^{ve}, a viable allele of *rho*, which causes the loss of most parts of the L5, L4, and the distal portion of the L3 vein (Figure 5I), no appreciable rescue of vein tissues was observed (Figure 5J). These data indicate that *bap170* genetically acts downstream or in parallel to *kni*, but upstream *rho* during vein specification. Together, the phenotype of *bap170* loss-of-function alleles and the genetic interactions with several members of the EGFR pathway suggest that PBAP participates in the negative regulation of EGFR signaling during eye and wing vein development.

Expression of EGFR targets and intervein genes in *bap170* mutant background: To further analyze the role of PBAP on EGFR signaling, we studied the expression of several genes involved in the regulation of veins and interveins development in *bap170* mutant background. According with epistatic analyses, we first looked at the expression of *rho*, as it behaves as a potential target of PBAP function. *rho* is expressed along the future vein cells just before the appearance of EGFR-induced Mapk activation (Gabay *et al.* 1997), (Figure 6, A and E). Upon its expression, *rho* becomes a trigger and subsequently a target of EGFR signaling, participating in a positive feedback loop that boosts EGFR activation (Roch *et al.* 1998). We found that, in $bap170^{hfl1}$ wing discs, *rho* is expressed at higher than normal levels along the entire normal *rho* expression pattern (presumptive veins and the wing margin), and ectopically at random locations in intervein areas (Figure 6, B and C). Partial depletion of Bap170 by RNAi (Figure 6G) essentially confirms this result. Activation of an UAS-*Bap170RNAi* transgene in the dorsal region of the wing disc, using the *MS1096*-GAL4 line, causes a high level of *rho* expression in the dorsal half of the wing pouch both in the veins, in the dorsal row at the D/V boundary, and, randomly, in some areas of interveins (compare Figure 6, E and F). Upregulation and ectopic expression of *rho* is also evident in eye discs of $bap170^{hfl1}$mutants, especially behind the morphogenetic furrow (compare Figure 6, D and H). Expression analysis of *Delta*, another gene activated in veins by EGFR, confirms the upregulation of EGFR signaling in $bap170^{hfl1}$ wing discs (Figure 6, I and J). Because the restriction of *rho* expression to the veins also depends on the transcriptional repressor Net, acting in the interveins (Brentrup *et al.* 2000), and on E(spl)mβ, acting at the boundaries between veins and interveins (de Celis *et al.* 1997; Sotillos and de Celis 2005), we asked whether the expression of these genes was compromised in *bap170* mutant discs. We also analyzed the expression of *blistered*, which is required for repression of *rho* in interveins and for intervein cell differentiation (Montagne *et al.* 1996). In wild-type discs, the expression of these genes is restricted to intervein cells in part as a consequence of their repression in veins by EGFR signaling (Figure 6, K, M, and O). We found that the maximum accumulation levels of *net* or *E(spl)m*β mRNAs, as well Bs protein, are not reduced by loss of Bap170 (compare Figure 6, K and L; Figure 6, M and N; Figure 6, O and P), indicating that the PBAP is not required for proper expression of these genes. A weak reduction of *net*, *E(spl)m*β, and *bs* expression is only detectable in the proximity of the

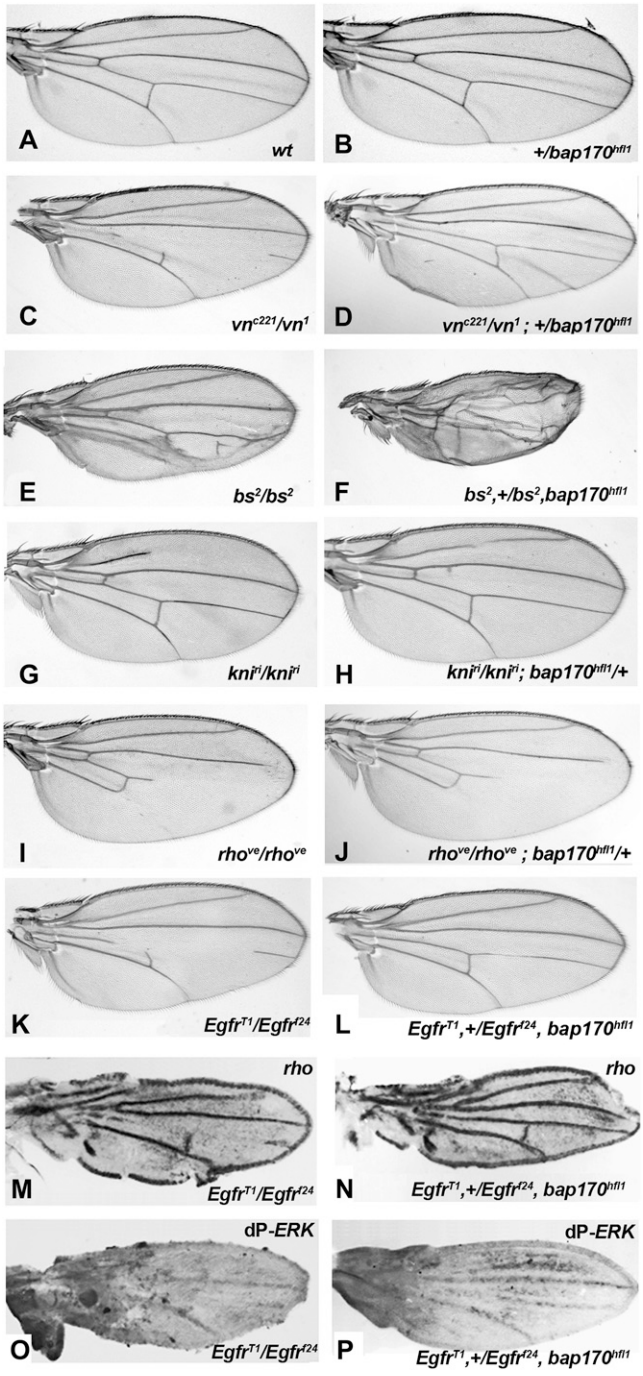

FIGURE 5.—*bap170* genetically interacts with components of the EGFR signaling in wing veins differentiation. Wings from wild-type (A) and heterozygous +/*bap170*^{hfl1} flies (B) show the normal veins patterning. The lack of the L4 vein caused by *vn*^{1}/*vn*^{c221} allelic combination of the EGFR ligand *vein* (C) is dominantly rescued in wings of *bap170*^{hfl1}/+; *vn*^{1}/*vn*^{c221} flies (90% of cases on 110 wings) (D). A *bs*^{2}/*bs*^{2} wing showing ectopic vein tissues in the distal region of the L3, L4, and L5 veins (E). Homozygous *bs*^{2} wing, which is also heterozygous for *bap170*^{hfl1}, shows enhancement of the *bs* phenotype (F). In wings of *knir*^{i}/*knir*^{i} homozygous flies the distal part of the L2 vein is missing (G), whereas in *knir*^{i}/*knir*^{i}; *bap170*^{hfl1}/+ wings the L2 vein is nearly complete (95% of cases on 100 wings) (H). In *rho*^{ve}/*rho*^{ve} adults the wings lack the distal portion of the L3 and most of the L4 and L5 veins (I). When these flies are also heterozygous for *bap170*^{hfl1} no appreciable rescue of vein differentiation can be detected (J). In *Egfr*^{T1}/*Egfr*^{f24} wings, most of the L4 vein is lost (K) by local deficit of EGFR signaling activity, as shown by the lack of *rho* transcription (M) and dP-ERK accumulation (O) in pupal wings. In *Egfr*^{T1},+/*Egfr*^{f24}, *bap170*^{hfl1} flies, the L4 vein differentiation is completely restored (85% of cases on 100 wings) (L), as well as *rho* expression (N) and dP-ERK levels (P) in L4.

veins, where, it is likely, that they are repressed by a localized ectopic activation of *rho*/EGFR signaling occurring in *bap170* mutant discs (Figure 6, L, N, and P). In addition, Net- and E(spl)mβ-mediated repression of *rho* is not functionally affected by loss of Bap170. Ectopically expressed Net can efficiently repress *rho* in both *bap170*^{hfl1} as well in wild-type wing discs (Figure 6, Q and R), and the same effect can be obtained by ectopic E(spl)mβ expression (not shown). This suggests that the PBAP complex is not required for Net or E(spl)mβ function. Another general inhibitor of EGFR signaling, the expression of which is also activated by the pathway, is the EGFR ligand-antagonist encoded by *argos* (SCHWEITZER *et al.* 1995; GOLEMBO *et al.* 1996). Interestingly, we found that, in wing discs of *bap170*^{hfl1} mutants, but also in several other tissues of mutant larvae such as eye discs and brains (not shown), the expression of the *argos* is severely compromised (Figure 6, S and T). This is surprising given that *argos* is normally activated by the EGFR pathway and that the high level of EGFR signaling in *bap170* wing discs should result in higher than normal levels of *argos* transcription. Quantitative real time RT–PCR performed on wing discs from *wild-type* and *bap170*^{hfl1} mutant larvae staged at −2 hr APF essentially confirm the upregulation of *rho* and the downregulation of *argos* expression in *bap170*^{hfl1} mutants (Figure 8) as determined by *in situ* approaches. The uncoupled response of *argos* from *rho* and *Dl* suggests that the PBAP complex, together with the EGFR pathway, are required for proper *argos* regulation. This observation suggests that the upregulation of *rho*/Dl/EGFR seen in *bap170* mutants might just be the consequence of insufficient Argos levels to antagonize the activity of the EGFR pathway. Since *argos* expression is not completely abolished by loss of Bap170 (Figure 6T), and given that the expression of an activated form of *Ras1* (*Ras*^{V12}) can efficiently induce *argos* transcription in *bap170* mutant wing discs (not shown), it can be argued that the PBAP complex might participate in the enhancement of *argos* expression rather than in its activation, perhaps by ensuring proper availability of argos regulatory regions to specific activators.

Bap170 can repress *rho* in condition of low EGFR activity: Although the deficit of *argos* expression could explain the range of phenotypes observed in *bap170* mutant eyes and wings, our epistatic analyses of vein phenotypes also suggested a direct role of PBAP as repressor of *rho*. To check this possibility we decided to perform overexpression experiments of Bap170. Local

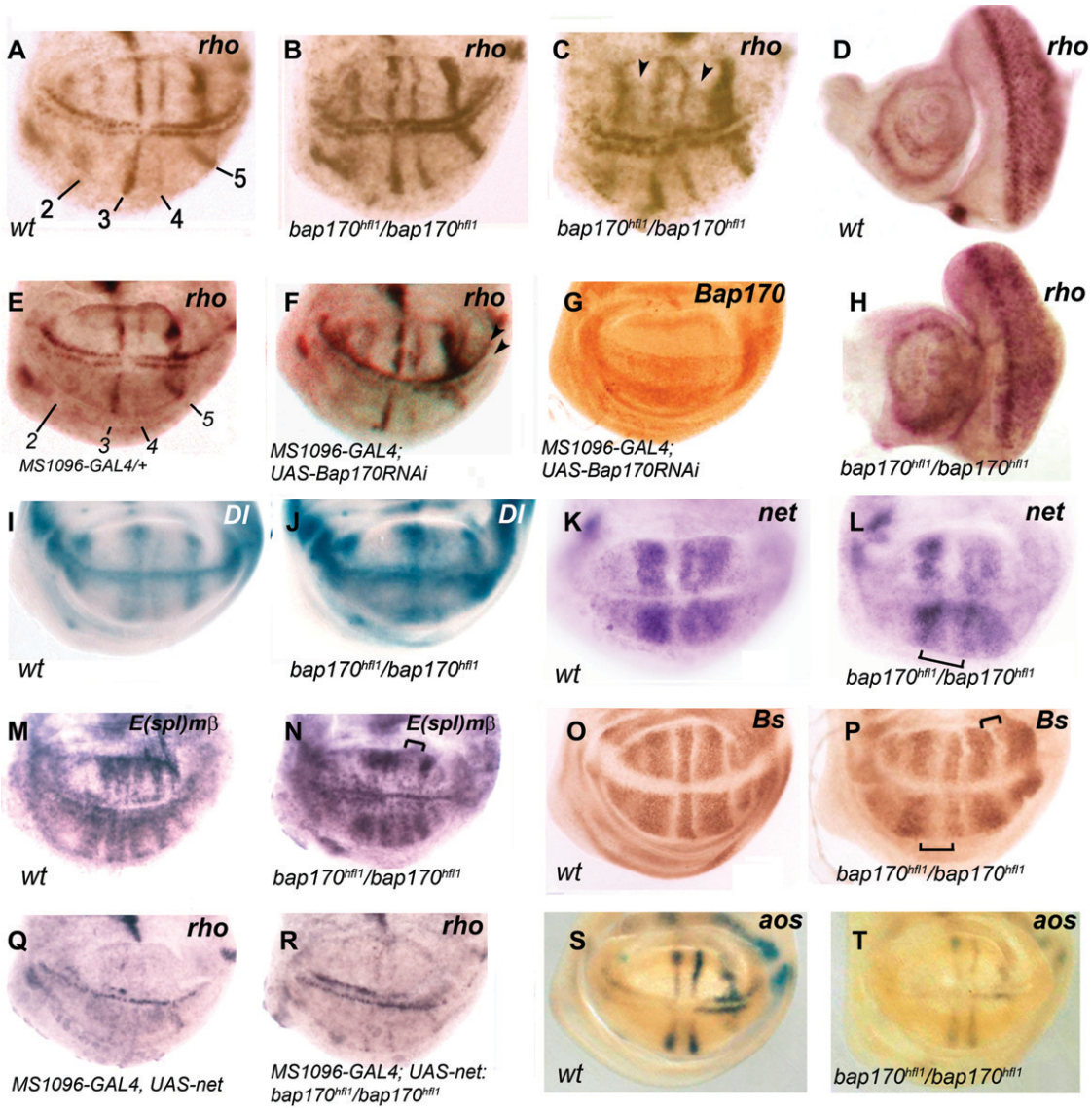

FIGURE 6.—Expression of EGFR targets and intervein genes in *bap170* mutant discs. Expression of *rhomboid* in wing discs of late third instar wild-type (A) and $bap170^{hfl1}/bap170^{hfl1}$ (B and C) larvae. (A) Typical expression pattern of *rhomboid* in the presumptive wing veins (L2–L5 are indicated), and at the wing margin. (B and C) Two wing discs from *bap170* mutants larvae showing *rho* expressed at higher than normal level in presumptive veins but also in intervein areas (arrowheads in C). In eye discs of $bap170^{hfl1}/bap170^{hfl1}$ third instar larvae (H) *rho* is expressed ectopically and at higher level than in wild-type eye discs (D). (E) Normal *rho* expression in wing disc of *MS1096-GAL4/+* mid third instar larva. (F and G) Wing discs of *MS1096*-GAL4/+; *UAS-bap170RNAi/+* larvae at mid third instar when the *MS1096*-GAL4 is strongly expressed at the dorsal compartment. (F) Partial depletion of *bap170* in the dorsal region of the wing disc causes dorsally restricted upregulation of *rho* in the veins, at the dorsal row of cells at the D/V boundary (dorsal and ventral rows are indicated by arrowheads) and ectopic expression in few intervein cells. (G) Staining with anti-BAP170 antibody, which shows the reduced level of BAP170 in the dorsal area of the wing pouch in *MS1096*-GAL4/+; *UAS-bap170RNAi/+* discs. (I and J) *Delta* expression monitored by β-gal staining in wing discs of control $Dl\text{-}LacZ^{05151}/+$ (I) and $bap170^{hfl1}/bap170^{hfl1}$; $Dl\text{-}LacZ^{05151}/+$ (J) third instar larvae, at −10 hr APF. Expressions of *net* (K and L), *E(spl)m*β (M and N), and Bs (O and P) in wing discs of wild-type (K, M, and O) or $bap170^{hfl1}/bap170^{hfl1}$ (L, N, and P) third instar larvae. The maximum expression levels of these genes are not compromised by loss of Bap170, except nearby the veins (brackets in L, N, and P) and in some intervein areas. (Q and R) *rho* expression in late third instar wing discs from *MS1096*-GAL4/+;UAS-*net*/+ (Q) or *MS1096*-GAL4/+; $bap170^{hfl1}/bap170^{hfl1}$; UAS-*net*/+ (R) larvae. At this stage the expression of the *MS1096*-GAL4 driver is extended in the entire wing pouch. Ectopic expression of Net efficiently represses *rho* transcription in both wild-type and $bap170^{hfl1}/bap170^{hfl1}$ genetic background. *argos* expression in wing disc of control $aos\text{-}lacZ^{05845}/+$ (S) and $bap170^{hfl1}/bap170^{hfl1}$; $aos\text{-}lacZ^{05845}/+$ (T) late third instar larvae. Identical results were obtained using the $aos\text{-}lacZ^{W11}$ enhancer trap line.

or ubiquitous overexpression of Bap170 failed to induce any visible phenotypes in wild-type flies, as a probable consequence of limited amounts of Sayp and/or Bap180 in wild-type context, which might physically prevent the Bap170 excesses to be functional. However, it is also possible that EGFR signaling might have a

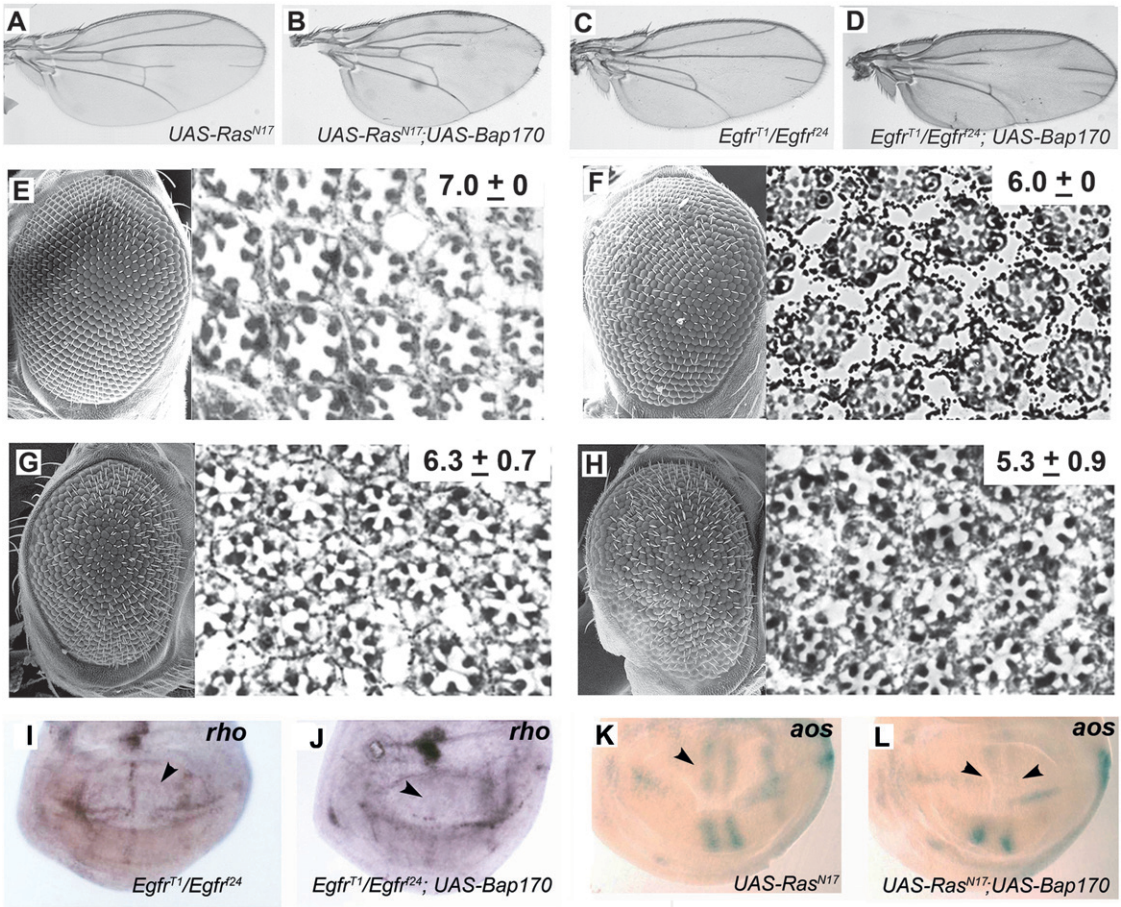

Figure 7.—Phenotype of Bap170 overexpression. (A) *omb*-GAL4/UAS-*Ras*^{N17} control female wing. Expression of the dominant-negative *Ras*^{N17} in the *omb* domain causes the loss of the central part of the L4 vein. (B) The simultaneous expression of *Ras*^{N17} and Bap170 in *omb* domain (*omb*-GAL4/UAS-*Ras*^{N17};*UAS-bap170/+* female wing) induces loss of the entire L4 vein emanating from the posterior crossvein and the lack of the distal end of the L3. A further reduction of wing surface and occasionally notching at the margins can be observed. (C) *MS1096*-GAL4/+; *Egfr*^{f24}/*Egfr*^{T1} control female wing showing the typical loss of most of the L4 vein characteristic of the *Egfr*^{f24}/*Egfr*^{T1} phenotype (compare with Figure 5K). (D) In *MS1096*-GAL4/+; *Egfr*^{f24}/*Egfr*^{T1}; *UAS-bap170/+* female wings, the L4 vein is completely abolished and the central region of the L3 does not differentiate. (E) *Ras85D*^{e1B}/*sev*-GAL4 control eyes showing a wild-type morphology and regular number of rhabdomeres (ANP = 7.0). (F) *Ras85D*^{e1B}/*sev*-GAL4; *UAS-bap170/+* flies displays rough eyes and loss of all R7 photoreceptors within ommatidia (ANP = 6.0). (G) *elav*-GAL4/+; *pnt*$^{\Delta 88}$/*pnt*^{1277} control eyes are rough with an average of 6.3 PR cells per ommatidium. (H) *elav*-GAL4/*UAS-bap170*; *pnt*$^{\Delta 88}$/*pnt*^{1277} eyes display increased roughness and further reduction of PR cells (ANP = 5.3). (I) Mid third instar expression of *rho* mRNA in wing discs of control *MS1096*-GAL4; *Egfr*^{f24}/*Egfr*^{T1} larvae. The lack of *rho* expression in L4 vein is indicated (arrowhead). (J) Wing discs from *MS1096*-GAL4; *Egfr*^{f24}/*Egfr*^{T1}; *UAS-bap170/+* larvae at the same stage. Bap170 overexpression in the dorsal half of the wing pouch by the *MS1096*-GAL4 driver causes a reduction of *rho* expression in the dorsal half of L3 vein (arrowhead) and in the dorsal row of cells at the wing margin. (K) Wing discs from *MS1096*-GAL4/*UAS-Ras*^{N17}; *aos-LacZ/+* third instar larvae showing the reduced *argos* reporter expression in the dorsal half of the wing blade. (L) In wing discs of *MS1096*-GAL4/*UAS-Ras*^{N17}; *aos-LacZ/UAS-bap170* third instar larvae argos reporter expression in the dorsal part of the wing pouch and at the D/V boundary is further reduced.

mechanism to relief PBAP mediated-repression of *rho* in EGFR-expressing cells. To verify this possibility, we decided to overexpress Bap170 in a genetic context of reduced EGFR signaling. When Bap170 was overexpressed in wing or eye discs with insufficient EGFR activity (*Ras*^{N17}, *Egfr*^{f24}/*Egfr*$^{(T1)}$, *Ras85D*^{e1B}/+ or *pnt*$^{\Delta 88}$/*pnt*^{1277}), it causes an increase of the typical EGFR downregulation phenotype (Figure 7). In the wing discs of the corresponding genetic combinations we observed extra repression of both *rho* (compare Figure 7, I and J) and *argos* (compare Figure 7, K and L). Downregulation of both *rho* and *argos* by overexpressed Bap170 in condition of low EGFR activity was also confirmed by quantitative RT–PCR analyses in wing discs of third instar larvae staged at −2 hr APF (Figure 8). These results can be explained considering that Bap170, in addition to its role to ensure a proper level of *argos* expression, can antagonize the EGFR signaling also by participating in the repression of *rho* transcription. The repression of *rho* by overexpressed Bap170 would be sufficient to reduce the activity of the entire EGFR signaling including the *argos* expression. These results

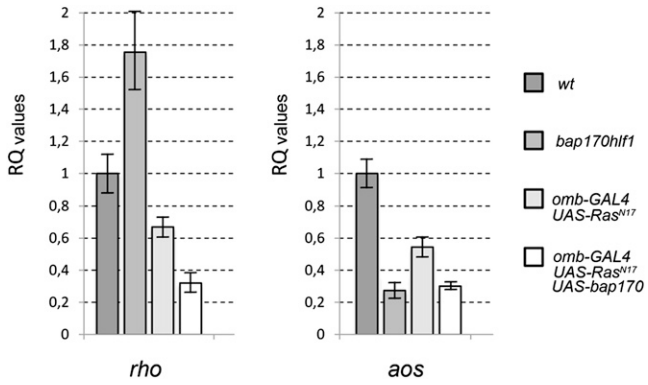

Figure 8.—Relative quantification by real time RT–PCR of *rho* and *argos* mRNA levels in wing discs of indicated genotypes referred to the expression of the ubiquitous expressed gene *mfl*. For each genotype (*wt*, *bap170^{hfl1}/bap170^{hfl1}*, *omb-GAL4, UAS-Ras^{N17}*, or *omb-GAL4, UAS-Ras^{N17}; UAS-Bap170*) three RNA samples (10 wing discs each) were collected and independently reverse transcribed with oligo-dT primers. Real-time PCRs were performed with using SYBR green-based quantification method (Applied Biosystems); PCR amplification efficiencies were determined for each gene and ΔΔCT relative quantification was done using *mfl* gene expression as internal control to normalize the results.

also suggest that the Bap170-mediated repression of *rho* is antagonized by the EGFR pathway, given that this effect is only detectable by reducing the EGFR activity. Hence, we propose that the EGFR-related phenotypes of Bap170 mutants are the result of the simultaneous failure of the PBAP complex to repress *rho* and activate *argos*. It remains to be established whether PBAP regulates these genes through direct binding to their regulatory sequences or indirectly by controlling the expression of other essential EGFR regulators.

CONCLUSIONS

Genetic analysis in flies has allowed definition of Brm complex requirement in specific developmental programs such as those mediated by segmentation genes (Brizuela and Kennison 1997; Treisman *et al.* 1997), homeotic genes (Tamkun *et al.* 1992; Vazquez *et al.* 1999), and patterning genes (Collins and Treisman 2000). Genetic links between the function of Brahma complex and developmental programs regulated by the EGFR signaling pathway have also been established. Analyses of the phenotypes generated by a dominant-negative form of Brm (*brm^{K804R}*) or by the temperature-sensitive allele of *snr1* (*snr1^{E1}*) demonstrated a key role of Brm complex in wing vein patterning and the differential ability of the complex to function as either activator or repressor of *rho* expression in wing cells (Elfring *et al.* 1998; Collins *et al.* 1999; Marenda *et al.* 2004). The recent identification of BAP and PBAP as different forms of the Brahma complex, displaying distinct but in part antagonistic functions, sheds light on the dual role played by the Brahma complex on EGFR signaling. BAP and PBAP share a common core complex, which includes Brahma and Snr1, but are distinguished by the addition of Osa for BAP, and of Bap170, Bap180/Polybromo, and Sayp for PBAP. Therefore, it is likely that previous analyses on *brm* and *snr1* mutants recorded the phenotypes arising from the simultaneous loss of BAP and PBAP. The recent finding that Osa is required for proper expression of EGFR target genes in wing disc has shown that, among the two complexes, BAP is the form that participates in the positive regulation of the EGFR pathway (Terriente-Felix and de Celis 2009). Our analysis of *bap170* mutants demonstrates that the PBAP has an opposite role, being involved in the negative regulation of EGFR signaling through the transcriptional sustainment of *argos* and the repression of *rhomboid*. A possible model to explain this dual role of PBAP is to assume that the complex might switch from the repression of *rho* to the activation of *argos* in response to the level of EGFR activity. In cells with low EGFR activity, the PBAP would participate in the repression of *rho* transcription, whereas in cells with hyperactivated EGFR, the signaling would relief the PBAP-mediated repression on *rho* but would "utilize" the PBAP complex for the positive regulation of *argos* expression. Through the control of *rho* and *argos* expressions the PBAP complex became part of the transcriptional machinery, which regulates the autoregulatory loops of the EGFR signaling. It is possible that high levels of the EGFR signaling might sequestrate/inactivate potential Bap170/PBAP interacting factor/s required for *rho* repression, whereas low EGFR activity will allow these factor/s to interact with the Bap170/PBAP and repress *rho*. In several developmental contexts the MAPK/EGFR signaling derepresses and stimulates target gene expression through the phosphorylation of the Yan repressor and Pnt-P2 activator, respectively. Although it is possible that in eye discs the switch of the PBAP function might be mediated by potential interactions between PBAP and Pnt-P2 or Yan, a clear role of these two transcriptional regulators in wing vein development has never been demonstrated. However, other regulators of the EGFR targets such as *groucho*, *capicua*, and *atro* might execute this function in wing discs. Finally, because all signature subunits of BAP and PBAP are ubiquitous in imaginal disc cells but the two complexes have opposite roles on the EGFR signaling regulation, it is evident that the activities of the two forms must be tightly regulated in space and time. Although further studies are required to delineate the functional relationship between BAP and PBAP on EGFR pathway, it is possible that the EGFR signaling itself, perhaps by modulating the phosphorylation levels of key components of the complexes, might regulate the shift between the two forms and/or between different states of the two complexes.

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Bap170, a Subunit of the Drosophila PBAP Chromatin Remodeling Complex, Negatively Regulates the EGFR Signaling

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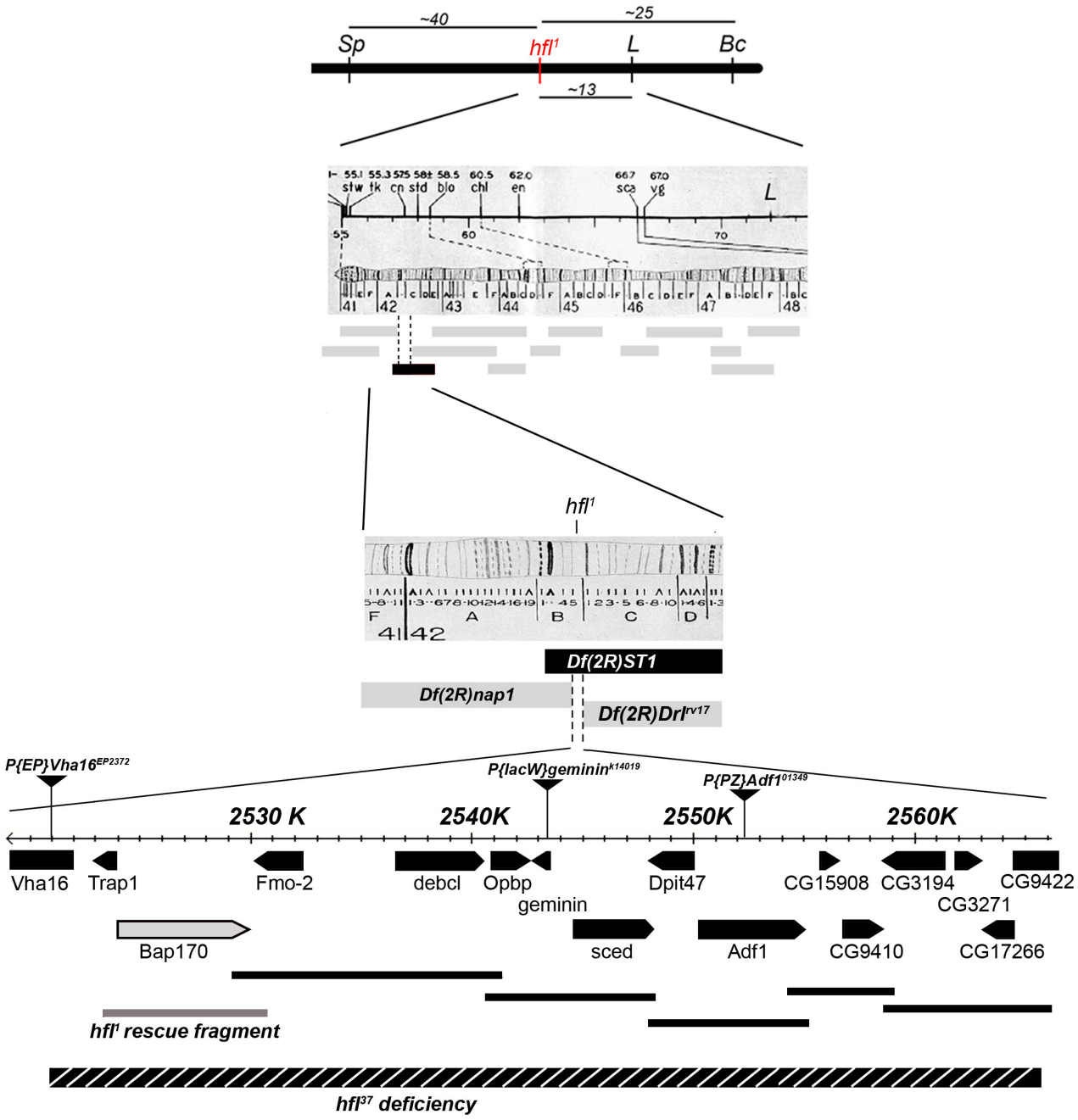

FIGURE S1.—Genetic and molecular approaches used to map the *hfl1* mutation. (Top) Genetic map of *hlf1* relative to the dominant alleles *Lobe* (*L*), *Black cells* (*Bc*) and *Sternopleural* (*Sp* or wg^{Sp1}). (Middle) Complementation analysis of *hlf1* with a series of chromosomal deficiencies covering the polytenic region between the 48 division and the centromere of the second chromosome. Grey bars represent the complementing deficiencies and the black bar indicates the non-complementing *Df(2R)ST1* deficiency. (Bottom) Molecular map of the putative interval between the distal breakpoint of *Df(2R)nap1* and the proximal breakpoint of the $Df(2R)Drl^{rv17}$. The black triangles on molecular map represent the positions of three of the five P-element insertions used to generate local deficiencies by P-mediated imprecise excision experiments. To obtain excision events, disgenic P/CyO; P(Δ2-3), Sb/+ flies (in ry^{506} or w^{1118} genetic background, depending on the P dominant marker) were crossed with a w^{1118}; *CyO/Sco* or *CyO/Sco;* ry^{506} mapping strain and, among the non-Sb progeny, single Cy non Sco flies, lacking the P dominant marker (excision event), were recovered and crossed again with the mapping strain to obtain a stock. About 40 excision events per P-element were recovered and each tested for their ability to complement *hfl1* mutation. Only one non-complementing excision event (obtained with the $P(EP)Vha16^{EP2372}$) was recovered and named hfl^{37}. Molecular mapping of hfl^{37} breakpoints (seee material and methods) allowed to determine its extension (represented at the bottom of the figure). The lines below the molecular map represent the genomic fragments used for transgene-mediated rescue of hlf1 mutants (see material and methods). Black lines represent the non-rescuing fragments; the grey line indicates the rescuing fragment containing the *bap170* gene.

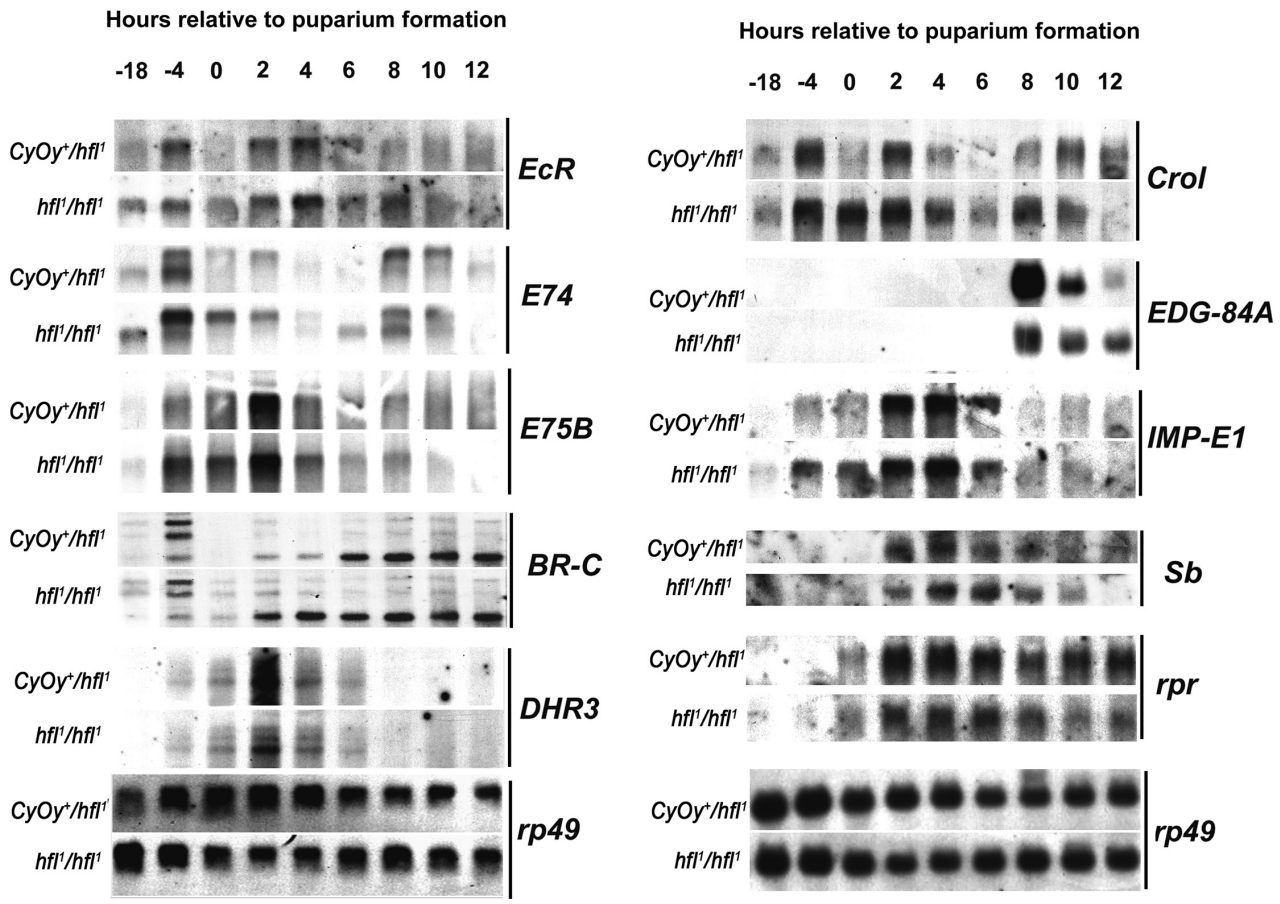

FIGURE S2.—*PBAP* is dispensable for transcriptional regulation of a subset of ecdysone primary-responsive genes during larval-prepupal period. A recent microarray analysis carried out in double $bap170^{D65}$/ $bap180^{D86}$ mutants at puparium formation showed altered expression of genes involved in morphogenesis and immune response, but no correlation with genes regulated by ecdysone receptor (Carrera et al. 2008). However, because the analysis was performed at the fixed stage of white prepupa (0h after puparium formation APF), it might have left unnoticed potential qualitative or quantitative variations for genes which possess dynamic expression throughout larval-prepupal period. In this period, many ecdysone regulated genes can switch to different mRNA species and/or display reiterative cycles of activation and repression (Segraves, 1994; Russel and Ashburner, 1996; Thummel, 1996; Richards, 1997). Using $Bap170^{hfl1}$ mutants, we performed time-course Northern blot experiments to analyze the expression of a subset of ecdysone responsive genes, including direct targets of EcR. We found that, during the larval-prepupal period the expression *BR-C*, *E74*, *E75B*, *DHR3*, *EcR*, *E78*, *crol*, *rpr*, *Sb*, *IMP-E1* and *EDG-84A* (Fig. S1), is substantially normal in $bap170^{hfl1}$ mutant background, thus confirming previous analyses in $bap170^{D65}$/ $bap180^{D86}$ mutants (Carrera et al. 2008).

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