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 *D. melanogaster* Brahma-chromatin remodelers. A common multisubunit-core, containing the Brahma ATP-ase, can associate either with Osa to form the BAP complex, or with Bap170, Bap180 and Sayp to constitute the PBAP one. 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 hyper-activation of the EGFR pathway, such as over-recruitment 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, being required for transcriptional repression of *rhomboid* and for efficient expression of *argos*. Interestingly, genetic evidences also indicate that Bap170-mediated repression of *rho* is inhibited by the EGFR signaling, suggesting a scenario of mutual antagonism between EGFR signaling and PBAP function.
INTRODUCTION

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 ligands-processing activity, represents the limiting factor regulating the spatial and temporal hyper-activation 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, the EGFR signaling usually involves a common cytoplasmic transduction cascade which includes Ras, Raf, MEK and the 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., 1995).
1994). This allows EGFR targets to be relieved from Yan-mediated repression, and be induced by the Pnt-P2 activation function.

Although much is known about the mechanisms which trigger the precise spatial and temporal activation of the EGFR signaling, still limited is the knowledge about the mechanisms which, 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 de-repression of a considerable number of genes in distinct developmental settings (Hasson et al., 2006).

Also, some connections between EGRF signaling and the function of another widespread used transcriptional machinery, the Brahma (Brm) chromatin remodeling complex, have been described in Drosophila. Thus, in addition to the documented role played by Brm complex as positive regulators of Hox genes (Kennison and Tamkun, 1988; Papoulas et al., 1998; Vazquez et al., 1999; Collins 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 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 Brm complex on EGFR signaling might be in part explained 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 role on EGFR target genes 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 genes regulation in wing discs have better defined the role of the 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 the 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 discs 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 hyper-activation of the EGFR signaling, such as over-recruitment 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 up-regulation of *rho* and down-regulation 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 proper level of *argos* expression. Interestingly, genetic evidences also indicate that the Bap170-mediated repression of *rho* is inhibited by the EGFR signaling, thus depicting a scenario of a reciprocal regulation between EGFR signaling and PBAP function.
Drosophila strains: The following GAL4 and UAS lines were used: UAS-RasV12 and UAS-RasN17 (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-RasN17 (Karim et al., 1996), sev-GAL4 (Basler et al. 1989), tub-GAL4 (Lee and Luo, 1999). The following alleles and enhancer trap lines were obtained from the Bloomington Stock Center: rhoλv, vnλ221, vn1, EgfrT1, EgfrJ24, bs2, kniλi-1, pnt1277, pntλ88, rafλ7M7, rasλe1B, aos-lacZλ5845, aos-lacZλW11, Dil-LacZλ5151, while bap170λ65 and bap170λ135 mutants were kindly provided by J. Treisman.

Genetic and molecular mapping of half life locus: hlfλ 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. hlfλ 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 to rescue the prepupal lethality, demonstrated that hlfλ mutation was not caused by the PZ- insertion in 25E1. A clean stock carrying hlfλ (CyO/hlfλ; ryλ506), 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/hlfλ; ryλ506 flies, we argued the hlfλ mutation might be 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. hlfλ was genetically mapped at
about 13 cM from \( L \) (\emph{Lobe}) and 23 cM from Bc (Black cells) mutations, toward the centromere (supplemental material Fig. S1). Complementation tests with deficiencies overlapping the region between \emph{Lobe} and the centromere were used to map \emph{hfl} \textsuperscript{i} mutation to the 42B3-C2 cytological region, between the proximal breakpoint of \textit{Df(2R)Drl} \textsuperscript{v17} and the distal breakpoint of the \textit{Df(2R)nap1}, within the \textit{Df(2R)ST1} deficiency. In order to better define the \emph{hfl} \textsuperscript{i} position we thought to mobilize individually single P-element insertions available in the 42B3-C2 region with the aim to generate, by imprecise excisions, small \emph{hfl} \textsuperscript{i}-no-complementing deficiencies. Among 40 excisions recovered for each used P-element (\emph{P[EP]}\textit{Vha16}\textsuperscript{EP2372}, \emph{P[lacW]}\textit{geminin}\textsuperscript{k14019}, \emph{P[PZ]}\textit{l(2)01289}\textsuperscript{01289}, \emph{P[PZ]}\textit{jing} \textsuperscript{01094}), a single event (named \emph{hfl} \textsuperscript{37}), which failed to complement \emph{hfl} \textsuperscript{i}, was obtained using the \emph{P[EP]}\textit{Vha16}\textsuperscript{EP2372} insertion. Complementation analyses with the P lethal insertions mapped in the same region showed that \emph{hfl} \textsuperscript{37} behaves genetically as a deficiency extending at least from the \textit{Vha16} (identified by the \emph{P[EP]}\textit{Vha16}\textsuperscript{EP2372}) and the \textit{Adf1} (identified by the \emph{P[PZ]}\textit{Adf} \textsuperscript{101349}) genes (see Fig.S1). Given that \emph{hfl} \textsuperscript{37} retained both extremity of the PZ elements, as determined by Southern blot hybridizations, inverse PCRs were used to map the \emph{hfl} \textsuperscript{37} limits. This showed that the proximal breakpoints of the \emph{hfl} \textsuperscript{37} deficiency maps within the \textit{Vha16} gene whereas the distal breakpoint is located within the first intron of the CG9422 gene thus extending \emph{hfl} \textsuperscript{37} approximately for 40Kb (Fig.S1). A detailed search at the Flybase genomic database identified, in the \emph{hfl} \textsuperscript{37} deficiency, at least 17 potential candidate gene. Since \emph{hfl} \textsuperscript{i} allele complemented lethal P insertions in the \textit{Vha16}, \textit{geminin} and \textit{Adf1}, these genes were excluded as \emph{hfl} candidate. Thus, to identify the \emph{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 \emph{hfl} \textsuperscript{i} homozygotes (Fig. S1). Only a bap170-containing transgene was able to fully rescue the \emph{hfl} \textsuperscript{i} lethality. The identity between \emph{half life} and \emph{bap170} was also confirmed by the rescue of \emph{hfl}/\emph{hfl} 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 blots analyses, and transgenic lines preparation was 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 -4hrs 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). 35 cycles of PCR amplification were carried out with primer pairs spanning the third (5'-CAGTTGCGGCTACGTTG-3') and the fourth exon (5'-GCGCCAGTGCTAGCTGCC-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 were first reverse transcribed with oligo-dT primers and subsequently PCR-amplified using SYBR green master mix (Applied Biosystems), and the following primers pairs relative to *argos*, *rho*, and, as internal normalizer, to *mfl* gene: aosFor, 5'-TGCATCGCCTCACTCAAGTG-3'; aosRev,5'-CATTTGTTGGCGATCGATT-3'; rhoFor, 5'-ACTGGCCCTGTTCACTCCTA-3'; rhoRev, 5'-GGAACGGGTAGCCGAAT-3'; mflFor, 5'-GCCATGTGGCTGACGAAA-3'; mflRev, 5'-GTAATCTTGCGCCATTAGCA-3'). PCR amplification efficiencies were determined for each gene and $\Delta \Delta CT$ relative quantification were 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 170bp 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 germ-line specific 5.0 kb species since it can be detected by RT-PCR only in early 0-2hrs 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 ARID domain (Fig.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 tub-GAL4 driver. Although we did not investigate the role of this female specific variant, it is likely that it may represent 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^{1135} 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 1.3Kb DNA fragment derived from the 5’ end of bap170 gene was linked in frame upstream the LacZ coding region in the pCasper-βgal vector (Thummel et al.1988). This fragment, which encompasses the genomic region between the exon 1 of trap1 gene to the BamHI site in the exon 2 of bap170 (Fig. 2D), was selected for its ability to rescue to wild type conditions bap170^{hfl1}/bap170^{hfl1} or bap170^{1135}/bap170^{1135} homozygous flies, when used to drive the expression of the bap170 cDNA. The derived Bap170-βGAL fused protein contains the first 194aa N-terminal residues of Bap170 joined to the complete β-galactosidase aminoacid 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 (0h APF) of control dll-GAL4/UAS-GFP and mutant dll-GAL4, bap170<sup>hfl1</sup>/UAS-GFP, bap170<sup>hfl1</sup> individuals. Scanning electron microscopy was performed as described by Kimmel et al. (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 (Wolff and Ready, 1991). Mutant bap170<sup>hfl1</sup> eyes were obtained from dll-GAL4,bap170<sup>hfl1</sup>/bap170<sup>hfl1</sup>;UAS-bap170/+ flies, in which the distalless-GAL4 driver were used to rescue BAP170 function in the distal region of the leg discs, wings margins, antennal but not eye primordia (Cohen et al., 1989, Dong et al., 2000 and Gorfinkiel et al., 1997). The average numbers of photoreceptors per ommatidium (ANP) was determined, for each genotype, on about 100 ommatidia derived from three eyes taken from different organisms. Cobalt sulphide staining of pupal retinas was performed as described by Wolff and Ready on pupae at +60h APF (Wolff and Ready, 1991). In genetic interaction analyses of wing phenotypes, identical results were obtained using either bap170<sup>hfl1</sup> or bap170<sup>1135</sup> allele, whereas bap170<sup>165</sup> mutation generated weak and sporadic interaction effects. Adult wings were dissected from 4-5 day old females of each genotype and mounted in Permount.

**Drosophila immunocytochemistry, in situ hybridizations, and X-gal staining:** Third instar larvae were staged based on 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 1h at 4° and permeabilized in PBS+0.3% Triton-X100 for 2h at 4°. Incubations of primary and secondary antibodies were all carried out at 4° for 12h. 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)mβ* 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-X100 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 in parallel in the same conditions the sample and control tissues (24h at 37°C for *aos-lacZ,* and 2h at 25°C 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 hrs of the prepupal period with no evidence of imaginal discs or head capsule eversion (Fig 1A and 1B). In vivo time-lapse imaging of fluorescently labeled leg and wing discs was used to compare the behavior of wild type and hfl\(^{1}\) mutants discs during the prepupal period (Fig. 1C). This analysis revealed that mutant leg and wing discs normally start the elongation phase (point +4h APF) but, as development proceeds (points +7 to +14 h 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 40kb genomic region between the Vha16 and CG9422 genes on the second chromosome (Materials and Methods and Fig.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 Fig.S1), a gene encoding a multi-domain nuclear protein of 170kD which includes a N-
terminal AT-rich interaction domain (ARID), a region with multiple LXXLL motifs, and two C-terminal C₂H₂ Zn-fingers (Mohrmann et al. 2004 and Fig.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 the 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 discs morphogenesis and the prepupal lethality observed in bap170^{h61} 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 blots analyses on poly(A)$^+$ RNA extracted at several stages during Drosophila development revealed that $bap170$ is constitutively expressed throughout the life cycle (Fig. 2B). No quantitative differences in mRNA accumulation or qualitative variations in the splicing pattern, that could potentially suggest a stage specific regulation of $bap170$, are evident during 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 1.3kb genomic fragment, containing the $bap170$ transcriptional regulatory sequences, to the LacZ gene (Fig. 2D and Materials and Methods). This fragment was selected for its ability to fully rescue the $bap170^{h81}$ 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 germ line 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 (Fig. 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. 2008). The sole exception to the generalized expression in imaginal tissues are the salivary glands cells, in which the β-gal activity is detectable at -18h APF, but disappears at -4h APF (Fig. 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 (Fig. 2C).
bap170^{hfl1} mutants lack both Bap170 and Bap180 subunit of PBAP complex: In a recent study performed to investigate the role of PBAP complex, deletion mutants of the \textit{bap170} and \textit{bap180} genes have been described (Carrera et al. 2008). Surprisingly, homozygous for the null \textit{bap180}^{\Delta86} allele are viable but female sterile. Conversely, two specific mutations have been obtained for \textit{bap170}. The first is the viable hypomorphic \textit{bap170}^{465} mutation, a deletion of the 5\textquotesingle end of the gene, which reduces adult viability and causes formation of ectopic wing vein material. Homozygous \textit{bap170}^{465} flies still encodes a mutated Bap170 protein lacking the ARID domain, which is sufficient to sustain some Bap180 accumulation (Carrera et al. 2008). Conversely, the second mutation, \textit{bap170}^{\Delta135}, a nearly complete deletion of \textit{bap170} gene, causes total loss of Bap170 and Bap180 and results in a fully penetrant prepupal lethality. We found that \textit{bap170}^{\Delta135}/\textit{bap170}^{\Delta135} organisms display an identical lethal phase and the same defects of discs elongation observed in \textit{bap170}^{hfl1}/\textit{bap170}^{hfl1} mutants. The similarity between \textit{bap170}^{hfl1} and \textit{bap170}^{\Delta135} mutations was also confirmed at molecular level. Through sequence analysis of \textit{bap170}^{hfl1} allele, we found that a 16 bp deletion, within the fourth exon of \textit{bap170} gene, introduced a frameshift which predicts the synthesis of a mutated Bap170 deleted of the last C-terminal 304aa containing the two Zn fingers (Fig. 2A). The transcription of the mutated gene is not affected, as determined by northern blot analysis (Fig. 2E). In contrast, western blots carried out on extracts of \textit{bap170}^{hfl1} homozygous larvae showed that the predicted 151kD \textit{Bap170}^{\DeltaZnF} form is undetectable (Fig. 2F). This indicates a critical role of the two Zn fingers domains for Bap170 stability. As expected, the lack of Bap170 is associated to a corresponding loss of Bap180 (Fig. 2F), demonstrating that \textit{bap170}^{hfl1}, like \textit{bap170}^{\Delta135}, is a null Bap170 allele. Thus, the prepupal lethality of \textit{bap170}^{hfl1} or \textit{bap170}^{\Delta135} 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 cells 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 larval-prepupal period (see supplemental material Fig. S2). With the aim to further investigate 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 such as 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*/*bap170* 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 methods) of *bap170*/*bap170* 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 Fig. 3A and D). In a Drosophila wild type eye section each ommatidium contains 7 photoreceptor (PR) cells (R1-R7 or R1-6 and R8), four cone cells and eight accessory cells arranged in a highly structured pattern (Fig.3B, C). Conversely, in *bap170*/*bap170* mutant eyes, the ommatidia often contain more than 7 PRs cells of comparable size, with an average number of 7.35 PRs per ommatidium (n=100) (Fig.3E). Some ommatidia show supernumerary outer photoreceptors, whereas others
have extra putative R7. Since the extra-PRs have not a fixed identity, we assumed that *bap170* mutation might cause abnormal activation of a general differentiation program common to all PRs cells, such as that induced by EGFR signaling. Cone cells recruitment, which also depends by the EGFR signaling, is also affected in *bap170<sup>hfl1</sup>* eyes, given that ommatidia often display more than 4 cone cells (compare Fig. 3C and F). All the described phenotypes of *bap170<sup>hfl1</sup>* mutant eyes can be restored to the normal condition in control flies in which Bap170 was expressed under the control of the ubiquitous tub-GAL4 driver (Fig 3G-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 Fig. 3K), a phenotype which is again reminiscent of an ectopic EGFR signaling activation. Two doses of the UAS-*bap170*RNAi transgene lead to a severe wings phenotype (Fig.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 veins 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 type of PRs, with the exception of the R8 (Dominguez et al., 1998; Freeman, 1996). To determine whether *bap170* genetically behaves as a negative regulator of the Egfr pathway in retinal differentiation, we tested if *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 $\text{Egfr}^{f24}/\text{Egfr}^{(T1)}$, generates adult flies with severe rough eyes which show a reduced number of PRs per ommatidium (Fig. 4B). By halving the amount of $\text{bap170}$ in these flies, the order of ommatidial facets, as well the average number of PRs, are partially rescued (Fig. 4C). $\text{bap170}^{hfl1}$ 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 ($\text{sev-Ras}^{N17}$) (Fig. 4D, 4E).

When tested with $\text{Raf}^{HM7}$, a temperature sensitive Raf allele, $\text{bap170}^{hfl1}$ dominantly rescues the severe lack of photoreceptors caused by $\text{Raf}^{HM7}$ at 18° (compare F and G in Fig. 4) and, partially, the lethality of $\text{Raf}^{HM7}/Y$ males at 25° (not shown). Finally, $\text{bap170}^{hfl1}$ can also dominantly suppresses the loss of PRs caused by a viable combination of $\text{pnt}$ alleles (compare H and I in Fig. 4). Therefore, PBAP appears to antagonize EGFR signaling during PRs recruitment. To test whether BAP170 antagonizes the Egfr signaling also in wing discs, we performed genetic interaction analyses between null $\text{bap170}$ alleles and mutations in genes involved in vein/intervein development (here we report only the results for $\text{bap170}^{hfl1}$, but $\text{bap170}^{\Delta 135}$ 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, $\text{vn}^{C221}/\text{vn}^1$, the L4 vein fails to differentiate (Fig. 5C). Conversely, $\text{bap170}^{hfl1}/+;\text{vn}^{C221}/\text{vn}^1$ flies develop almost completely the L4 veins (Fig. 5D). Similarly, the lack of L4 vein caused by allelic combination of Egf receptor, $\text{Egfr}^{f24}/\text{Egfr}^{(T1)}$ (Fig. 5K), is fully rescued in $\text{Egfr}^{f24}$, $\text{bap170}^{hfl1}/\text{Egfr}^{(T1)},+$ flies (Fig. 5L). This rescue is associated with recovered expression of $\text{rho}$ (Fig. 5M-N) and MAPK activation in L4 (Fig. 5O-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 $\text{blistered}$, a gene required to repress
Egfr activity in the interveins. Thus, both the extra veins phenotype and wing size reduction characteristic of bs<sup>2</sup>/bs<sup>2</sup> mutants are enhanced by halving the dose of bap170 (compare Fig. 5E and F). The interaction with knirps supports the view that PBAP might antagonize vein-promoting activity downstream 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<sup>ri1</sup> 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 (Fig. 5G) (Lunde et al. 2003). Conversely, in bap170<sup>hfl1/+;kni<sup>ri-1</sup>/kni<sup>ri-1</sup> flies, the L2 vein is nearly completely restored (Fig. 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 rho<sup>ve</sup>, a viable allele of rho, which causes the loss of most part of the L5, L4 and the distal portion of the L3 vein (Fig.5I), no appreciable rescue of vein tissues was observed (Fig. 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 analyse 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), (Fig. 6A, 6E). Upon its expression, rho becomes a trigger and subsequently a target of
EGFR signaling, participating in a positive feedback loop that boosts EGFR activation (Martín-Blanco et al., 1999). We found that, in bap170\textsuperscript{hfl/l} 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 location in interveins areas (Fig. 6B and C). Partial depletion of Bap170 by RNAi (Fig. 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 high level of rho expression in 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 Fig. 6E and F). Upregulation and ectopic expression of rho is also evident in eye discs of bap170\textsuperscript{hfl/l} mutants, especially behind the morphogenetic furrow (compare Fig. 6D and H). Expression analysis of Delta, another gene activated in veins by EGFR, confirms the upregulation of EGFR signaling in bap170\textsuperscript{hfl/l} wing discs (Fig. 6I 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 were compromised in bap170 mutant discs. We also analysed the expression of blistered which is required for repression of rho in interveins and for intervein cells 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 (Fig. 6K, M, O). We found that the maximum accumulation levels of net or E(spl)mβ mRNAs, as well Bs protein are not reduced by loss of Bap170 (compare Fig. 6K and L; Fig. 6M and N; Fig. 6O and P), indicating that the PBAP is not required for proper expression of these genes. A weak reduction of net, E(spl)mβ and bs expression is only detectable in the proximity of the veins, where, it is likely, that they are repressed by a localized ectopic activation of rho/EGFR signaling occurring in bap170 mutant discs (Fig.
6L, N, 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 bap170hfl1 as well in wild type wing discs (Fig. 6Q and R), and the same effect can be obtained by ectopic E(spl)mβ expression (not shown). This suggests that PBAP complex is not required for Net or E(spl)mβ function. Another general inhibitor of Egfr signaling which expression 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 bap170hfl1 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 (Fig. 6S and T). This is surprising given that argos is normally activated by 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-PCRs performed on wing discs from wild type and bap170hfl1 mutant larvae staged at -2h APF essentially confirm the up-regulation of rho and the down-regulation of argos expression in bap170hfl1 mutants (Fig. 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 up-regulation 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 (Fig. 6T), and given that the expression of an activated form of Ras1 (RasV12) 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 over-expression experiments of Bap170. Local or ubiquitous over-expression 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 the EGFR signaling might have a mechanism to relief PBAP mediated-repression of rho in EGFR expressing cells. To verify this possibility, we decided to over-express Bap170 in a genetic context of reduced EGFR signalling. When Bap170 was over-expressed in wing or eye discs with insufficient EGFR activity (Ras\textsuperscript{N17}, Egfr\textsuperscript{124}/Egfr\textsuperscript{T1}, Ras85D\textsuperscript{e1B}/+ or pnt\textsuperscript{88}/pnt\textsuperscript{1277}), it causes an increase of the typical EGFR down-regulation phenotype (Fig. 7). In the wing discs of the corresponding genetic combinations we observed extra repression of both rho (compare Fig. 7I and J) and argos (compare Fig.7K and L). Down-regulation of both rho and argos by over-expressed Bap170 in condition of low EGFR activity was also confirmed by quantitative RT-PCR analyses in wing discs of third instar larvae staged at -2h APF (Fig. 8). These results can be explained considering that Bap170, in addition to its role to ensure proper level of argos expression, can antagonize the EGFR signaling also by participating in the repression of rho transcription. The repression of rho by over-expressed Bap170 would be sufficient to reduce the activity of the entire EGFR signaling including the argos expression. These 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 analyses in flies allowed to define Brm complex requirement in specific developmental programs such as those mediated by segmentation genes (Treisman et al., 1997; Brizuela and Kennison, 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<sup>K804R</sup>) or by the temperature-sensitive allele of snr1 (snr1<sup>E1</sup>) 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 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 showed that, among the two complexes, BAP is the form which 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 the 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 hyper-activated 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 auto-regulatory 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 de-represses and stimulates target genes 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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FIGURES LEGENDS

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 40kb which removes several genes nearby the half-life locus, and hence results in embryonic lethality. (B) Time course of the morphological changes during the 12h of the prepupal period (time is relative to puparium formation). hfl^1 homozygous display a normal phenotype until +6h APF. At +24h 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 h APF show that leg (lg) and wing (w) discs 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).
FIGURE 2. Expression analysis of bap170 and molecular characterization of bap170<sup>hfl1</sup> allele. (A) Diagram of the bap170 genomic region with indicated the 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 16bp deletion within the bap170<sup>hfl1</sup> 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<sup>hfl1</sup> (BAP170<sup>ΔZnF</sup>) is depicted below the wild type protein. Grey 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 0-12h 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 -18h or -4h APF: (Sg) salivary glands, (Wd) wing discs, (Fb) fat bodies. As control, RT-PCRs were 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 Methods). Below, dissected tissues from bap170-lacZ transgenic larvae after histochemical 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 -18h fades at -4h 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<sup>hfl1</sup>/bap170<sup>hfl1</sup> mutant larvae on
poly(A)+ RNA samples prepared from larvae at -18 and -4h APF. Northern 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 brains tissues from wild type, bap170^{135} or bap170^{hi1} homozygous mutant larvae at about -10hrs 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 75kD 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,G); apical sections through adult retinas (B,E,H); pupal retinas stained with cobalt sulphide (C,F,I). Wild type tissues (A,B,C); mutant bap170^{hi1} tissues (D,E,F), and eye tissues from bap170^{hi1} mutants rescued by ubiquitous expression of Bap170 (G,H,I). bap170 mutant eyes were obtained from bap170^{hi1}/bap170^{hi1}; dll-GAL4/UAS-bap170 flies in which Bap170 function was rescued in some tissues but not in eye primordia (see methods), whereas rescued control eyes were obtained from bap170^{hi1}/bap170^{hi1}; tub-GAL4/UAS-bap170 flies. bap170^{hi1} 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^{hi1} 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^{hi1} 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 veins phenotype (L).

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 around 750 ommatidia arranged in a highly ordered scheme and a characteristic pattern of seven rhabdomeres within each ommatidium (A). Egfr^{T1}/Egfr^{I24} 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 cells number of raf^{HM7}/Y flies raised at 18°C (ANP=5.3)(F) are both significantly recovered in raf^{HM7}/Y; bap170^{hfl1}/+ adult flies (ANP=6.5)(G). Ommatidia within adult pnt^{Δ88}/pnt^{1277} eyes display an average of 6.3 PRs/ommatidium (H), which is rescued to 6.75 in bap170^{hfl1}/+; pnt^{Δ88}/pnt^{1277} adult eyes.

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^{I}/vn^{c221} allelic combination of the Egfr ligand vein (C) is dominantly rescued in wings of bap170^{hfl1}/+; vn^{I}/vn^{c221} flies (90% 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, that is also heterozygous for bap170^{hfl1}, show enhancement of the bs phenotype (F). In wings of
knf\(^f\)/knf\(^f\) homozygous flies the distal part of L2 vein is missing (G), whereas in knf\(^f\)/knf\(^f\); 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\(^j24\) 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\(^j24\), bap170\(^hfl1\) flies, the L4 vein differentiation is completely restored (85% of cases on 100 wings) (L), as well rho expression (N) and dP-ERK levels (P) in L4.

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 to L5 are indicated), and at the wing margin. (B, C) Two wing discs from bap170 mutants larvae showing rho expressed at higher than normal level in presumptive veins but also in interveins 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 interveins 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, J) *Delta* expression monitored by β-gal staining in wing discs of control *Dl-LacZ*05151/+ (I) and *bap170hfl1/bap170hfl1*; *Dl-LacZ*05151/+ (J) third instar larvae, at -10h APF. Expressions of net (K,L), *E(spl)mβ* (M,N) and Bs (O,P) in wing discs of wild type (K,M,O) or *bap170hfl1/bap170hfl1* (L,N,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,P) and in some interveins areas. (Q,R) *rho* expression in late third instar wing discs from *MS1096-GAL4/+;UAS-net/+* (Q) or *MS1096-GAL4/+; bap170hfl1/bap170hfl1*; 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 *bap170hfl1/bap170hfl1* genetic background. *argos* expression in wing disc of control *aos-lacZ*05845/+ (S) and *bap170hfl1/bap170hfl1*; *aos-lacZ*05845/+ (T) late third instar larvae. Identical results were obtained using the *aos-lacZ*W11 enhancer trap line.

FIGURE 7. Phenotype of Bap170 over-expression. (A) *omb-GAL4/UAS-RasN17* control female wing. Expression of the dominant negative *RasN17* in the *omb* domain causes the loss of the central part of the L4 vein. (B) The simultaneous expression of *RasN17* and Bap170 in *omb* domain (*omb-GAL4/UAS-RasN17;UAS-bap170/+* female wing) induces loss of 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/+; EgfrT24/EgfrT1* control female wing showing the typical loss of the most part of the L4 vein characteristic of the *EgfrT24/EgfrT1* phenotype (compare with Fig. 5K). (D) In *MS1096-GAL4/+; EgfrT24/EgfrT1; UAS-bap170/+* female wings, the L4 vein is completely abolished and the central region of the L3 does not differentiate. (E) *Ras85De1B/sev-GAL4* control eyes showing a wild-type morphology and regular number of rhabdomeres (ANP=7.0). (F) *Ras85De1B/sev-GAL4; UAS-bap170/+* flies displays rough
eyes and loss of all R7 photoreceptors within ommatidia (ANP=6.0). (G) elav-GAL4/+; pnt^{A88/pnt^{1277}} control eyes are rough with an average of 6.3 PR cells per ommatidium. (H) elav-GAL4/UAS-bap170; pnt^{A88/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^{J24}/Egfr^{T1} larvae. The lack of rho expression in L4 vein is indicated (arrowhead). (J) Wing discs from MS1096-GAL4; Egfr^{J24}/Egfr^{T1}; UAS-bap170/+ larvae at the same stage. Bap170 over-expression 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 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.

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 ∆∆CT relative quantification were done using mfl gene expression as internal control to normalize the results.
Fig. 1

A. Percent survival

B. Images at different time points

C. Images showing fluorescence and everted head
Fig. 6
Fig. 8

![Graphs showing RQ values for rho and aos with different conditions: wt, bap170hil1, omb-GAL4 UAS-RasN17, omb-GAL4 UAS-RasN17 UAS-bap170.](image-url)