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Identification of Novel Ras-Cooperating Oncogenes in *Drosophila melanogaster*: A RhoGEF/Rho-Family/JNK Pathway Is a Central Driver of Tumorigenesis

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ABSTRACT

We have shown previously that mutations in the apico-basal cell polarity regulators cooperate with oncogenic Ras (Ras^{ACT}) to promote tumorigenesis in *Drosophila melanogaster* and mammalian cells. To identify novel genes that cooperate with Ras^{ACT} in tumorigenesis, we carried out a genome-wide screen for genes that when overexpressed throughout the developing Drosophila eye enhance Ras^{ACT}-driven hyperplasia. Ras^{ACT}-cooperating genes identified were *Rac1* Rho1, *RhoGEF2*, *pbl*, *rib*, and *east*, which encode cell morphology regulators. In a clonal setting, which reveals genes conferring a competitive advantage over wild-type cells, only *Rac1*, an activated allele of *Rho1* ($Rho1^{ACT}$), *RhoGEF2*, and *pbl* cooperated with Ras^{ACT}, resulting in reduced differentiation and large invasive tumors. Expression of *RhoGEF2* or *Rac1* with Ras^{ACT} upregulated Jun kinase (JNK) activity, and JNK upregulation was essential for cooperation. However, in the whole-tissue system, upregulation of JNK alone was not sufficient for cooperation with Ras^{ACT}, while in the clonal setting, JNK upregulation was sufficient for Ras^{ACT}-mediated tumorigenesis. JNK upregulation was also sufficient to confer invasive growth of Ras^{V12}-expressing mammalian MCF10A breast epithelial cells. Consistent with this, $HER2^{+}$ human breast cancers (where human epidermal growth factor 2 is overexpressed and Ras signaling upregulated) show a significant correlation with a signature representing JNK pathway activation. Moreover, our genetic analysis in Drosophila revealed that Rho1 and Rac are important for the cooperation of RhoGEF2 or Pbl overexpression and of mutants in polarity regulators, Dlg and aPKC, with Ras^{ACT} in the whole-tissue context. Collectively our analysis reveals the importance of the RhoGEF/Rho-family/JNK pathway in cooperative tumorigenesis with Ras^{ACT}.

CANCER is a multistep process, and transformation from a normal cell to an invasive/metastatic cancer has been considered to involve in six steps: (1) activation of mitogen-signaling pathways to allow growth factor independence; (2) elimination of cell-cycle inhibitors, (3) prevention of apoptosis, (4) promotion of invasion and metastasis, (5) acquiring unlimited replicative ability (upregulation of Telomerase), and (6) activation of angiogenesis (reviewed by Hanahan and Weinberg 2000). In addition, there is strong evidence that normal cells surrounding tumor cells (tumor microenvironment) can significantly affect the growth and development of the tumor and that the tumor and stroma (surrounding normal cells) evolve together in the development of the tumor (reviewed by Bissell and Radisky 2001). The vinegar fly, *Drosophila melanogaster*, presents an excellent genetically amenable system with which to model the first four of these cancer hallmarks, as well as the interaction of tumor cells with their microenvironment (reviewed by Brumby and Richardson 2005).

Genetic analyses in Drosophila have revealed many genes that when deregulated induce or contribute to tumorigenesis. Drosophila tumor suppressors have been classed as hyperplastic (such as those of the Salvador/Warts/Hippo, SWH, pathway), which result in increased proliferation or survival but do not disrupt tissue structure or differentiation, or neoplastic (such as Dlg, Scrib, and Lgl), which lead to loss of tissue structure, differentiation defects, and failure to exit the cell cycle (reviewed by Brumby and Richardson 2005; Hariharan and Bilder 2006). Dlg, Scrib, and Lgl act antagonistically to the apical polarity modules, the atypical protein kinase C (aPKC), and Crumbs (Crb) complexes, to regulate epithelial apico-basal cell polarity and limit

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proliferation (reviewed by Humbert *et al.* 2008). We have recently shown that deregulation of Lgl, aPKC, or Crb promotes tissue growth without affecting cell polarity by deregulation of the SWH pathway (Grzeschik *et al.* 2010). However, homozygous mutant epithelial tissue from *scrib, lgl,* or *dlg* mutant larvae that has lost apico-basal cell polarity shows all four hallmarks of cancer that can be modeled in the fly; the tissue continues to proliferate, does not die, fails to differentiate, and is capable of invasive behavior (Gateff and Schneiderman 1969; Gateff 1978; Woodhouse *et al.* 1998; Bilder and Perrimon 2000; Bilder *et al.* 2000).

By contrast, when *scrib* or *lgl* mutant tissue is generated in the context of wild-type tissue in the developing Drosophila eye using clonal analysis, it exhibits only some of the hallmarks of cancer. While both *lgl* and *scrib* mutant clones are unable to cease proliferation, showing increased expression of the key G1-S-phase cell-cycle regulator cyclin E (Richardson *et al.* 1993, 1995; Knoblich *et al.* 1994) and ectopic cell cycles, they are still capable of differentiation, thereby preventing overgrowth (Brumby and Richardson 2003; Grzeschik *et al.* 2007). In addition, *scrib* mutant cells are eliminated by Jun kinase (JNK)-mediated cell death that is induced by the surrounding wild-type tissue (Brumby and Richardson 2003). However, when activated Ras or Notch oncogenes are expressed in *scrib* mutant clones, cell survival is dramatically increased and invasive/metastatic behavior is observed (Brumby and Richardson 2003; Pagliarini and Xu 2003). This includes the breakdown of the basement membrane and invasion/migration of mutant cells to distant sites. Thus *scrib* loss-of-function shows many hallmarks of cancer and exhibits the ability to cooperate with oncogenic Ras or Notch in tumor progression.

The cooperation of *scrib* loss-of-function with Ras^{ACT} and activated *Notch* ($Notch^{intra}$ or $Notch^{ACT}$) in tumorigenesis most likely depends on the loss of cell polarity, as mutations in other apico-basal cell polarity regulators of the Scrib, aPKC, or Crb complexes can also cooperate with oncogenic Ras in tumorigenesis in Drosophila eye epithelial tissues (Pagliarini and Xu 2003). Furthermore, overexpression of Crb, which results in a loss of apico-basal cell polarity, cooperates with Ras^{ACT} in tumorigenesis (Leong *et al.* 2009). One important factor that contributes to Ras^{ACT}-mediated cooperative tumorigenesis with *scrib*$^-$, revealed by our and other studies, is the JNK signaling pathway (Brumby and Richardson 2003; Igaki *et al.* 2006; Uhlirova and Bohmann 2006; Leong *et al.* 2009). Blocking JNK function in *scrib*$^-$ Ras^{ACT} tumors reestablishes differentiation and reduces the tumor's invasive properties. Downregulation of the E-cadherin–β-catenin complex in apico-basal polarity mutants also contributes to tumorigenesis (Igaki *et al.* 2006). Whether JNK activation and E-cadherin–β-catenin downregulation are the only events downstream of apico-basal polarity mutants contributing to Ras^{ACT}-cooperative tumorigenesis is unclear. We envisioned that insight might be gained on the nature of other critical functions that are affected by loss of cell polarity for Ras^{ACT}-cooperative tumorigenesis, by identifying other genes that cooperate with oncogenic Ras. In this study, we present the results of a genetic screen to identify genes that when overexpressed enhance a Ras^{ACT}-induced hyperplastic eye phenotype. We identified key regulators of the actin cytoskeleton and cell morphology, including Rho1-family GTPases and RhoGEFs as Ras^{ACT}-cooperating proteins. We show that JNK pathway activation underlies the cooperation of these actin cytoskeletal regulators with Ras^{ACT}. Moreover, we show that JNK and Ras signaling cooperate to promote invasive growth in normal human mammary epithelial cells and reveal by bioinformatics analysis that JNK signaling correlates with upregulation of Ras in human breast cancer. Our studies reveal a RhoGEF/Rho-family/JNK pathway as an important factor in oncogenic Ras mediated tumorigenesis.

MATERIALS AND METHODS

Fly stocks, conditions of culture, overexpression, and clonal analysis: For the screening of *GS* lines, a recombinant of *ey-GAL4* and $UAS\text{-}Ras85D^{V12}$ ($ey{>}Ras^{ACT}$) was generated. Potential interacting *GS* lines were retested against $ey{>}Ras^{ACT}$ and also to *ey-GAL4* to assess the effect of expression of the gene alone on the adult eye. At least 50 progeny were analyzed for each cross, and representative images are shown. All flies were raised on a standard cornmeal agar food at 25°.

Validating transgenes used were: *UAS-rib* (Deborah Andrew), $UAS\text{-}Rho1^{CFP2a}$ (Greco *et al.* 2001), $UAS\text{-}Rho1^{ACT}$ (Billuart *et al.* 2001), *UAS-RhoGEF2* (Udo Hacker, Widmann and Dahmann 2009), *UAS-east* (Wasser and Chia 2000), *UAS-pbl-GFP#3,* and *UAS-pbl-GFP#8* (Robert Saint, Somers and Saint 2003), *UAS-Rac1* (Luo *et al.* 1994).

The MARCM (mosaic analysis with repressible cell marker) system (Lee and Luo 2001) with *FRT82B, ey-FLP,* and *UAS-GFP* (*ey-FLP1, UASmCD8-GFP;;Tub-GAL4 FRT82B Tub-GAL80;* Lee and Treisman 2001) was used to induce GFP positively marked clones.

Other stocks used were: *dlg-RNAi 4689 C2V* (gift from B. Dickson, Dietzl *et al.* 2007), validated for knockdown of Dlg and specificity (Grzeschik *et al.* 2010), msn^{06946} (*msn-lacZ*) (Mattila *et al.* 2005); *UAS-P35* (Hay *et al.* 1994); $UAS\text{-}bsk^{K53R}$ ($UAS\text{-}bsk^{DN}$) (Weber *et al.* 2000), $UAS\text{-}aPKC^{\Delta N}$ (Betschinger *et al.* 2003); $UAS\text{-}aPKC^{CAAX\text{-}DN}$ (Sotillos *et al.* 2004); $UAS\text{-}Ras85D^{V12}$ ($UAS\text{-}Ras^{ACT}$) (Karim and Rubin 1998); $UAS\text{-}Rac1^{N17}$ ($UAS\text{-}Rac1^{DN}$) (Luo *et al.* 1994); $UAS\text{-}Rho1^{RNAi}$ #12734 [Vienna Drosophila Resource Center (VDRC), Dietzl *et al.* 2007] and $scrib^1$ (D. Bilder).

Immunocytochemistry for analysis of Drosophila tissues: For analysis of third-instar larval eye-antenna discs, the discs were dissected in PBS, fixed in 4% PFA, washed in PBT (0.1% TritonX-100), and blocked in PBT + 2% normal goat serum. BrdU labeling for the detection of S phase cells was carried out as previously described (Leong *et al.* 2009). Antibodies used were mouse Elav (Developmental Studies Hybridoma Bank, DSHB, 1:20), mouse β-galactosidase (Rockland, 1:500), and mouse anti-BrdU (Becton-Dickinson, 1:50). Secondary antibodies were: anti-mouse Alexa647 (Invitrogen; 1:400) or anti-mouse Alexa488 (Invitrogen; 1:400). F-actin was detected with phalloidin-

tetramethylrhodamine isothiocyanate (Rhodamine; Sigma, 0.3 mM).

Matrigel invasion assay for mammalian MCF10A cells: Parental MCF10A cell lines were retrovirally co-infected with JNK1a1, MKK4, and MKK7 overexpression constructs and H-Ras^{V12cherry} selected with puromycin, sorted for GFP/cherry on a FACSVantage SE-DiVa flow cytometer (Becton Dickinson, Franklin Lakes, NJ), and maintained as previously described (Dow *et al.* 2008). MCF10A derivative cell lines stably expressing candidate genes were quantified for invasive phenotypes in 3D organotypic cultures as previously described (Dow *et al.* 2008) using growth factor-reduced Matrigel (BD Biosciences, Franklin Lakes, NJ) and the standard overlay method (Debnath *et al.* 2003; Dow *et al.* 2008). After 7 days in culture, individual acini were classified as "normal" acini, defined as those with a contiguous acini boundary with no cellular extensions, or "invasive," defined as acini with disorganized boundary structures showing cellular protrusions or cellular spikes invading into the surrounding matrix. Cell lines were plated in duplicate wells for each experiment and 100 acini were scored per well.

Constructs and antibodies for mammalian cell analysis: JNK1a1, MKK4, and MKK7 overexpression constructs were PCR amplified from Addgene plasmids 13798 (pcDNA3-Flag-JNK1a1), 14615 (pcDNA3-Flag-MKK4), and 14538 (pcDNA3-Flag-MKK7a1) and inserted into pMSCV^{puro} (clontech, PT3303-5) or MSCV-IRES-GFP. MSCV-cherry-IRES-H-Ras^{V12} and MSCV control vectors were already available (Dow *et al.* 2008).

The following primary antibodies were used for probing Western blots: α-tubulin (no. T5168; Sigma-Aldrich), Mouse anti-MKK7 (no. 32-7000, Invitrogen), monoclonal mouse Anti-Flag (F3165, Sigma), rabbit anti-SEK1/MKK4 (no. 9152, cell signaling), rabbit anti-Ras (cloneMC57, no. 05-775, Upstate). Peroxidase-labeled horse anti-mouse IgG (S1018, vector laboratories, Burlingame, CA) and goat anti-rabbit IgG (no. 170-6515, Bio-Rad laboratories, Hercules, CA) secondary antibodies were used for LiCor Western blotting.

Scanning electron microscopy and imaging: Fluorescent labeled samples were mounted in 80% glycerol and analyzed by Confocal microscopy (Bio-Rad MRC1000 or Olympus FV1000) and images were processed using Confocal Assistant^{R} and Fluorview software and assembled using Adobe Photoshop CS2 and Adobe Illustrator CS2. Adult eyes were imaged with a Scitec Infinity1 camera. Scanning electron micrographs of adult eyes were carried out as previously described (Richardson *et al.* 1995), except that the samples were gold coated before imaging and were imaged on a Philips XL30 FEG field-emission scanning electron microscope, at 2 V, and working distance 10 mm.

Breast Cancer Gene expression data sets: Breast cancer data sets used are publicly available and were downloaded from the authors' websites (see supporting information, File S1). We used normalized data (log^2 intensity in single-channel platforms or log^2 ratio in dual-channel platforms) and probe sets were mapped to Entrez-Gene IDs to merge data across the various datasets. Breast cancer subtypes were defined using a two-dimensional clustering model previously described on the basis of two module scores, ESR1 and HER2, representing ER and HER2 phenotypes, respectively. Gene sets representing JNK and RAS signaling were combined to compute a gene signature score defined as the weighted linear combination of the log^2 expression values of the genes in the signature. Kruskal–Wallis tests were used to determine differences in expression between classes and Spearman's Rho correlations were used to assess correlations between the signatures. For further information see File S1.

RESULTS

A screen for *Ras^{ACT}*-cooperating genes in the developing Drosophila eye reveals cell morphology regulators: To identify novel genes able to cooperate with activated *Ras85D* (*Ras^{ACT}*), we first sought to generate a hyperplastic phenotype mediated by *Ras^{ACT}* that could be used in an F1 screen. Expression of *Ras^{ACT}* via the *eyeless-GAL4* (*ey-GAL4*) driver in the developing eye has been previously shown to result in hyperplasia during larval development and generates an overgrown adult eye phenotype (Karim and Rubin 1998). Therefore, we generated a stock containing *ey-GAL4* and *UAS-Ras^{ACT}* (*ey>Ras^{ACT}*), which resulted in overgrown adult eyes that was more obvious in males than females (Figure 1, A and B, and Figure S1). At the larval stage, the expression of *Ras^{ACT}* led to enlarged eye discs with enlarged ommatidia and greater spacing between ommatidial clusters (Figure S3 and Figure S4), consistent with the documented role of *Ras^{ACT}* in cell growth and proliferation (Karim and Rubin 1998; Prober and Edgar 2000). To validate that the phenotype of *ey>Ras^{ACT}* was responsive to genes known to cooperate with *Ras^{ACT}* in tumorigenesis, we tested if knocking down the junctional neoplastic tumor suppressors, *dlg, scrib,* or *lgl,* by RNAi, could enhance the phenotype. Indeed, *dlg* knockdown enhanced the *ey>Ras^{ACT}* hyperplastic eye phenotype at the adult stage and resulted in subtly larger eye discs than *Ras^{ACT}* alone, with greater spacing between ommatidial clusters (Figure 1D, Figure S3, and Figure S4), but had no obvious defects when expressed alone (Figure 1C, see Figure S3, and Figure S4). *lgl* or *scrib* knockdown had only mild effects on the *ey>Ras^{ACT}* phenotype (data not shown), perhaps due to a lower level of knockdown achieved with these RNAi lines. To determine whether the *ey>Ras^{ACT}* adult eye phenotype was sensitive to increased activity of polarity regulators, we then tested whether overexpression of an activated version of the apical cell polarity regulator aPKC (aPKC$^{\Delta N}$), which alone does not affect the adult eye, could enhance the *ey>Ras^{ACT}* phenotype. Indeed *ey>Ras^{ACT} aPKC$^{\Delta N}$* adult females exhibited strongly enhanced hyperplastic eyes (Figure 1, E and F, and Figure S1), whereas no males eclosed (presumably reflecting the stronger phenotype of *Ras^{ACT}* expression exhibited in males). Furthermore, overexpression of the apical cell polarity regulator Crb, via the *ey* driver, resulted in an ablated eye phenotype alone, but was pupal lethal with *Ras^{ACT}* (data not shown). Thus, these data show that deregulation of polarity regulators can enhance the *Ras^{ACT}* phenotype and validate the use of the *ey>Ras^{ACT}* adult eye phenotype as a system suitable for screening for genes that when overexpressed can cooperate with oncogenic Ras, to increase hyperplasia or result in pupal lethality.

To identify novel genes that when overexpressed cooperate with *Ras^{ACT}*, we screened the *GS* line collection

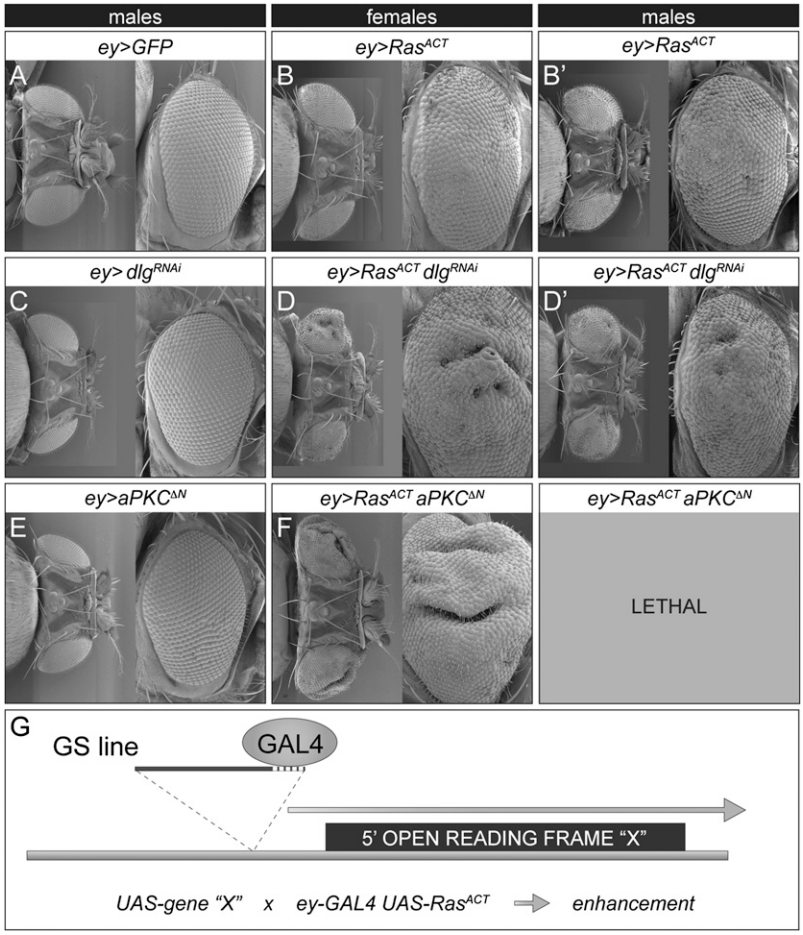

FIGURE 1.— Strategy for Ras^{ACT}-cooperating gene screen: scanning electron micrographs of adult eyes (dorsal and lateral views) from female or males flies expressing oncogenic Ras (Ras^{ACT}) and activated aPKC ($aPKC^{\Delta N}$) or knockdown of Dlg (dlg^{RNAi}), compared with Ras^{ACT} alone and controls. Posterior is to the left and dorsal is to the top in the lateral views, in this and all other adult eye figures. (A) *ey-GAL4 UAS-GFP* (*ey>GFP*), (B) *ey-GAL4 UAS-*Ras^{ACT} (*ey>*Ras^{ACT}), (C) *ey-GAL4 UAS-*dlg^{RNAi} (*ey>*dlg^{RNAi}), (D) *ey-GAL4 UAS-*Ras^{ACT} *UAS-*dlg^{RNAi} (*ey>*Ras^{ACT} dlg^{RNAi}), (E) *ey-GAL4 UAS-*$aPKC^{\Delta N}$ (*ey>*$aPKC^{\Delta N}$), (F) *ey-GAL4 UAS-*Ras^{ACT} $aPKC^{\Delta N}$ (*ey>*Ras^{ACT} $aPKC^{\Delta N}$). Ras^{ACT} expression via the *ey-GAL4* driver results in hyperplastic eyes. Expression of $aPKC^{\Delta N}$ or dlg^{RNAi} via *ey-GAL4* results in slight roughening. Expression of $aPKC^{\Delta N}$ or dlg^{RNAi} with Ras^{ACT} via *ey-GAL4* results in enhanced overgrowth of the Ras^{ACT} hyperplastic adult eye. Males expressing Ras^{ACT} and $aPKC^{\Delta N}$ die at the pupal stage. (G) Diagram of *ey>*Ras^{ACT} screening strategy. *ey-GAL4 UAS-*Ras^{ACT} flies were crossed to a library of enhancer *P* (*GS*) lines, expressing via *UAS(GAL4)* adjacent genes (*UAS-gene "X"*), and those which enhanced the *ey>*Ras^{ACT} phenotype were selected for further analysis.

of enhancer *P* lines (TOBA *et al.* 1999; AIGAKI *et al.* 2001). The map position of these lines in the genome, as well as the tagged gene (the gene that is overexpressed by the line) has, in most cases, been determined and a database established to enable ready access to this information (http://gsdb.biol.metro-u.ac.jp/%7Edclust/). This enhancer *P* transgenic set has been successfully used in several screens to identify interacting genes (AIGAKI *et al.* 2001; KANUKA *et al.* 2005; LAVIOLETTE *et al.* 2005; ZHANG *et al.* 2006). To identify enhancers of Ras^{ACT}, we carried out an F1 screen, scoring for lines that enhanced the mild hyperplastic phenotype of *ey>*Ras^{ACT}. Approximately 5000 *GS* lines were screened and lines that scored as moderate or strong enhancers were retested against *ey>*Ras^{ACT}. Confirmed interacting *GS* lines (Table 1 and see Table S1) were then validated by testing whether independent enhancer *P* lines or transgenes could also enhance the *ey>*Ras^{ACT} phenotype. The interactors were also analyzed for whether they resulted in hyperplasia alone by crossing to *ey-GAL4*. Those that were unable to be confirmed by independent enhancer *P* lines or transgenes, or produced only mild enhancement with an independent line, were not pursued (see Table S1). A possible reason why some of these could not be validated by an independent transgene or enhancer *P* line is that the *GS* line was inserted in the open reading frame of the gene, and therefore a truncated neomorphic protein may be produced (see Table S1). Alternatively, the level of expression of the gene may be critical for cooperation with Ras^{ACT} and the *GS* line may express the protein at a different level than the independent lines. An example of this is Src, which has previously been described as promoting hyperplasia at a lower level of expression, but inducing cell death and tissue ablation when expressed at a higher level (VIDAL *et al.* 2007).

One validated enhancer was Delta, which is a ligand for Notch; however, as has been previously described (BAONZA and FREEMAN 2005; FERRES-MARCO *et al.* 2006), it also showed a hyperplastic eye phenotype when expressed alone (data not shown) and was not further analyzed. Validated enhancers exhibited phenotypes ranging from eyes with regions of aberrant differentiation (cuticle and bristles within the eye field), morphological defects, and male lethality at the pupal stage to enlarged, overgrown adult eyes (Table 1 and Figure 2). Strikingly, the majority of the cooperating proteins fell into the category of Rho-family GTPases, Rho1 and Rac1 (SETTLEMAN 2001), and Rho1 regulators, RhoGEF2 (BARRETT *et al.* 1997; HACKER and PERRIMON 1998) and Pbl (PROKOPENKO *et al.* 1999) (see below). The other two cooperating proteins were the BTB/POZ

TABLE 1

Validated *ey>Ras^{ACT}*-cooperating genes, function, and human homologs

GS line	Potential overexpressed genes, position of GS insertion	Validating transgene	Validated gene Function	Closest human homolog
13019 (GSV6)	CG2248-RA, −2385 CG9149-RA, +2119 (antisense)	*UAS-Rac1*	CG2248 (*Rac*1) Rho-family GTPase. Actin cytoskeleton regulation.	Rac1 (*e*–101)
12503 (GSV6)	CG8416-RA, −125	*UAS-Rho1-CFP2a* *UAS-Rho1^{ACT}*	CG8416 (*Rho*1) Rho-family GTPase. Actin cytoskeleton regulation.	RhoA (*e*–94)
45 (GSV1)	CG9635-RD, −122 CG6829-RB, +7054 (anti-sense)	*UAS-RhoGEF2*	CG9635 (*RhoGEF2*) Rho family activator. Actin cytoskeleton regulation.	p115-RhoGEF (*e*–47) LARG (*e*–50) PDZ-RhoGEF (*e*–53)
9792 (GSV6)	CG8114-RA, +344 (5′-UTR)	*UAS-pbl-GFP#8*	CG8114 (*pbl*) Rho family activator (RhoGEF)	Ect2 (*e*–144)
14458 (GSV6)	CG8114-RA, −484	*UAS-pbl-GFP#8*	Actin cytoskeleton regulation, cytokinesis, migration	Ect2 (*e*–144)
9641 (GSV6)	CG7230-RA, −6758	*UAS-rib*	CG7230 (*rib*) BTB/POZ transcription factor.	ZFP161 (*e*–8)
1106 (GSV1)	CG4399-RB, −163	*UAS-east*	CG4399 (*east*) metallopeptidase M14	None
1211 (GSV1)	CG4399-RB, −706	*UAS-east*	Carboxypeptidase A domain and putative proteolysis activity, nuclear endoskeleton regulation	None

and Psq domain nuclear localized protein, Ribbon (Rib), required for cell shape changes and epithelial morphogenesis (Bradley and Andrew 2001; Shim *et al.* 2001), and the nuclear cytoskeletal regulator, East (Wasser and Chia 2000) (Figure 2, see Figure S1, and Figure S2).

The effects of the *Ras^{ACT}*-cooperating genes on cell survival, proliferation, differentiation, and morphology: Expression of the *Ras^{ACT}*-cooperating genes alone did not result in hyperplasia, and indeed *rib, Rho1,* and *RhoGEF2* resulted in small eyes with morphological defects, suggesting that they were inducing cell death (see Figure S1 and Figure S2). Since activation of Ras inhibits apoptosis (Bergmann *et al.* 1998; Kurada and White 1998), it was possible that *Ras^{ACT}* was cooperating with these genes by preventing cell death. However, expression of the cell-death inhibitor, P35 (a caspase inhibitor, Hay *et al.* 1994), with the *Ras^{ACT}*-cooperating genes did not lead to increased hyperplasia (see Figure S1 and Figure S2), although the male lethality of *Rho1* was rescued by expressing *p35* (see Figure S2). Thus, Ras^{ACT} does not cooperate by simply blocking cell death, although it is possible that its cell survival function could contribute to the cooperative effects. Therefore, Ras^{ACT} must be providing other functions, such as promoting cell growth and proliferation or affecting cell–cell adhesion, as has been previously reported (Prober and Edgar 2000; Prober and Edgar 2002; O'keefe *et al.* 2007), and we have observed in cooperative tumorigenesis with *scrib* mutants (Brumby and Richardson 2003).

As detailed below, while all of the *Ras^{ACT}*-cooperating genes enhanced *Ras^{ACT}* tissue growth, a spectrum of cooperative effects were observed: *pbl, Rac1,* and *east* enhanced *Ras^{ACT}* tissue growth, *RhoGEF2* enhanced the effect of *Ras^{ACT}* on tissue growth, as well as affected cell morphology and differentiation, and *Rho1* or *rib* (which alone had strong morphology defects, differentiation defects, and disorganized proliferation patterns) cooperated with *Ras^{ACT}* by enhancing tissue growth, as well as affecting cell morphology and differentiation (see Figure S1, Figure S2, Figure S3, and Figure S4).

Rac1: The *GS* line and an independent transgene for *Rac1* (Luo *et al.* 1994) showed similar hyperplastic phenotypes with *Ras^{ACT}* (Figure 2, C and D). In the larval eye disc, expression of *Rac1* alone did not affect eye development; however, with *Ras^{ACT}* it resulted in an increased tissue growth and morphological defects, although differentiation still occurred, albeit aberrantly patterned (see Figures S3 and Figure S4).

Rho1: The *Rho1 GS* line showed a strong effect with *ey>Ras^{ACT}* resulting in male lethality (Figure 2E); however, expression of several *Rho1* transgenes did not enhance the *ey>Ras^{ACT}* phenotype to the same extent as the *GS* line, although *UAS-Rho1^{CFP2a}* (Greco *et al.* 2001) showed slight to moderately increased hyperplasia (see

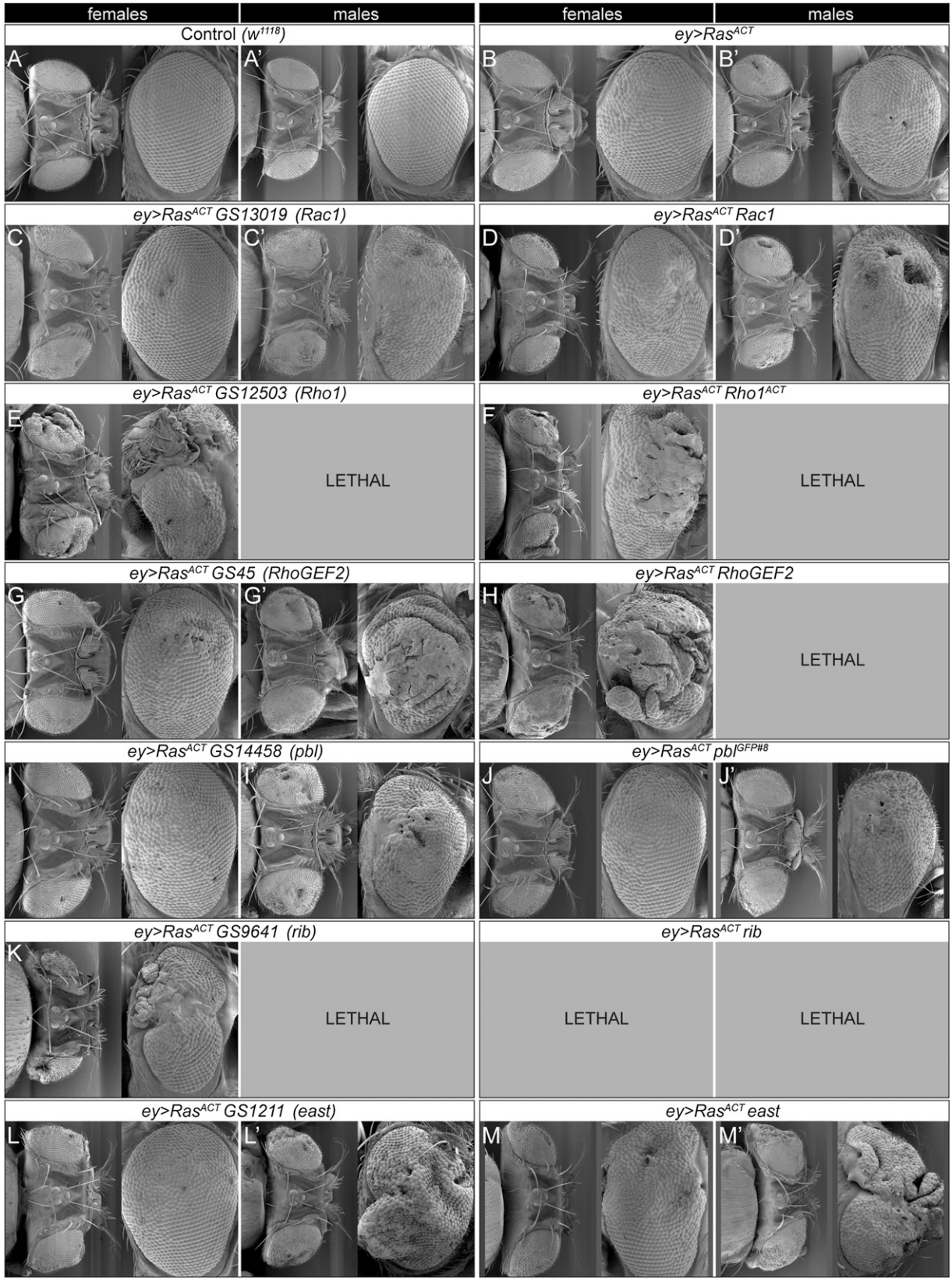

FIGURE 2.— Interaction of cooperating *GS* lines with *ey>Ras*^{ACT} and validation: scanning electron micrographs of adult eyes (dorsal and lateral views) from female or male flies expressing *Ras*^{ACT} and cooperating *GS* lines or *UAS* transgenes via the *ey* driver, compared with *ey>Ras*^{ACT} alone and wild-type control eyes. (A) Control (*w*^{1118}), (B) *ey>Ras*^{ACT}, (C) *ey>Ras*^{ACT} *GS13019 (Rac1),* (D) *ey>Ras*^{ACT} *Rac1,* (E) *ey>Ras*^{ACT} *GS12503 (Rho1),* (F) *ey>Ras*^{ACT} *Rho1*^{ACT}, (G) *ey>Ras*^{ACT} *GS45 (RhoGEF2),* (H) *ey>Ras*^{ACT} *RhoGEF2,* (I) *ey>Ras*^{ACT} *GS14458 (pbl),* (J) *ey>Ras*^{ACT} *pbl*$^{GFP\#8}$, (K) *ey>Ras*^{ACT} *GS9641 (rib),* (L) e*y>Ras*^{ACT} *GS1211 (east),* (M) *ey>Ras*^{ACT} *east.* Expression of *ey>Ras*^{ACT} with *GS9641 (rib), GS12503 (Rho1),* or *RhoGEF2* was male lethal, and with *rib* was male and female lethal.

Figure S2). Expression of the *Rho1 GS* line alone via the *ey* driver led to male lethality and females had very reduced eyes with differentiation defects, but *ey>Rho1^{CFP2a}* did not noticeably affect the adult eye (see Figure S2). It is possible that the wild-type *Rho1* transgenes tested did not express Rho1 to the same level as the *GS* line, and therefore could not accumulate sufficient levels of active GTP-bound Rho to show cooperation with *Ras^{ACT}*. Therefore, we tested an activated allele of *Rho1*, *Rho1^{V14}* (*Rho1^{ACT}*) (Lee *et al.* 2000). *Rho1^{ACT}* alone was male lethal, but female eyes were not as severely affected as with *Rho1^{GS12503}* (see Figure S2). Expression of *Rho1^{ACT}* with *Ras^{ACT}* strongly enhanced the *ey>Ras^{ACT}* phenotype (Figure 2F, and see Figure S2), indicating that activated Rho was required for cooperation with Ras^{ACT}. Consistent with the effect on the adult eyes, *Rho1* or *Rho1^{ACT}* alone resulted in very small eye discs, although S phases were observed throughout the eye disc, and exhibited altered cell morphology and reduced differentiation (see Figure S3 and Figure S4). Coexpression of *Ras^{ACT}* with *Rho1* or *Rho1^{ACT}* resulted in larger eye discs relative to these genes alone; however, proliferation and differentiation were similarly affected (see Figure S3 and Figure S4).

RhoGEF2: The *GS* line targeting *RhoGEF2* and an independent *RhoGEF2* transgene (Mulinari *et al.* 2008) cooperated with *ey>Ras^{ACT}* (Figure 2, G and H, and see Figure S2). However, the *RhoGEF2* transgene showed more severe effects than the *GS* line, resulting in greater hyperplasia in females and male lethality at the pupal stage (Figure 2H; see Figure S2; and data not shown). When expressed alone the *RhoGEF2* transgene was also more severe than the *GS* line, resulting in ablation of eye tissue (Figure S2). Consistent with these effects on the adult eye, in the larval eye discs, *RhoGEF2* alone resulted in aberrant proliferation patterns, tissue morphology (see Figure S3), and partially blocked differentiation (see Figure S4), and when expressed with Ras^{ACT} they strongly affected tissue morphology and blocked differentiation (see Figure S4).

Pbl: GS lines targeting *pbl*, and two out of five independent *pbl* transgenes (Somers and Saint 2003), enhanced the *ey>Ras^{ACT}* phenotype (Figure 2, I and J, and see Figure S2; and data not shown). Of the independent transgenes, *UAS-pbl$^{GFP\#8}$* showed a stronger effect than *UAS-pbl$^{GFP\#3}$* (data not shown). Although we have not tested it directly, it is possible that the level of *pbl* expression is critical for the cooperative effects with *Ras^{ACT}*. In the larval eye disc, expression of *pbl* alone did not prevent differentiation nor did it substantially affect the pattern of S phases or tissue morphology (see Figure S3 and Figure S4). Coexpression of *Ras^{ACT}* with *pbl* resulted in an enhancement of the tissue growth effect of *Ras^{ACT}*, as well as morphological defects, although differentiation still occurred, albeit in an aberrant pattern (see Figure S3 and Figure S4).

Rib: An independent transgene of *rib* (Bradley and Andrew 2001) resulted in a more extreme phenotype than the *GS* line with *ey>Ras^{ACT}*, since it was lethal in both males and females (data not shown). Expression of *rib GS* line alone via the *ey* driver resulted in reduced adult eyes with differentiation defects in both males and females (see Figure S1), while the *rib* transgene was male and female lethal when expressed with *ey-GAL4*. Consistent with the adult phenotypes, expression of *rib* alone resulted in very small eye discs, although S phases were observed throughout the eye disc, that had altered cell morphology and reduced differentiation (see Figure S3 and Figure S4). Coexpression of *Ras^{ACT}* with *rib* resulted in larger eye discs relative to *rib* expression alone; however, proliferation and differentiation were similarly affected (see Figure S3 and Figure S4).

East: The cooperation of *east* with *Ras^{ACT}* was confirmed by expression of a *UAS-east* transgene (Wasser and Chia 2000) (Figure 2, L and M). In larval eye discs, expression of *east* alone did not prevent differentiation (see Figure S4) or obviously affect the pattern of S phases or tissue morphology (see Figure S3), but with *Ras^{ACT}* it enhanced the tissue growth effect of *Ras^{ACT}* and led to morphological and differentiation defects (see Figure S3 and Figure S4).

The requirement of Rac or Rho1 activity for cooperation with Ras^{ACT}: Since Pbl and RhoGEF2 are known actin cytoskeletal regulators that function through the Rho-family GTPase, Rho1 (Hacker and Perrimon 1998; O'keefe *et al.* 2001; Padash Barmchi *et al.* 2005; Patch *et al.* 2009), we reasoned that other Ras^{ACT}-cooperating genes may work in a common pathway via Rho1 or Rac1 in their cooperation with Ras^{ACT}. To address this, we assessed the requirement of Rho1 or Rac1 on the ability of the *Ras^{ACT}*-cooperating genes for the cooperation with *Ras^{ACT}* in a whole-tissue setting. To block Rac1 function we expressed a dominant negative allele, blocked in the inactive GDP-bound state, *Rac1^{N17}* (*Rac1^{DN}*) (Luo *et al.* 1994). Three Rac genes in Drosophila have overlapping functions and it is likely that the *Rac1* dominant-negative allele interferes with the function of all *Rac* genes (Hakeda-Suzuki *et al.* 2002). To reduce Rho1 function, we used a RNAi transgene (*Rho1^{RNAi}*), which has been shown to effectively knockdown Rho1 protein levels and function (Massarwa *et al.* 2009; Yan *et al.* 2009). While expression of *Rac1^{DN}* or *Rho1^{RNAi}* showed no discernable effects alone (data not shown) or on the *ey>Ras^{ACT}* phenotype (see Figure S5), *Rac1^{DN}* suppressed the cooperation with *Rac1* and *Ras^{ACT}*, and *Rho1^{RNAi}* suppressed *Rho1^{GS12503}* and *Rho1^{ACT}* cooperation with *Ras^{ACT}*, as expected (Table 2; see Figure S6). Both *Rac1^{DN}* and *Rho1^{RNAi}* showed suppression of the *ey>Ras^{ACT} dlg^{RNAi}* and *ey>Ras^{ACT} aPKC$^{\Delta N}$* phenotypes (Table 2; see Figure S5). *Rho^{RNAi}* suppressed *RhoGEF2* and *pbl* cooperation with *Ras^{ACT}* (Table 2; see Figure S7), as expected. Interestingly, *Rac1^{DN}* suppressed *pbl* and showed partial suppression of *RhoGEF2* cooperation

TABLE 2

Summary of genetic interactions of *Rho1*, *Rac1*, *aPKC*, or *bsk* with *ey>Ras^{ACT}* + cooperating genes

	ey>Ras^{ACT}	*ey>Ras^{ACT} Rac1^{DN}*	*ey>Ras^{ACT} Rho1^{RNAj}*	*ey>Ras^{ACT} aPKC^{DN}*	*ey>Ras^{ACT} bsk^{DN}*
UAS-dlg^{RNAj}	Enhanced	Slight suppression	Slight suppression	Slight suppression	Suppression
UAS-aPKC$^{\Delta N}$	Enhanced	Suppression	Suppression	No suppression	Suppression
UAS-Rac1	Enhanced	Suppression	No suppression	No suppression	Suppression
GS12503(Rho1)	Enhanced	No suppression	Suppression	No suppression	Slight suppression
UAS-Rho1^{ACT}	Enhanced	No suppression	Suppression	No suppression	Suppression
UAS-RhoGEF2	Enhanced	Slight suppression	Suppression	No suppression	Suppression
UAS-pbl$^{GEF\#8}$	Enhanced	Slight suppression	Slight suppression	No suppression	Suppression
GS9641(rib)	Enhanced	No suppression	Slight suppression	No suppression	No suppression
UAS-rib	Enhanced	No suppression	No suppression	No suppression	No suppression
UAS-east	Enhanced	No suppression	No suppression	No suppression	No suppression

with *ey>Ras^{ACT}*, but did not alter the ability of *Rho1^{GS12503}*, *Rho1^{ACT}*, *rib*, or *east* to cooperate with *Ras^{ACT}* (Table 2; see Figure S6 and Figure S7). *Rho^{RNAi}* partially suppressed *rib^{GS9641}* (although not the stronger *rib* transgene; data not shown), but not *Rac1* or *east* (Table 2; see Figure S6 and Figure S7). Collectively, these genetic interactions are consistent with the notion that Dlg, aPKC, RhoGEF2, and Pbl act upstream of Rho1 and Rac in their cooperative effects with *Ras^{ACT}*. These results also suggest that in their cooperation with *Ras^{ACT}*, Rib acts upstream of Rho1 and East acts downstream or independently of Rho1 and Rac.

The requirement of aPKC activity for the cooperation with Ras^{ACT}: Since we have previously shown that the *scrib* mutant clonal phenotype depends on aPKC and that aPKC contributes to the cooperative tumorigenesis of *scrib* mutants with *Ras^{ACT}* or *Notch^{ACT}* (Leong *et al.* 2009), we tested whether the *Ras^{ACT}*-cooperating genes also required aPKC for their cooperative effects. We blocked aPKC activity by expression of a kinase-dead (dominant-negative) transgene (*aPKC^{DN}*) (Sotillos *et al.* 2004), which in clones can suppress the defects of *scrib* or *lgl* mutants (Grzeschik *et al.* 2007; Leong *et al.* 2009). *aPKC^{DN}* exhibited no effect upon the *ey>Ras^{ACT}* phenotype (see Figure S5); however, it partially suppressed the cooperative effect of *dlg^{RNAi}* with *Ras^{ACT}* (Table 2 and see Figure S5), consistent with the antagonistic relationship between these proteins (as described previously). Surprisingly, *aPKC^{DN}* did not suppress the *ey>Ras^{ACT} aPKC$^{\Delta N}$* phenotype (Table 2 and see Figure S5), perhaps due to high expression of *aPKC$^{\Delta N}$*, although it can suppress weaker activated aPKC phenotypes due to expression of a membrane-tethered aPKC construct (*aPKC$^{CAAX\text{-}WT}$*; Sotillos *et al.* 2004 and data not shown). *aPKC^{DN}* was unable to suppress the cooperative effects of any of the other *Ras^{ACT}*-cooperating genes (Table 2, see Figure S6 and Figure S7), suggesting that aPKC acts upstream or independently of these genes.

JNK is upregulated and is required for the cooperative effect of Rho-GTPases and Rho-family regulators with Ras^{ACT}: Activation of JNK is critical for cooperative tumorigenesis of *scrib* mutants with *Ras^{ACT}* or *Notch^{ACT}* (Leong *et al.* 2009). To determine the involvement of JNK signaling in the cooperation of *ey>Ras^{ACT}* with *RhoGEF2* and *Rac1*, we first tested whether JNK activity was increased in these eye discs, using the *msn-lacZ* reporter to monitor JNK pathway activity (Mattila *et al.* 2005). Expression of *Ras^{ACT}* via *ey-GAL4* resulted in a weak induction of *msn-lacZ* in some cells in the eye disc (Figure 3, A and B), which was expected because of previous findings on the regulation of Jun and Fos activity via the Ras-MAPK signaling pathway (Kockel *et al.* 1997; Ciapponi *et al.* 2001). However, coexpression of *Rac1* or *RhoGEF2* with *Ras^{ACT}* resulted in a more consistent and stronger upregulation of *msn-lacZ* throughout the eye disc (Figure 3, C and D). Thus, in the *ey>Ras^{ACT}* system, JNK activity is induced by *Rac1* or *RhoGEF2* expression.

We then tested if blocking JNK signaling, by expression of kinase-dead (dominant negative) transgene (*bsk^{DN}*), could affect the cooperation of the *Ras^{ACT}*-cooperating genes with *Ras^{ACT}* on the adult eye phenotypes. As expected on the basis of our findings in the clonal setting (Leong *et al.* 2009), *bsk^{DN}* strongly suppressed the cooperation of *Ras^{ACT}* with *dlg^{RNAi}* or *aPKC$^{\Delta N}$*, but did not affect the *ey>Ras^{ACT}* phenotype (Table 2 and see Figure S5). Expression of *bsk^{DN}* also suppressed the cooperation of *RhoGEF2, pbl, Rac1,* and *Rho1^{ACT}* and partially suppressed the stronger phenotype of *Rho1^{GS12503}* with *Ras^{ACT}* (Table 2, see Figure S6, and Figure S7). Consistent with this, expression of *bsk^{DN}* resulted in a suppression of the ectopic S phases observed in posterior region of *ey>Ras^{ACT}* + *Rac1* or *RhoGEF2* eye discs (Figure 3, E–H). Thus, in the *ey>Ras^{ACT}* system, JNK activity is required for the increased proliferation observed in *Rac1* or *RhoGEF2* + *Ras^{ACT}* eye discs. However, *bsk^{DN}* failed to suppress the cooperative effects of *east* or *rib* with *Ras^{ACT}* (Table 2, see Figure S7). Since it is conceivable that *bsk^{DN}* could function by acting on other MAPK-family signaling pathways, such as p38, to confirm that these interactions were due specifically to blocking the JNK signaling pathway, we also tested whether reducing the dosage of *bsk* would

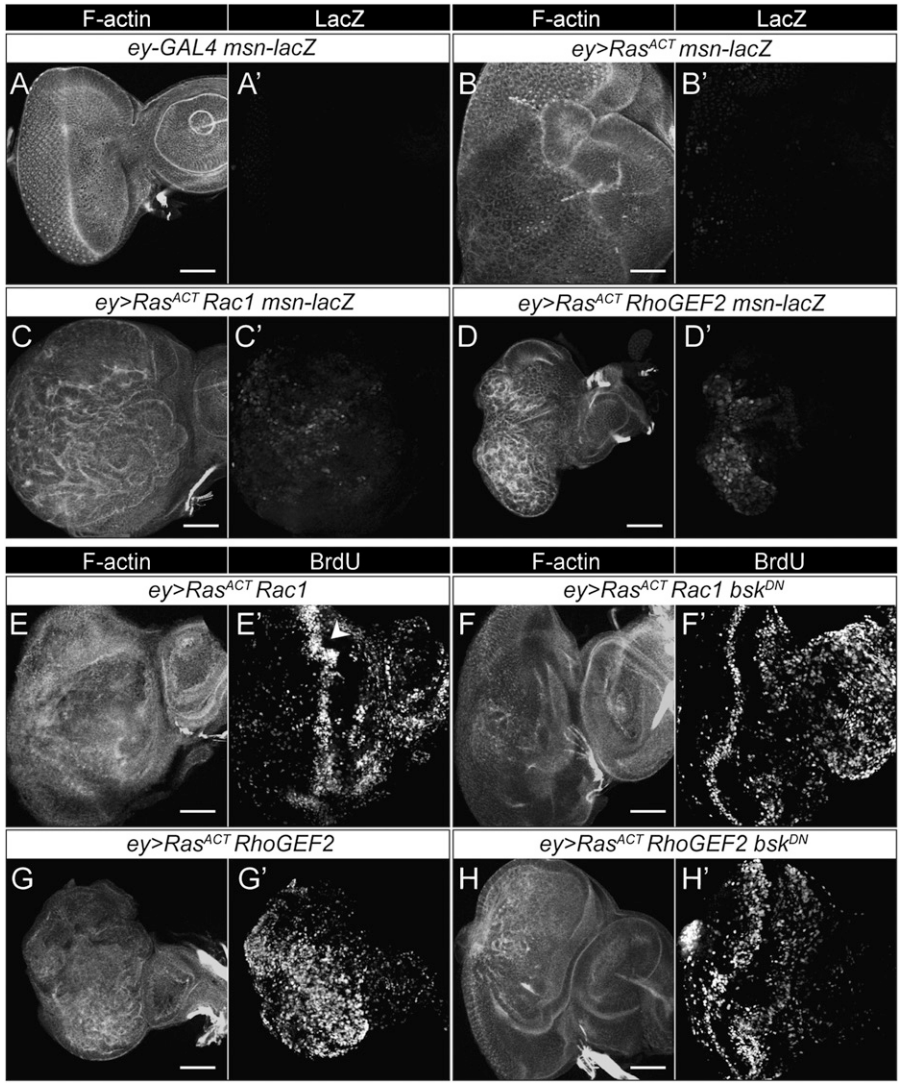

FIGURE 3.— The JNK pathway is upregulated in *ey>Ras^{ACT}* + *Rac1* or *ey>Ras^{ACT}* + *RhoGEF2*, and blocking JNK reduces the overgrowth by decreasing the number of S phases: (A–D) LacZ staining of eye discs from female larvae of: (A) *ey-GAL4 msn-lacZ*, (B) *ey>Ras^{ACT} msn-lacZ*, (C) *ey>Ras^{ACT} Rac1 msn-lacZ*, (D) *ey>Ras^{ACT} RhoGEF2 msn-lacZ*. Rac1 or RhoGEF2-expressing eye discs show increased LacZ staining (*msn* expression). (E–H) BrdU labeling of eye discs from: (E) *ey>Ras^{ACT} Rac1*, (F) *ey>Ras^{ACT} Rac1 bsk^{DN}*, (G) *ey>Ras^{ACT} RhoGEF2*, (H) *ey>Ras^{ACT} RhoGEF2 bsk^{DN}*. The ectopic S phases are suppressed by blocking JNK signaling with *bsk^{DN}*. In E, the arrowhead marks the second mitotic wave. Scale bars, 50 μm.

suppress the *ey>Ras^{ACT}* + *RhoGEF2* or *Rac1* phenotypes. Indeed, *bsk^2*/+ suppressed the cooperative overgrowth phenotypes of *Rac1* or *RhoGEF2* with *Ras^{ACT}* (see Figure S8). Collectively, these data suggest that *RhoGEF2, pbl, Rac1,* and *Rho1* require JNK activity for their cooperation with *Ras^{ACT}*, but that *east* and *rib* cooperate with *Ras^{ACT}* independently of JNK.

Finally, to determine whether upregulation of JNK signaling was sufficient for cooperation with *Ras^{ACT}*, we coexpressed *Ras^{ACT}* (via the *ey* driver) with various transgenes encoding components of the JNK signaling pathway (STRONACH 2005); Bsk (JNK), Hep (JNK kinase), Hep^{ACT} (activated version of Hep), Msn (JNK kinase kinase kinase), and Eiger (tumor necrosis factor, TNF, homolog, which signals through the JNK pathway). We also knocked down a negative regulator of the pathway, the JNK phosphatase, Puc (using *UAS-puc^{RNAi}*), in the *ey>Ras^{ACT}* background. Expression of these transgenes or RNAi had no discernable effect when expressed alone (data not shown) and did not enhance the *ey>Ras^{ACT}* phenotype (see Figure S8 and data not shown). Thus, JNK signaling is required, but is not sufficient, for the cooperation with *Ras^{ACT}* in the whole eye tissue setting.

In a clonal setting, *Rac1*, *Rho1^{ACT}*, *RhoGEF2*, and *pbl* cooperate with *Ras^{ACT}* in tumorigenesis: Mutations in genes, such as *scrib*, that affect cell morphology, result in tumors when the whole tissue is mutant, but are unable to do so when mutant cells are generated in clones surrounded by wild-type tissue (BRUMBY and RICHARDSON 2003; PAGLIARINI and XU 2003). This phenomenon is due to induction of cell-competitive mechanisms leading to JNK-mediated cell death (ADACHI-YAMADA and O'CONNOR 2004; IGAKI 2009; JOHNSTON 2009). However, while *Ras^{ACT}* itself in clones results in some hyperplasia and ectopic differentiation in the eye field relative to wild type (Figure 4, A–D), when it is expressed in *scrib*$^-$ clones in the eye disc, mutant clones outgrow the wild-type tissue forming massive neoplastic tumors that invade between the brain lobes (BRUMBY and RICHARDSON 2003; PAGLIARINI and XU 2003; LEONG *et al.* 2009). On the basis of these findings, we wished to test whether the

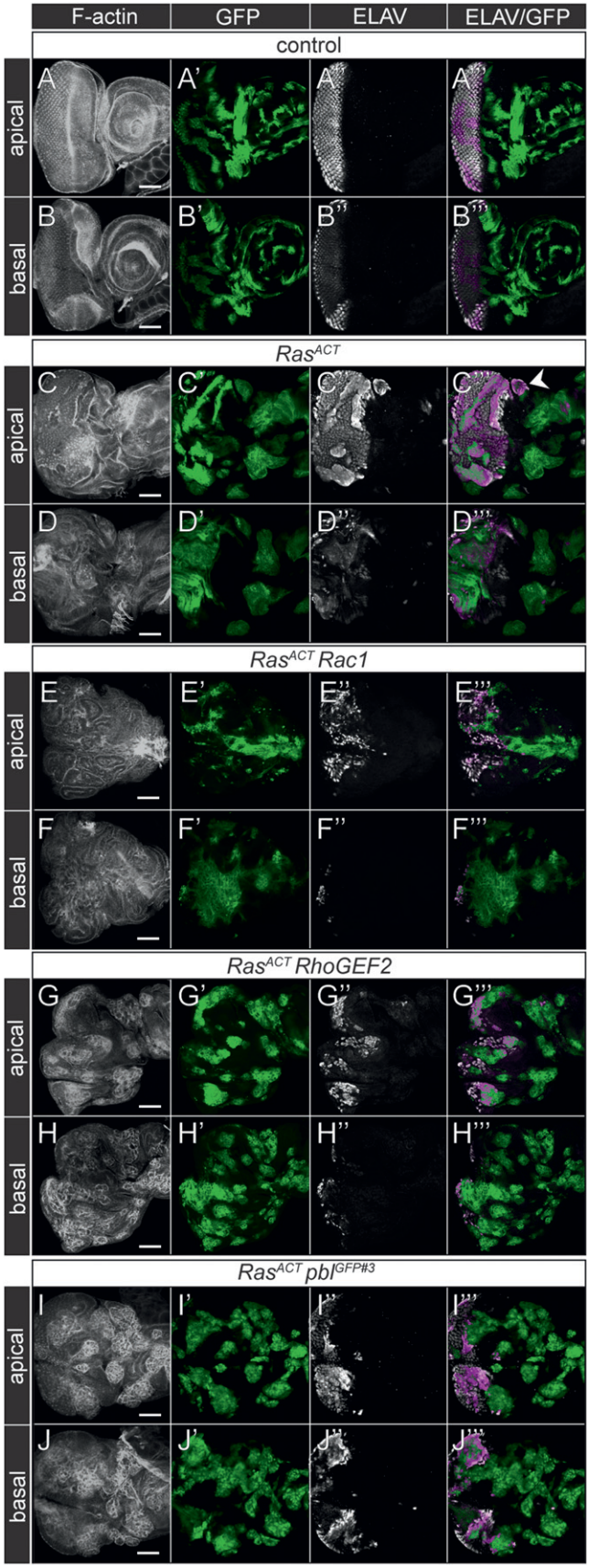

FIGURE 4.— Analysis of the cooperation of *Rac1, RhoGEF2,* and *pbl* with Ras^{ACT} in eye disc clones: expression of Ras^{ACT} with cooperating oncogenes in clones compared with controls from day 5 staged larvae stained for F-actin and ELAV. Clones are positively marked by GFP. (A) *FRT82B* control, apical section; (B) *FRT82B* control, basal section; (C) Ras^{ACT} clones, apical section; (D) Ras^{ACT} clones, basal section; (E) Ras^{ACT} + *Rac1*, apical section; (F) Ras^{ACT} + *Rac1*, basal section; (G) Ras^{ACT} + *RhoGEF2*, apical section; (H) Ras^{ACT} + *RhoGEF2*, basal section; (I) Ras^{ACT} + $pbl^{GFP\#3}$, apical section; (J) Ras^{ACT} + $pbl^{GFP\#3}$, basal section. In C, the arrowhead points to a patch of ectopic differentiation. Expression of *Rac1, RhoGEF2,* or $pbl^{GFP\#3}$ with Ras^{ACT} results in large overgrowths, more prominently in basal sections. Non-cell-autonomous overgrowth is also observed around the clones as evidenced by tissue folding seen by F-actin staining. Scale bars, 50 μm.

ey>Ras^{ACT} cooperating genes could cooperate with Ras^{ACT} in a clonal setting.

Rac1: When expressed alone, *Rac1* showed many small clones that were basally excluded with pyknotic features, suggesting that cells were dying or being outcompeted (see Figure S9). *Rac1* cooperated with Ras^{ACT} to form large neoplastic tumors, particularly in the basal sections, and differentiation was largely blocked (Figure 4, E and F). Larvae harboring these tumors showed an extended larval lifetime, over which the tumors continued to grow, reaching massive sizes, similar to *scrib*$^-$ + Ras^{ACT} tumors (see Figure S10).

Rho1: $Rho1^{GS12503}$ expression resulted in very small clones, suggesting that they were dying or being outcompeted; however, coexpression of Ras^{ACT} with $Rho1^{GS12503}$ did not improve clonal survival (see Figure S11). Since activated Rho1 ($Rho1^{ACT}$) was able to cooperate better than wild-type Rho1 when expressed in the whole eye tissue (Figure 2, D and E), we envisaged that $Rho1^{ACT}$ may be able to cooperate with Ras^{ACT} in clones. Indeed, while $Rho1^{ACT}$ alone resulted in small clones and morphological defects, $Rho1^{ACT}$ + Ras^{ACT} tumors showed overgrowth during the extended larval lifetime forming invasive tumors, as scored by invasion between the brain lobes (see Figure S12).

RhoGEF2: Expression of *RhoGEF2* alone resulted in small clones exhibiting features of dying cells (see Figure S9). *RhoGEF2* cooperated with Ras^{ACT} to form large neoplastic tumors, particularly in the basal sections, with reduced differentiation (Figure 4, G–H), and the tumors increased in size over the extended larval life span, although were not as large as *scrib*$^-$ + Ras^{ACT} tumors (see Figure S10).

Pbl: Expression of *pbl* alone produced wild-type sized clones, although some basally extruded differentiated cells were observed (see Figure S9). Similar to *RhoGEF2* + Ras^{ACT}, *pbl* cooperated with Ras^{ACT} to form large neoplastic tumors, with reduced differentiation (Figure 4, I–J) and showed massive overgrowth over the extended larval stage (see Figure S10).

Rib: rib expression via the transgene of *GS* line resulted in very small clones, suggesting that they were dying or being outcompeted (see Figure S11; data not shown). Coexpression of Ras^{ACT} with *rib* mildly increased *rib* clonal size, but did not lead to tumor formation (see

Figure S11). Interestingly, *rib* + Ras^{ACT} eye discs showed non-cell-autonomous overgrowth effects, suggesting that Ras^{ACT} may impart "un-dead" cell characteristics to the *rib*-expressing cells, allowing the release of morphogens that promote compensatory proliferation of the surrounding wild-type tissue, as has been previously described (reviewed by Fan and Bergmann 2008).

East: east-expressing clones alone in the eye disc did not appear to show any morphological or differentiation abnormities and coexpression of *east* with Ras^{ACT} resulted in a similar phenotype to Ras^{ACT} alone (see Figure S11). Thus, unlike the situation in the whole eye disc, East did not cooperate with Ras^{ACT} to promote hyperplasia or neoplasia in the clonal system.

Taken together, these data show that *Rac1*, an activated allele of *Rho1* ($Rho1^{ACT}$), *RhoGEF2*, and *pbl*, but not *Rho1*, *rib*, or *east*, were capable of cooperating with Ras^{ACT} in a clonal setting. The differences observed between cooperative effects of these genes in the whole tissue *vs.* the clonal setting highlight the context dependent nature of Ras^{ACT}-mediated cooperative tumorigenesis.

JNK is upregulated in eye disc clones of Ras^{ACT} + *Rac1* or *RhoGEF2*, and is required and sufficient for cooperative neoplastic overgrowth: We then tested whether the JNK pathway was upregulated in eye disc clones upon the expression of *Rac1* or *RhoGEF2* with Ras^{ACT} by monitoring the expression JNK pathway reporter, *msn-lacZ*. In Ras^{ACT} + *Rac1* or *RhoGEF2* + Ras^{ACT}-expressing clones, in either apical or basal sections, high levels of JNK signaling were observed (Figure 5, B, C, F, G) compared with Ras^{ACT}-expressing clones alone (Figure 5A) or wild-type discs (see Figure 3A). Indeed, in Ras^{ACT} + *Rac1*-expressing clones, high levels of *msn-lacZ* expression were also observed in the tissue invading between the brain lobes (Figure 5D), consistent with a role for JNK in promoting cell migration and invasion. The increased expression of *msn-lacZ* in the *RhoGEF2* + Ras^{ACT}-expressing clones (Figure 5, F and G), compared with Ras^{ACT} clones alone, likely reflected increased levels of JNK activation due to RhoGEF2 activity, since expression of *RhoGEF2* alone in clones also exhibited an upregulation of *msn-lacZ* expression (Figure 5E). This is likely to also be the case for *Rac1*, although we were unable to analyze the expression of *msn-lacZ* in clones expressing *Rac1* alone, since in this genetic background the clones were poorly viable (data not shown).

To determine the importance of JNK on the cooperative overgrowth in the clonal setting, we blocked the JNK pathway, using bsk^{DN}, in *Rac1* + Ras^{ACT} or *RhoGEF2* + Ras^{ACT}-expressing clones (Figure 6). Indeed, expression of bsk^{DN} increased differentiation and restored pupation of both *Rac1* + Ras^{ACT} (Figure 6, C and D compared with Figure 6, A and B) and *RhoGEF2* + Ras^{ACT} (Figure 6, G and H, compared with Figure 6, E and F)-expressing clones. Furthermore, bsk^{DN} reduced the invasive cell morphology of *Rac1* + Ras^{ACT}-expressing clones and decreased the invasive properties of the tumor (Figure 6, C and D, compared with Figure 6, A and B and data not shown). Furthermore, the expression of bsk^{DN} in $Rho1^{ACT}$ + Ras^{ACT}-expressing clones also restored pupation, increased differentiation, and prevented invasion between the brain lobes (see Figure S13). Collectively, these data show that the activation of JNK is essential to preventing differentiation, for blocking pupation, and for the invasive behavior of *RhoGEF2* + Ras^{ACT}, *Rac1* + Ras^{ACT}, or $Rho1^{ACT}$ + Ras^{ACT} tumors. However, at least in the case of *Rac1* + Ras^{ACT} + bsk^{DN} the tumors were still larger than Ras^{ACT} clones alone. These data indicate that in *Rac1* + Ras^{ACT} tumors a JNK-independent signal appears to drive additional overgrowth. This is in contrast to the whole-tissue system in which the increased proliferation of *Rac1* + Ras^{ACT} eye discs was JNK dependent (Figure 3, E and F). *RhoGEF2* + Ras^{ACT} + bsk^{DN} or $Rho1^{ACT}$ + Ras^{ACT}+ bsk^{DN} tumors were more similar to Ras^{ACT} alone, so in these cases (and possibly also *Rac1* + Ras^{ACT} tumors) a JNK-dependent signal is required for additional overgrowth. The requirement for JNK in this additional overgrowth is likely to relate to JNKs ability to block differentiation and pupation in these Ras^{ACT}-expressing clones, thereby enabling tumor overgrowth during an extended larval phase (see Discussion).

Finally, to examine whether activation of JNK was sufficient to cooperate with Ras^{ACT} in a clonal setting, we expressed a *UAS-bsk* transgene alone or in combination with *UAS-*Ras^{ACT} in eye disc clones and analyzed clonal growth with time (see Figure S14). Expression of *bsk* alone in clones resulted in small clone size and many cells exhibited a pyknotic phenotype, suggesting that cells were undergoing apoptosis or being outcompeted (see Figure S14). By contrast, expression of Ras^{ACT} with *bsk* rescued the cell-death phenotype of *bsk*-expressing clones and at day 5, eye discs were similar to Ras^{ACT} expression alone (see Figure S14). However, some *bsk* + Ras^{ACT} mosaic larvae exhibited an extended larval phase in which the tumor overgrew the surrounding wild-type tissue (see Figure S14). The tissue overgrowth was associated with altered cell morphology and aberrant differentiation. Moreover, in older larvae, tumor invasion was observed between the brain lobes (see Figure S14). Collectively, our data show that in a clonal setting, activation of JNK is sufficient to block pupation, promote Ras^{ACT}–mediated proliferation, disrupt differentiation, and induce invasive properties.

Cooperation of Ha-Ras^{V12} and JNK signaling in mammalian breast epithelial cells and in human cancer: Given our findings of the importance of JNK signaling in Drosophila Ras^{ACT}-mediated cooperative tumorigenesis with actin cytoskeletal regulators, we sought to investigate the requirement of JNK signaling for cooperation with oncogenic Ras in mammalian cell models and in human cancer. To explore the cooperation of JNK with activated Ras, we utilized MCF10A normal

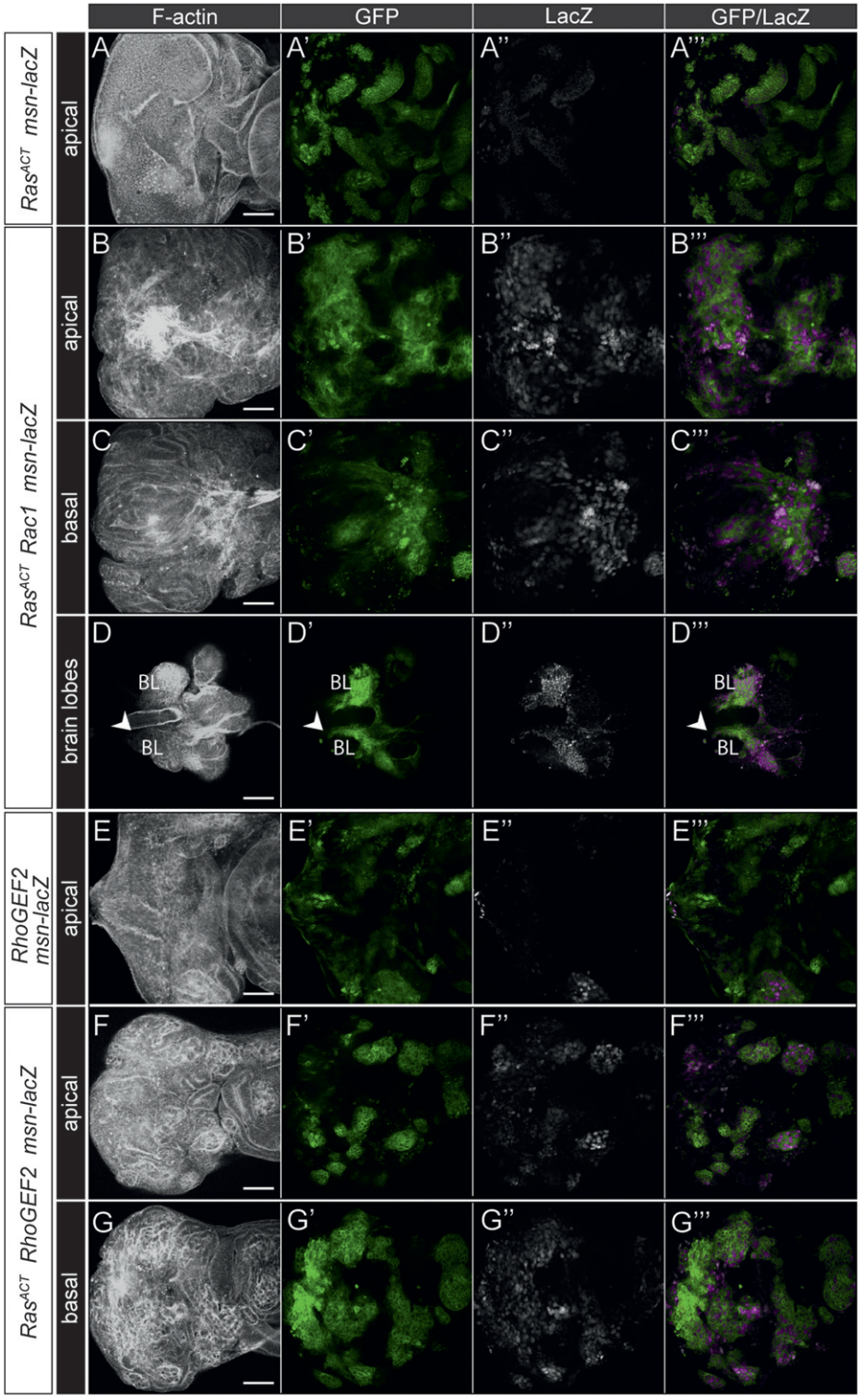

FIGURE 5.— *Rac1* or *RhoGEF2* expression in clones results in upregulation of the JNK pathway: LacZ staining, to detect *msn-lacZ* expression, and F-actin staining of mosaic discs expressing *Rac1* or *RhoGEF2* +/− Ras^{ACT}. Clones are positively marked by GFP. (A) Ras^{ACT} clones in *msn-lacZ*/+ discs, (B) Ras^{ACT} + *Rac1* clones in *msn-lacZ*/+ discs, apical view, (C) Ras^{ACT} + *Rac1* clones in *msn-lacZ*/+ discs, basal view, (D) Ras^{ACT} + *Rac1* clones in *msn-lacZ*/+ discs with brain lobes, showing invasion of GFP+ tissue between the brain lobes, BL, (E) *RhoGEF2* clones in *msn-lacZ*/+ discs, (F) Ras^{ACT} + *RhoGEF2* clones in *msn-lacZ*/+ discs, apical view, (G) Ras^{ACT} + *RhoGEF2* clones in *msn-lacZ*/+ discs, basal view. In D, the arrowhead points to GFP^+ tissue invading between the brain lobes. Ras^{ACT} results in mild ectopic *msn-lacZ* expression. Ras^{ACT} + *RhoGEF2* and Ras^{ACT} + *Rac1* clones show extensive upregulation of *msn-lacZ*. In some *RhoGEF2*-expressing clones, high levels of *msn-lacZ* expression is observed. Scale bars, A–C, E–G, 50 μm; D, 200 μm.

breast epithelial cells grown in 3D matrigel cultures. MCF10A cells form acini in matrigel; however, upon low-level expression of activated Harvey–Ras (Ha-Ras^{V12}) the lumens become filled with cells and with the concomitant knockdown of cell polarity regulators, such as hScrib, cells form invasive clusters (Dow *et al.* 2008)—thus this system is a useful model with which to examine cooperative tumorigenesis. We established MCF10A cell populations overexpressing *JNK1a1* and the JNK kinase genes, *MKK4* or *MKK7*, with or without *Ha-*Ras^{V12} and examined their behavior in matrigel (Figure 7, A–C). MCF10A lines expressing *JNK1a1, MKK4,* or *MKK7* alone were similar to controls, while low-level *Ha-*Ras^{V12} expression alone showed some invasive acini. Coexpression of *JNK1a1, MKK4,* or *MKK7* with *Ha-*Ras^{V12}

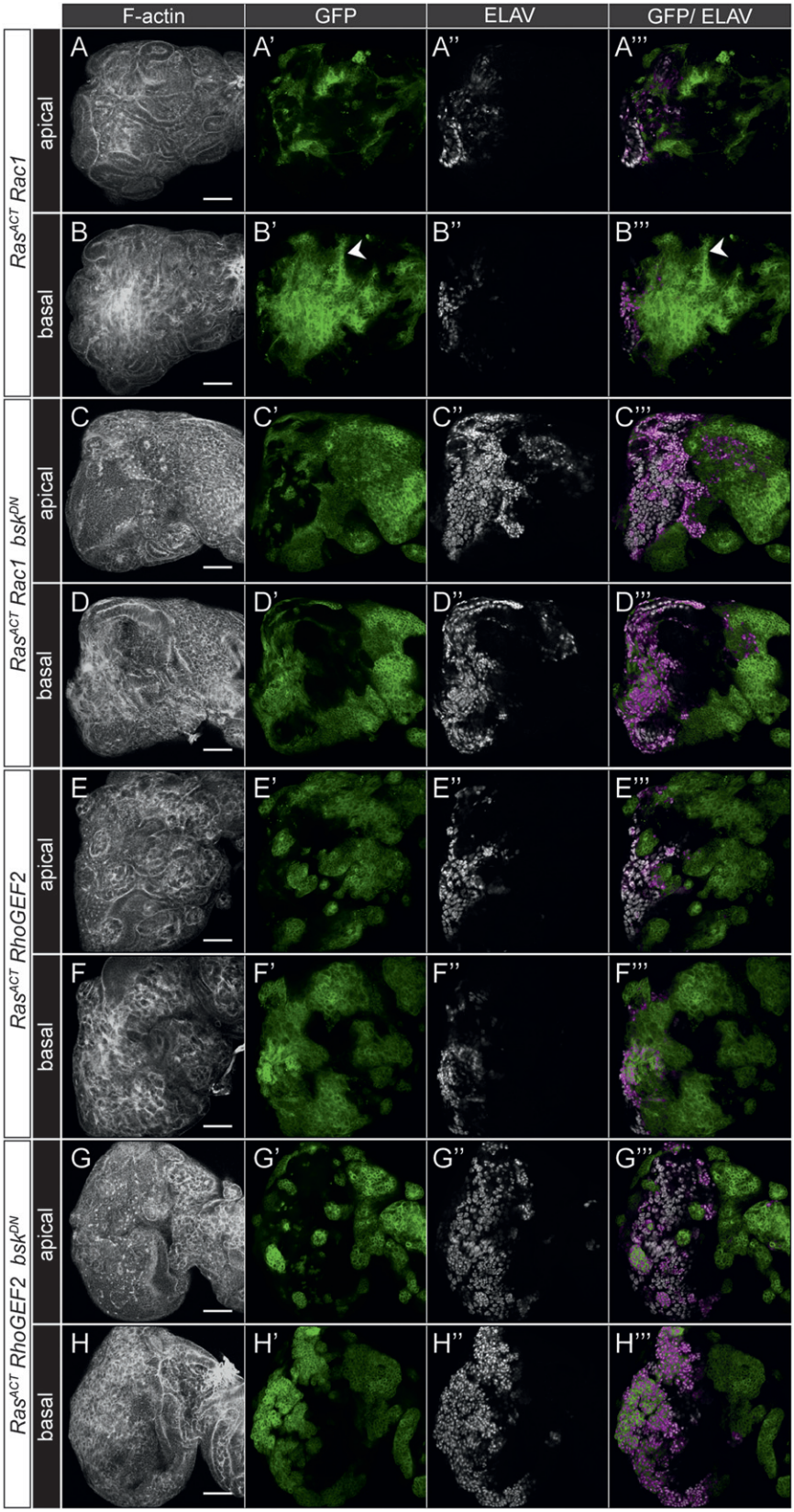

FIGURE 6.— JNK signaling is required for cooperation of *Rac1* or *RhoGEF2* with Ras^{ACT} in clones: clones expressing Ras^{ACT} with *Rac1* or *RhoGEF2* +/− bsk^{DN} at day 5, stained for F-actin and ELAV. Clones are positively marked by GFP. (A) Ras^{ACT} + *Rac1* clones, apical view; (B) Ras^{ACT} + *Rac1* clones, basal view; (C) Ras^{ACT} + *Rac1* + bsk^{DN} clones, apical view; (D) Ras^{ACT} + *Rac1* + bsk^{DN} clones, basal view; (E) Ras^{ACT} + *RhoGEF2* clones, apical view; (F) Ras^{ACT} + *RhoGEF2* clones, basal view; (G) Ras^{ACT} + *RhoGEF2* + bsk^{DN} clones, apical view; (H) Ras^{ACT} + *RhoGEF2* + bsk^{DN} clones, basal view. In B, the arrowhead points to tissue with an invasive morphology. Expression of bsk^{DN} rescues the cooperation of *Rac1* or *RhoGEF2* with Ras^{ACT} by increasing differentiation and reducing the invasive phenotype of *Rac1* + Ras^{ACT} clones. Scale bars, 50 μm.

resulted in a ~4-fold, ~3-fold and ~2.5-fold increase in invasive acini relative to *Ha-Ras*^{V12} alone, respectively. Thus, similar to Drosophila, upregulation of JNK in mammalian epithelial cells cooperates with Ras^{V12} to promote invasive properties upon normal human epithelial cells.

We also examined the effect of expressing *JNK1a1, MKK4,* or *MKK7* in MCF10A cells on anchorage-

independent growth (growth in soft agar; see Figure S15). Expression of these genes alone could not confer anchorage-independent growth to MCF10A cells or modify the ability of *Ha-Ras^{V12}* to promote growth in soft agar. In 2D cultures, expression of *JNK1a1, MKK4,* or *MKK7* also did not enhance the proliferation rate alone or in combination with *Ha-Ras^{V12}*, and indeed *MKK4* resulted in a decreased proliferation rate of *Ha-Ras^{V12}*-expressing cells (see Figure S15). These data indicate that upregulation of JNK signaling cooperates with Ras^{V12} in 3D cultures to promote invasion, but does not enhance cell proliferation rates in 2D cultures or promote anchorage independent growth.

To further examine the relevance of our findings to human cancer, we investigated a gene signature related to JNK signaling (Han *et al.* 2002) for its association with gene expression in breast cancer using publicly available data sets (see File S1). Breast cancers are now divided into three major molecular subtypes, according to estrogen receptor and HER2 expression, for clinical and research purposes (Sorlie *et al.* 2001; Sorlie *et al.* 2003; Sotiriou *et al.* 2003), which are recognized to have different biological mechanisms of tumor growth and progression. We found that in the breast cancer subtype that overexpresses the human epidermal growth factor receptor (HER2$^+$), there was a moderate and positive correlation with the JNK signature relative to the other breast cancer subtypes (HER2$^+$ Spearman's $\rho = 0.15$, $P < 0.001$; ER$^+$/HER2$^-$ $\rho = 0.05$, $P = 0.05$; ER$^-$/HER2$^-$ $\rho = 0.02$, $P = 0.6$; Figure 7D). As HER2 upregulation is known to activate Ras/Erk signaling ($P < 0.0001$; Figure 7E), this observation is in agreement with our data, highlighting cooperation between Ras and JNK signaling. The association of a high JNK signature in ER$^+$/HER2$^-$ ("luminal" subtype) breast cancers is also consistent with reports from previous clinical studies and xenograft models of tamoxifen resistance, which have reported a positive association with activated/phosphorylated JNK (Johnston *et al.* 1999; Schiff *et al.* 2000), although these tumors do not show high expression of the Ras signature (Figure 7E). While Ras is not an established oncogene in breast cancer, Ras pathway upregulation is recognized to be important for breast cancer growth and tumorigenesis (reviewed by Whyte *et al.* 2009), and our data support a link between Ras and JNK signaling in HER2$^+$ breast cancers. Together these data support further investigation into the relationship between JNK and Ras signaling in human cancers.

DISCUSSION

In a genome-wide overexpression screen for Ras^{ACT}-cooperating genes in the developing eye, we have identified *Rac1, Rho1, RhoGEF2, pbl, rib,* and *east*, which all have roles in regulation of cell morphology. We showed that in a clonal setting, which reveals the competitive ability of mutant tissue, that *Rac1*, an activated allele of *Rho1* (*Rho1^{ACT}*), *RhoGEF2*, and *pbl* exhibit cooperativity with *Ras^{ACT}*. Our studies reveal that JNK signaling is required for the cooperation of these genes with *Ras^{ACT}*; however, the role of JNK is gene and context dependent. In a whole-tissue setting, we show that (1) expression of *Rac1* + *Ras^{ACT}* or *RhoGEF2* + *Ras^{ACT}* leads to upregulation of the JNK-Jun/Fos (AP-1) target gene, *msn*, (2) that JNK signaling is required for the increased proliferative potential of *Rac1* or *RhoGEF2* with *Ras^{ACT}*, and (3) that the eye phenotypes of *Rac1, Rho1 RhoGEF2,* and *pbl* require JNK, but (4) JNK is not sufficient for cooperation. By contrast in a clonal setting, upregulation of JNK is both necessary and sufficient for cooperative tumorigenesis of *Rac1, Rho1^{ACT},* or *RhoGEF2* with Ras^{ACT}: (1) JNK is upregulated in *Rac1* + *Ras^{ACT}* or *RhoGEF2* +/− *Ras^{ACT}* clones, (2) blocking JNK reduces the tumorigenic potential of *Rac1, RhoGEF2,* or *Rho1^{ACT}* with *Ras^{ACT}*, and (3) upregulation of JNK alone cooperates with *Ras^{ACT}*, although was less aggressive than *scrib*, *Rac1, Rho1^{ACT}, RhoGEF2*, or *pbl* with *Ras^{ACT}*. This role for JNK is conserved in mammalian cells, since JNK upregulation cooperates with activated *Ha-Ras* to promote invasive growth of MCF10A normal breast epithelial cells in 3D cultures, and upregulation of the JNK signature correlates with HER2$^+$ human breast cancers, where Ras signaling is upregulated. However, upregulation of JNK signaling in mammalian cells did not increase the proliferation or anchorage-independent growth properties of *Ha-Ras^{V12}*, consistent with our analysis that JNK was not sufficient to promote hyperproliferation in the *ey>Ras^{ACT}* system. Collectively, our data reveal the importance of the RhoGEF/Rho-family/JNK pathway for cooperative tumorigenesis with *Ras^{ACT}*. Moreover, our data reveal that the cooperation of JNK with oncogenic Ras in tumorigenesis is conserved between Drosophila and humans and highlights the relevance of Drosophila screens and genetic analysis to human cancer biology.

Context dependent effects of JNK activation on cell behavior: Our analysis revealed that the *Ras^{ACT}*-cooperating genes resulted in different effects in different contexts; when expressed alone within the whole eye tissue the spectrum of phenotypes ranged from little effect (*Rac1, pbl, east*) to reduced eyes with morphological and differentiation defects (*Rho1, Rho1^{ACT}, RhoGEF2, rib*), and with *Ras^{ACT}* from increased hyperplasia (*Rac1, pbl, east*) or more severe morphological and differentiation defects (*Rho1, Rho1^{ACT}, RhoGEF2, rib*), while in the clonal setting expression of the *Ras^{ACT}*-cooperating genes alone ranged from little effect (*pbl, east*) to small clones with evidence of apoptosis (*Rac1, Rho1, Rho1^{ACT}, RhoGEF2, rib*), and with *Ras^{ACT}* either did not cooperate (*Rho1, rib* and *east*) or resulted in neoplastic invasive tumors (*Rac1, Rho1^{ACT}, RhoGEF2, pbl*). We hypothesize that these spectrums of phenotypic outcomes are related to the severity of cell-morphology disruption and to different levels of Rho1

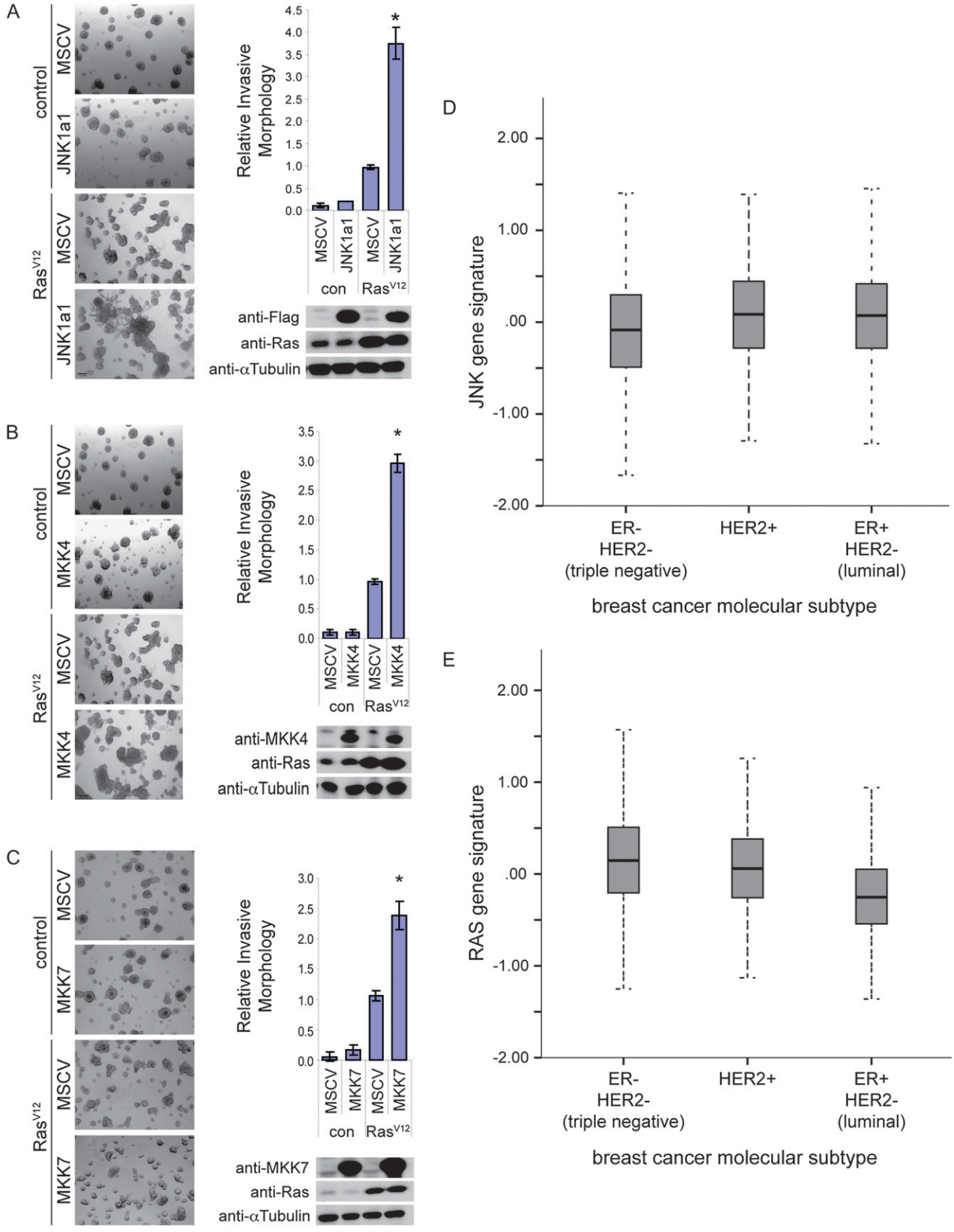

FIGURE 7.— Cooperation of JNK and Ras signaling in normal mammalian epithelial cells and human cancer: (A–C) *JNK1a1, MKK4,* and *MKK7* cooperate with *Ha-Ras^{V12}* in promoting invasion of MCF10A cells in 3D matrigel cultures. A representative of three independent experiments with three independently derived MCF10A cell line sets overexpressing *JNK1a1* (A), *MKK4* (B), or *MKK7* (C) in the context of *Ha-Ras^{V12}* expression is shown. Bright-field images of acini morphology (left) (scale bar, 100 μm). Invasive morphology quantitation expressed relative to Ha-Ras^{V12} control [$*P < 0.05$; Student's *t*-test, two tailed, unpaired; error

and JNK signaling, although we have not been able to measure this directly due to the absence of reliable Drosophila reagents for Western analysis.

The reduced eye phenotype of *Rho1, Rho1^{ACT}, RhoGEF2,* and *rib* when expressed alone in the whole eye tissue, is consistent with strong activation of JNK, since *ey*-driven expression of *hep^{ACT}* also results in reduced eyes (data not shown). Furthermore, in cooperation with *Ras^{ACT}* in the whole eye disc, *Rac1, Rho1, Rho1^{ACT}, RhoGEF2,* and *pbl* required JNK. Indeed, *Rac1* + *Ras^{ACT}* and *RhoGEF2* + *Ras^{ACT}* eye discs upregulated Jun/Fos (AP-1) activity and JNK was required for the increased numbers of S phase cells in these discs. Thus, *RhoGEF2* and *Rac1* require the activation of JNK to cooperate with *Ras^{ACT}* to result in increased hyperplasia. A role for JNK in promoting proliferation has recently been revealed in tissue regeneration after wounding (Bergantinos *et al.* 2010), and the SWH tissue growth control pathway has been implicated in this process (Grusche *et al.* 2011; Sun and Irvine 2011). Whether the SWH pathway is also required for cooperation of JNK with *Ras^{ACT}* to increase hyperplasia remains to be determined.

In the clonal setting the cooperation of *Rac1, Rho1^{ACT}, RhoGEF2,* and *pbl*, but not *Rho1, rib* or *east*, with *Ras^{ACT}* could be related to their ability to upregulate JNK to an appropriate level. Indeed the degree of overgrowth and invasive properties may be related to the level of JNK upregulation; *Rac1* + *Ras^{ACT}* and *scrib^{-}* + *Ras^{ACT}* tumors show a more consistent upregulation of JNK (revealed by *msn-lacZ* expression) than in *RhoGEF2* + *Ras^{ACT}* tumors, which correlates with the more severe overgrowth and invasion of *Rac1* + *Ras^{ACT}* or *scrib^{-}* + *Ras^{ACT}* tumors. Moreover, the expression of *bsk* (*jnk*) alone was sufficient to cooperate with *Ras^{ACT}* to produce large neoplastic tumors, consistent with the previous report that upregulation of JNKK (Hep) expression can also cooperate with *Ras^{ACT}* (Uhlirova and Bohmann 2006). Uhlirova and Bohmann also showed that the level of JNK pathway activation appears to be important for this cooperation, since overexpression of an activated version of *hep* (*hep^{ACT}*), which in contrast to *bsk* or *hep* upregulation, promotes high levels of cell death when expressed in clones (Brumby and Richardson 2003; Leong *et al.* 2009), was unable to cooperate with *Ras^{ACT}* (Uhlirova and Bohmann 2006). These observations may explain why *Rho1* and *rib* (situations in which we hypothesize that JNK signaling may be higher) did not cooperate with *Ras^{ACT}* in the clonal situation; the high levels of cell death triggered by strong JNK activation may not be able to be overcome by expression of *Ras^{ACT}*. Upregulation of the Ras–MAPK signaling pathway blocks apoptosis via phosphorylation of the cell-death inducer, Hid, as well as downregulation of *hid* transcription (Bergmann *et al.* 1998; Kurada and White 1998). When high levels of JNK activity are induced, the activation of Hid or other cell death inducers may not be able to be blocked by *Ras^{ACT}*. By contrast, since expression of *east* alone in clones did not exhibit signs of cell death, *east* may be unable to induce sufficient activation of JNK in the clonal setting to enable cooperation with *Ras^{ACT}*.

In a clonal setting, we showed that JNK is required to block differentiation and pupation and to promote the invasive phenotypes of *RhoGEF2, Rac1,* and *Rho1^{ACT}* in cooperation with Ras^{ACT}, although not the cell-morphology defects. The effect of JNK on invasion has been shown to be due to upregulation of targets important in cell migration, such as Paxillin, and in breakdown of the extracellular matrix, such as MMP1 (Beaucher *et al.* 2006; Uhlirova and Bohmann 2006; Srivastava *et al.* 2007; Leong *et al.* 2009), but how JNK blocks differentiation and pupation is currently unknown. Expression of *bsk^{DN}* also reduced tumor overgrowth to a level commensurate with *Ras^{ACT}* alone for all except *Rac1* + *Ras^{ACT}*. The reduced differentiation and delayed pupation mediated by JNK most likely contributes to the overgrowth phenotypes, since the overgrowth manifests during the extended larval phase. The JNK-mediated overgrowth in these tumors may depend upon the JAK–STAT pathway, since JNK signaling in *scrib^{-}* cells has been shown to induce expression of the cytokine, Unpaired (Upd), which can lead to activation of the JAK–STAT tissue growth control signaling pathway in *scrib^{-}* cells, but also in adjacent cells wild type (Wu *et al.* 2010). *Rac1, Rho1^{ACT}, RhoGEF2,* and *pbl* + *Ras^{ACT}* mosaic discs exhibited some non-cell-autonomous tissue growth, suggesting that such a mechanism involving JAK–STAT signaling may be occurring.

For *Rac1* + *Ras^{ACT}* + *bsk^{DN}* the tumors were still larger than *Ras^{ACT}* alone, suggesting that a JNK-independent mechanism must be triggered to drive the overgrowth of these tumors and their competitive advantage over the surrounding wild-type tissue. This is similar to what occurs in *scrib^{-}* + *Ras^{ACT}* tumors when JNK signaling is blocked; although the overgrowth is reduced, tumors are still considerably larger than with *Ras^{ACT}* alone (Leong *et al.* 2009). Pertinent to this is that while activation of JNK alone can cooperate with Ras^{ACT} (this

bars represent standard deviation] (right). Western blot of whole-cell lysates probed with antibodies as indicated on the right side. α-Tubulin was used as loading control. (D, E) Box-plots representing expression of a gene signature of JNK activation and Ras activation in human breast cancers. The relative expression of JNK signature genes (D) and Ras signature genes (E) were compared using gene expression data and divided according to the three major molecular breast cancer subtypes: ER^{-}/HER2^{-} (triple negative), HER2^{+}, and ER^{+}/HER2^{-} (luminal). The correlation with high JNK signature expression in HER2^{+} and ER^{+}/HER2^{-} is higher than in the other breast cancer subtypes, and of these the Ras signature is higher in the HER2^{+} subtype ($P < 0.0001$).

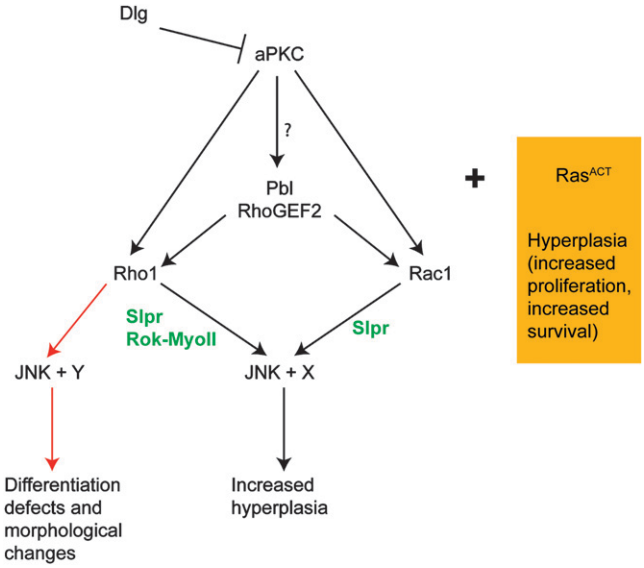

Figure 8.—Model of interactions of Ras^{ACT}-cooperating genes: genetic epistasis tests reveal a possible model of how the Ras^{ACT}-cooperating genes may function in a pathway to lead to the upregulation of JNK in the *ey-GAL4* system. Expression of Ras^{ACT} alone via *ey-GAL4* leads to hyperplasia. Pathways shown in black lead to increased hyperplasia, while those in red lead to differentiation defects and morphological changes, which we propose is due to higher levels of Rho1 and JNK signaling. Proteins shown in green are hypothesized to be required for the effects, on the basis of information in other systems (see text). Since JNK is required, but not sufficient, to cooperate with *ey>*Ras^{ACT}, we hypothesize that other factors (X or Y) are altered by Rho1 or Rac1 signaling to enable cooperation with Ras^{ACT}. Whether knockdown of Dlg or activation of aPKC requires Pbl or RhoGEF2 is not known (indicated by ?). See text for further details.

study; Uhlirova and Bohmann 2006), the cooperative effect is not as potent as with *Rac1* + Ras^{ACT} or *scrib*$^{-}$ + Ras^{ACT}, raising the possibility that these genes are affecting other processes to mediate cooperative overgrowth. This may include the integrity of the E-cadherin–β-catenin complex, which has been revealed to also contribute to Ras^{ACT}-mediated cooperative tumourigenesis (Igaki *et al.* 2006). Furthermore, we and others have recently shown that the SWH pathway, which inhibits both cell proliferation and survival, is deregulated by loss-of-function of the polarity regulator, Lgl, in the eye disc (Grzeschik *et al.* 2010), and in *lgl*$^{-}$ + Ras^{V12} clones in the wing disc (Menendez *et al.* 2010). Therefore deregulated SWH signaling could contribute to the increased proliferative potential of *Rac1* + Ras^{ACT} or *scrib*$^{-}$ + Ras^{ACT} tumors independently of JNK. Other factors, such as the relative level of the Myc cell growth protein, which has been shown to affect the survival of *lgl*$^{-}$ clones in the wing disc (Froldi *et al.* 2010), or the recently discovered membrane protein isoform, Flower-Lose, which is associated with dying cells in cell competition (Rhiner *et al.* 2010), may also be involved in the overgrowth of *Rac1* + Ras^{ACT} or *scrib*$^{-}$ + Ras^{ACT} tumors.

Hierarchical relationships between the Ras^{ACT}-cooperating genes: Our genetic analysis of the Ras^{ACT}-cooperating genes with *Rho1, Rac1, aPKC,* and *bsk* (*jnk*) in the whole eye (Table 2) has revealed an interaction relationship between these genes (Figure 8). We found that blocking aPKC, with the kinase-dead (dominant-negative) form, partially suppressed the dlg^{RNAi} + Ras^{ACT} cooperative phenotype, but not other cooperative interactions, suggesting that aPKC acts downstream of Dlg (consistent with the antagonistic relationship between basal-lateral cell polarity regulators and aPKC, reviewed by Humbert *et al.* 2008). Analysis of the genetic interactions of the Ras^{ACT}-cooperating genes with JNK, revealed that JNK acts downstream of dlg^{RNAi}, $aPKC^{\Delta N}$, *Rac1, Rho1* (and $Rho1^{ACT}$), *RhoGEF2*, and *pbl* in cooperation with Ras^{ACT} (Table 2 and Figure 8). The cooperation of *east* with Ras^{ACT} was epistatic to *rho1, rac1, bsk,* and *aPKC*, and therefore *east* must act downstream or independently of these genes in its cooperation with Ras^{ACT} to result in increased hyperplasia.

Analysis of the epistatic relationships of the Ras^{ACT}-cooperating genes (Table 2) revealed that *RhoGEF2* and *pbl* required both Rac1 and Rho1 activity for their cooperation with Ras^{ACT} (Figure 8). The requirement for Rho1 is consistent with previous studies (Barrett *et al.* 1997; Hacker and Perrimon 1998; O'keefe *et al.* 2001); however, the requirement of Rac1 for RhoGEF2 or Pbl function is novel and may be manifest only in the presence of Ras^{ACT}. We also found that dlg^{RNAi} and $aPKC^{\Delta N}$ cooperation with Ras^{ACT} required Rac1 and Rho1 function (Table 2 and Figure 8); however, whether their cooperation with Ras^{ACT} requires *RhoGEF2* or *pbl* remains to be determined. The mammalian homolog of Pbl (Ect2) can bind to the aPKC (PKCζ)/Par3/Par6 complex, but Ect2 was shown to regulate aPKC activity (Liu *et al.* 2004) rather than vice versa, as we would predict.

The cooperation of *rib* with Ras^{ACT} was not suppressed by bsk^{DN}, but was suppressed by $Rho1^{RNAi}$ such that the female eyes now exhibited a hyperplastic phenotype and male lethality was rescued (Table 2). It is difficult to explain in relation to the model (Figure 8) why the *rib* + Ras^{ACT} phenotype was suppressed by $Rho1^{RNAi}$, but not bsk^{DN}. It is possible that JNK is upregulated so highly by *rib* expression that it cannot be blocked by the bsk^{DN} transgene, but the $Rho1^{RNAi}$ transgene is more effective in downregulating Rho1. The genetic interaction of *rib* + Ras^{ACT} cooperation by $Rho1^{RNAi}$ suggests that Rho1 acts downstream of Rib to mediate cooperation with Ras^{ACT}. Since Rib is a nuclear-localized protein, thought to be a transcription factor (Bradley and Andrew 2001; Shim *et al.* 2001), it is possible that Rib may upregulate the expression of *Rho1* or Rho1 regulators to mediate its known effects on epithelial migration and morphogenesis (Jack and Myette 1997; Blake *et al.* 1998), as well as in cooperation with Ras^{ACT}. Interestingly, Rib has been shown to

upregulate the expression of the apical polarity regulator, Crb, and downregulate Moesin (Moe) to facilitate salivary gland and tracheal tube elongation (Kerman *et al.* 2008). Since Moe is a negative regulator of Rho1 (Speck *et al.* 2003), upregulation of Rib would be predicted to increase Rho1 activity to mediate its affects on cell morphology. Since Rib also upregulates *crb* transcription (Kerman *et al.* 2008), and we and others have shown that high Crb levels deregulates the SWH signaling pathway to promote tissue growth (Grzeschik *et al.* 2010; Robinson *et al.* 2010), it is possible that suppression of Rho1 activity in *rib* + Ras^{ACT} eye discs unleashes this cryptic activity of Rib in tissue growth via its effect on Crb.

Taken together, our genetic interaction data are consistent with a model whereby RhoGEF2 and Pbl act through Rho1 and Rac1 to activate JNK and increase hyperplasia or promote morphological and differentiation defects in a whole-tissue context. However, our data have revealed that while JNK is required, it is not sufficient for this cooperation with Ras^{ACT}, suggesting that Rho1 or Rac1 require another unidentified factor (X or Y) for cooperation with Ras^{ACT} to promote hyperplasia or morphological and differentiation defects (Figure 8). In the case of dlg^{RNAi}, $aPKC^{\Delta N}$, *pbl*, and *Rac1* this interaction results in a hyperplastic response through JNK activation and the effector, X. By contrast, $Rho1^{GS12503}$ and $Rho1^{ACT}$ result in morphological and differentiation defects via JNK activation and the Rho1 effector Y (which may be equivalent to X), while RhoGEF2 has intermediary effects. We propose that stronger activation of Rho1 and therefore JNK in *Rho1*-expressing eye discs leads to induction of a different effector, Y, or to a higher induction of effector, X. High activation of JNK could also contribute to the cell-morphology changes and differentiation defects observed for *Rho1*, since JNK has a well-described role in cell-morphology changes during Drosophila development (Martin-Blanco 1997; Harden 2002) and has been shown to contribute to loss of apico-basal cell polarity in wing imaginal discs in *lgl* mutants (Zhu *et al.* 2010).

Whether this hierarchical pathway linking polarity regulators, through Rho1 and Rac1, to JNK activation (Figure 8) occurs in a clonal setting remains to be determined. JNK is activated in $scrib^{-}$ clones by upregulation of Eiger (Igaki *et al.* 2009), and the Drosophila macrophage-like blood cells (hemocytes) are able to recognize the mutant cells and provide the source of Eiger (Cordero *et al.* 2010). However, since Rho1 and Rac1 can activate JNK during morphogenesis in Drosophila development (Settleman 2001), deregulation of cell polarity regulators may also lead to JNK activation cell intrinsically, via activation of Rho-family regulators, to contribute to Ras^{ACT}-mediated tumorigenesis. Indeed, Rho1 may directly activate JNK signaling, since it can form a complex with Slipper (Slpr, a JNKKK) in imaginal discs to active JNK-mediated cell death (Neisch *et al.* 2010). Moreover, Rho1, via Rho kinase (Rok) and Myosin II (Zipper), activates JNK to mediate compensatory proliferation in imaginal discs (Warner *et al.* 2010). Rac1 can also activate the JNK pathway directly in dorsal closure via Slpr (Garlena *et al.* 2010). Whether the Ras^{ACT}-cooperating genes lead to JNK activation in either the whole-tissue or clonal context via these mechanisms remains to be determined.

Cooperation of oncogenic Ras and JNK in mammalian cancer: Our analysis has revealed that the importance of JNK activation for oncogenic Ras-mediated tumorigenesis extends to mammalian cells, since upregulation of JNK1a or its activators MKK4 or MKK7 cooperates with Ha-Ras^{V12} in the MCF10A normal breast epithelial cell line, to induce invasive growth in 3D matrigel cultures. However, upregulation of the JNK signaling pathway did not cooperate with Ha-Ras^{V12} to increase anchorage-independent growth or cell proliferation in culture. Thus in this context, JNK upregulation is acting simply by promoting the invasive properties of $Ha\text{-}Ras^{V12}$-expressing MCF10A cells. Our previous studies have shown that in this system, the cooperation of *scrib* loss-of-function with $Ha\text{-}Ras^{V12}$ is due to further upregulation of Ras signaling (Dow *et al.* 2008). Whether this is also the case for JNK pathway upregulation in cooperation with $Ha\text{-}Ras^{V12}$ will require further analysis.

Our analysis has also revealed a correlation of the JNK signaling signature with the $HER2^{+}$ breast cancer subtype, which shows upregulation of Ras signaling. This finding provides evidence that upregulation of Ras with JNK may be important for the development of certain types of human cancer. In mammalian cells and human cancer, the role of JNK signaling is complex and context dependent (Kennedy and Davis 2003; Weston and Davis 2007; Whitmarsh and Davis 2007; Wagner and Nebreda 2009). However, our experiments support previous evidence that JNK pathway activation can cooperate with oncogenic Ras in mammalian cell transformation (Nielsen *et al.* 2007; Zhang *et al.* 2007; Ke *et al.* 2010); therefore, our analysis, together with these findings, highlights the need for further research into the association of Ras and JNK status in cancer cell lines and the involvement of JNK signaling in Ras-dependent tumors.

Mammalian homologs exist for Pbl (Ect2), RhoGEF2 (LARG, PDZ-RhoGEF, and p115-RhoGEF), and Rac1/Rho1-family proteins (Table 1). Upregulation of these proteins have been shown to induce cell transformation and are associated with human cancer (Miki *et al.* 1993; Fukuhara *et al.* 1999; Fukuhara *et al.* 2000; Fukuhara *et al.* 2001; Sahai and Marshall 2002; Ridley 2004; Gomez Del Pulgar *et al.* 2005; Titus *et al.* 2005). Indeed, upregulation of Rho-family proteins has been shown to cooperate with oncogenic Ras in enabling cell transformation, by overcoming Ras-induced cellular senescence due to upregulation of the cell-cycle inhibitor

p21 (Olson *et al.* 1998; Coleman *et al.* 2004). Recently, the Rac1 effector, Pak1, has been found to cooperate with ErbB2–MAPK and PI3K signaling in promoting growth factor-independent proliferation in 3D cultures and to be associated with estrogen-receptor positive human breast cancers (Arias-Romero *et al.* 2010). Whether JNK signaling is also involved in these cases has not been investigated. Given our findings from this study, further analysis of the association of mammalian Ect2, RhoGEF2-related proteins, and Rac1/Rho1-family members with JNK signaling, in Ras-dependent human cancers, is warranted. Since the Ras signaling pathway is upregulated in 30% of human cancers, but Ras itself is not sufficient for tumorigenesis due to induction of cellular senescence (Olson *et al.* 1998; Coleman *et al.* 2004; DeNicola and Tuveson 2009), our identification of the importance of JNK for cooperation of Ras with Rho-family regulators suggests new avenues of investigation in the understanding and treatment of Ras-dependent cancers.

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LITERATURE CITED

Adachi-Yamada, T., and M. B. O'Connor, 2004 Mechanisms for removal of developmentally abnormal cells: cell competition and morphogenetic apoptosis. J. Biochem. **136:** 13–17.

Aigaki, T., T. Ohsako, G. Toba, K. Seong and T. Matsuo, 2001 The gene search system: its application to functional genomics in *Drosophila melanogaster.* J. Neurogenet. **15:** 169–178.

Arias-Romero, L. E., O. Villamar-Cruz, A. Pacheco, R. Kosoff, M. Huang *et al.*, 2010 A Rac–Pak signaling pathway is essential for ErbB2-mediated transformation of human breast epithelial cancer cells. Oncogene **29:** 5839–5849.

Baonza, A., and M. Freeman, 2005 Control of cell proliferation in the Drosophila eye by Notch signaling. Dev. Cell **8:** 529–539.

Barrett, K., M. Leptin and J. Settleman, 1997 The Rho GTPase and a putative RhoGEF mediate a signaling pathway for the cell shape changes in Drosophila gastrulation. Cell **91:** 905–915.

Beaucher, M., E. Hersperger, A. Page-McCaw and A. Shearn, 2006 Metastatic ability of Drosophila tumors depends on MMP activity. Dev. Biol. **303:** 625–634.

Bergantinos, C., M. Corominas and F. Serras, 2010 Cell death-induced regeneration in wing imaginal discs requires JNK signalling. Development **137:** 1169–1179.

Bergmann, A., J. Agapite, K. McCall and H. Steller, 1998 The Drosophila gene hid is a direct molecular target of Ras-dependent survival signaling. Cell **95:** 331–341.

Betschinger, J., K. Mechtler and J. A. Knoblich, 2003 The Par complex directs asymmetric cell division by phosphorylating the cytoskeletal protein Lgl. Nature **422:** 326–330.

Bilder, D., and N. Perrimon, 2000 Localization of apical epithelial determinants by the basolateral PDZ protein Scribble. Nature **403:** 676–680.

Bilder, D., M. Li and N. Perrimon, 2000 Cooperative regulation of cell polarity and growth by Drosophila tumor suppressors. Science **289:** 113–116.

Billuart, P., C. G. Winter, A. Maresh, X. Zhao and L. Luo, 2001 Regulating axon branch stability: the role of p190 RhoGAP in repressing a retraction signaling pathway. Cell **107:** 195–207.

Bissell, M. J., and D. Radisky, 2001 Putting tumours in context. Nat. Rev. Cancer **1:** 46–54.

Blake, K. J., G. Myette and J. Jack, 1998 The products of ribbon and raw are necessary for proper cell shape and cellular localization of nonmuscle myosin in Drosophila. Dev. Biol. **203:** 177–188.

Bradley, P. L., and D. J. Andrew, 2001 *ribbon* encodes a novel BTB/POZ protein required for directed cell migration in *Drosophila melanogaster.* Development **128:** 3001–3015.

Brumby, A. M., and H. E. Richardson, 2003 *scribble* mutants cooperate with oncogenic Ras or Notch to cause neoplastic overgrowth in Drosophila. EMBO J. **22:** 5769–5779.

Brumby, A. M., and H. E. Richardson, 2005 Using *Drosophila melanogaster* to map human cancer pathways. Nat. Rev. Cancer **5:** 626–639.

Ciapponi, L., D. B. Jackson, M. Mlodzik and D. Bohmann, 2001 Drosophila Fos mediates ERK and JNK signals via distinct phosphorylation sites. Genes Dev. **15:** 1540–1553.

Coleman, M. L., C. J. Marshall and M. F. Olson, 2004 RAS and RHO GTPases in G1-phase cell-cycle regulation. Nat. Rev. Mol. Cell Biol. **5:** 355–366.

Cordero, J. B., J. P. Macagno, R. K. Stefanatos, K. E. Strathdee, R. L. Cagan *et al.*, 2010 Oncogenic Ras diverts a host TNF tumor suppressor activity into tumor promoter. Dev. Cell **18:** 999–1011.

Debnath, J., S. K. Muthuswamy and J. S. Brugge, 2003 Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures. Methods **30:** 256–268.

DeNicola, G. M., and D. A. Tuveson, 2009 RAS in cellular transformation and senescence. Eur. J. Cancer **45**(Suppl. 1): 211–216.

Dietzl, G., D. Chen, F. Schnorrer, K. C. Su, Y. Barinova *et al.*, 2007 A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila. Nature **448:** 151–156.

Dow, L. E., I. A. Elsum, C. L. King, K. M. Kinross, H. E. Richardson *et al.*, 2008 Loss of human Scribble cooperates with H-Ras to promote cell invasion through deregulation of MAPK signalling. Oncogene **27:** 5988–6001.

Fan, Y., and A. Bergmann, 2008 Apoptosis-induced compensatory proliferation: the Cell is dead. Long live the Cell! Trends Cell Biol. **18:** 467–473.

Ferres-Marco, D., I. Gutierrez-Garcia, D. M. Vallejo, J. Bolivar, F. J. Gutierrez-Avino *et al.*, 2006 Epigenetic silencers and Notch collaborate to promote malignant tumours by Rb silencing. Nature **439:** 430–436.

Froldi, F., M. Ziosi, F. Garoia, A. Pession, N. A. Grzeschik *et al.*, 2010 The lethal giant larvae tumour suppressor mutation requires dMyc oncoprotein to promote clonal malignancy. BMC Biol. **8:** 33.

Fukuhara, S., C. Murga, M. Zohar, T. Igishi and J. S. Gutkind, 1999 A novel PDZ domain containing guanine nucleotide exchange factor links heterotrimeric G proteins to Rho. J. Biol. Chem. **274:** 5868–5879.

Fukuhara, S., H. Chikumi and J. S. Gutkind, 2000 Leukemia-associated Rho guanine nucleotide exchange factor (LARG) links heterotrimeric G proteins of the G(12) family to Rho. FEBS Lett. **485:** 183–188.

Fukuhara, S., H. Chikumi and J. S. Gutkind, 2001 RGS-containing RhoGEFs: The missing link between transforming G proteins and Rho? Oncogene **20:** 1661–1668.

Garlena, R. A., R. L. Gonda, A. B. Green, R. M. Pileggi and B. Stronach, 2010 Regulation of mixed-lineage kinase activation in JNK-dependent morphogenesis. J. Cell Sci. **123:** 3177–3188.

Gateff, E., 1978 Malignant neoplasms of genetic origin in *Drosophila melanogaster*. Science **200:** 1448–1459.

Gateff, E., and H. A. Schneiderman, 1969 Neoplasms in mutant and cultured wild-tupe tissues of Drosophila. Natl. Cancer Inst. Monogr. **31:** 365–397.

Gomez del Pulgar, T., S. A. Benitah, P. F. Valeron, C. Espina and J. C. Lacal, 2005 Rho GTPase expression in tumourigenesis: evidence for a significant link. BioEssays **27:** 602–613.

Greco, V., M. Hannus and S. Eaton, 2001 Argosomes: a potential vehicle for the spread of morphogens through epithelia. Cell **106:** 633–645.

Grusche, F. A., J. L. Degoutin, H. E. Richardson and K. F. Harvey, 2011 The Salvador/Warts/Hippo pathway controls regenerative tissue growth in *Drosophila melanogaster*. Dev. Biol. **350:** 255–266.

Grzeschik, N. A., N. Amin, J. Secombe, A. M. Brumby and H. E. Richardson, 2007 Abnormalities in cell proliferation and apico-basal cell polarity are separable in Drosophila lgl mutant clones in the developing eye. Dev. Biol. **311:** 106–123.

Grzeschik, N. A., L. M. Parsons, M. L. Allott, K. F. Harvey and H. E. Richardson, 2010 Lgl, aPKC, and Crumbs regulate the Salvador/Warts/Hippo pathway through two distinct mechanisms. Curr. Biol. **20:** 573–581.

Hacker, U., and N. Perrimon, 1998 DRhoGEF2 encodes a member of the Dbl family of oncogenes and controls cell shape changes during gastrulation in Drosophila. Genes Dev. **12:** 274–284.

Hakeda-Suzuki, S., J. Ng, J. Tzu, G. Dietzl, Y. Sun *et al.*, 2002 Rac function and regulation during Drosophila development. Nature **416:** 438–442.

Han, S. Y., S. H. Kim and L. E. Heasley, 2002 Differential gene regulation by specific gain-of-function JNK1 proteins expressed in Swiss 3T3 fibroblasts. J. Biol. Chem. **277:** 47167–47174.

Hanahan, D., and R. A. Weinberg, 2000 The hallmarks of cancer. Cell **100:** 57–70.

Harden, N., 2002 Signaling pathways directing the movement and fusion of epithelial sheets: lessons from dorsal closure in Drosophila. Differentiation **70:** 181–203.

Hariharan, I. K., and D. Bilder, 2006 Regulation of imaginal disc growth by tumor-suppressor genes in Drosophila. Annu. Rev. Genet. **40:** 335–361.

Hay, B. A., T. Wolff and G. M. Rubin, 1994 Expression of baculovirus P35 prevents cell death in Drosophila. Development **120:** 2121–2129.

Humbert, P. O., N. A. Grzeschik, A. M. Brumby, R. Galea, I. Elsum *et al.*, 2008 Control of tumourigenesis by the Scribble/Dlg/Lgl polarity module. Oncogene **27:** 6888–6907.

Igaki, T., 2009 Correcting developmental errors by apoptosis: lessons from Drosophila JNK signaling. Apoptosis **14:** 1021–1028.

Igaki, T., R. A. Pagliarini and T. Xu, 2006 Loss of cell polarity drives tumor growth and invasion through JNK activation in Drosophila. Curr. Biol. **16:** 1139–1146.

Igaki, T., J. C. Pastor-Pareja, H. Aonuma, M. Miura and T. Xu, 2009 Intrinsic tumor suppression and epithelial maintenance by endocytic activation of Eiger/TNF signaling in Drosophila. Dev. Cell **16:** 458–465.

Jack, J., and G. Myette, 1997 The genes raw and ribbon are required for proper shape of tubular epithelial tissues in Drosophila. Genetics **147:** 243–253.

Johnston, L. A., 2009 Competitive interactions between cells: death, growth, and geography. Science **324:** 1679–1682.

Johnston, S. R., B. Lu, G. K. Scott, P. J. Kushner, I. E. Smith *et al.*, 1999 Increased activator protein-1 DNA binding and c-Jun NH2-terminal kinase activity in human breast tumors with acquired tamoxifen resistance. Clin. Cancer Res. **5:** 251–256.

Kanuka, H., T. Hiratou, T. Igaki, H. Kanda, E. Kuranaga *et al.*, 2005 Gain-of-function screen identifies a role of the Sec61alpha translocon in Drosophila postmitotic neurotoxicity. Biochim. Biophys. Acta **1726:** 225–237.

Karim, F. D., and G. M. Rubin, 1998 Ectopic expression of activated Ras1 induces hyperplastic growth and increased cell death in Drosophila imaginal tissues. Development **125:** 1–9.

Ke, H., R. Harris, J. L. Coloff, J. Y. Jin, B. Leshin *et al.*, 2010 The c-Jun NH2-terminal kinase 2 plays a dominant role in human epidermal neoplasia. Cancer Res. **70:** 3080–3088.

Kennedy, N. J., and R. J. Davis, 2003 Role of JNK in tumor development. Cell Cycle **2:** 199–201.

Kerman, B. E., A. M. Cheshire, M. M. Myat and D. J. Andrew, 2008 Ribbon modulates apical membrane during tube elongation through Crumbs and Moesin. Dev. Biol. **320:** 278–288.

Knoblich, J. A., K. Sauer, L. Jones, H. Richardson, R. Saint *et al.*, 1994 Cyclin E controls S phase progression and its down-regulation during Drosophila embryogenesis is required for the arrest of cell proliferation. Cell **77:** 107–120.

Kockel, L., J. Zeitlinger, L. M. Staszewski, M. Mlodzik and D. Bohmann, 1997 Jun in Drosophila development: redundant and nonredundant functions and regulation by two MAPK signal transduction pathways. Genes Dev. **11:** 1748–1758.

Kurada, P., and K. White, 1998 Ras promotes cell survival in Drosophila by downregulating hid expression. Cell **95:** 319–329.

Laviolette, M. J., P. Nunes, J. B. Peyre, T. Aigaki and B. A. Stewart, 2005 A genetic screen for suppressors of Drosophila NSF2 neuromuscular junction overgrowth. Genetics **170:** 779–792.

Lee, J. D., and J. E. Treisman, 2001 The role of Wingless signaling in establishing the anteroposterior and dorsoventral axes of the eye disc. Development **128:** 1519–1529.

Lee, T., and L. Luo, 2001 Mosaic analysis with a repressible cell marker (MARCM) for Drosophila neural development. Trends Neurosci. **24:** 251–254.

Lee, T., C. Winter, S. S. Marticke, A. Lee and L. Luo, 2000 Essential roles of Drosophila RhoA in the regulation of neuroblast proliferation and dendritic but not axonal morphogenesis. Neuron **25:** 307–316.

Leong, G. R., K. R. Goulding, N. Amin, H. E. Richardson and A. M. Brumby, 2009 scribble mutants promote aPKC and JNK-dependent epithelial neoplasia independently of Crumbs. BMC Biol. **7:** 62.

Liu, X. F., H. Ishida, R. Raziuddin and T. Miki, 2004 Nucleotide exchange factor ECT2 interacts with the polarity protein complex Par6/Par3/protein kinase Czeta (PKCzeta) and regulates PKCzeta activity. Mol. Cell Biol. **24:** 6665–6675.

Luo, L., Y. J. Liao, L. Y. Jan and Y. N. Jan, 1994 Distinct morphogenetic functions of similar small GTPases: Drosophila Drac1 is involved in axonal outgrowth and myoblast fusion. Genes Dev. **8:** 1787–1802.

Martin-Blanco, E., 1997 Regulation of cell differentiation by the Drosophila Jun kinase cascade. Curr. Opin. Genet. Dev. **7:** 666–671.

Massarwa, R., E. D. Schejter and B. Z. Shilo, 2009 Apical secretion in epithelial tubes of the Drosophila embryo is directed by the Formin-family protein Diaphanous. Dev. Cell **16:** 877–888.

Mattila, J., L. Omelyanchuk, S. Kyttala, H. Turunen and S. Nokkala, 2005 Role of Jun N-terminal Kinase (JNK) signaling in the wound healing and regeneration of a Drosophila melanogaster wing imaginal disc. Int. J. Dev. Biol. **49:** 391–399.

Menendez, J., A. Perez-Garijo, M. Calleja and G. Morata, 2010 A tumor-suppressing mechanism in Drosophila involving cell competition and the Hippo pathway. Proc. Natl. Acad. Sci. USA **107:** 14651–14656.

Miki, T., C. L. Smith, J. E. Long, A. Eva and T. P. Fleming, 1993 Oncogene ect2 is related to regulators of small GTP-binding proteins. Nature **362:** 462–465.

Mulinari, S., M. P. Barmchi and U. Hacker, 2008 DRhoGEF2 and diaphanous regulate contractile force during segmental groove morphogenesis in the Drosophila embryo. Mol. Biol. Cell **19:** 1883–1892.

Neisch, A. L., O. Speck, B. Stronach and R. G. Fehon, 2010 Rho1 regulates apoptosis via activation of the JNK signaling pathway at the plasma membrane. J. Cell Biol. **189:** 311–323.

Nielsen, C., J. Thastrup, T. Bottzauw, M. Jaattela and T. Kallunki, 2007 c-Jun NH2-terminal kinase 2 is required for Ras transformation independently of activator protein 1. Cancer Res. **67:** 178–185.

O'Keefe, D. D., D. A. Prober, P. S. Moyle, W. L. Rickoll and B. A. Edgar, 2007 Egfr/Ras signaling regulates DE-cadherin/Shotgun localization to control vein morphogenesis in the Drosophila wing. Dev. Biol. **311:** 25–39.

O'Keefe, L., W. G. Somers, A. Harley and R. Saint, 2001 The pebble GTP exchange factor and the control of cytokinesis. Cell. Struct. Funct. **26:** 619–626.

Olson, M. F., H. F. Paterson and C. J. Marshall, 1998 Signals from Ras and Rho GTPases interact to regulate expression of p21Waf1/Cip1. Nature **394:** 295–299.

Padash Barmchi, M., S. Rogers and U. Hacker, 2005 DRhoGEF2 regulates actin organization and contractility in the Drosophila blastoderm embryo. J. Cell Biol. **168:** 575–585.

Pagliarini, R. A., and T. Xu, 2003 A genetic screen in Drosophila for metastatic behavior. Science **302:** 1227–1231.

Patch, K., S. R. Stewart, A. Welch and R. E. Ward, 2009 A second-site noncomplementation screen for modifiers of Rho1 signaling during imaginal disc morphogenesis in Drosophila. PLoS One **4:** e7574.

Prober, D. A., and B. A. Edgar, 2000 Ras1 promotes cellular growth in the Drosophila wing. Cell **100:** 435–446.

Prober, D. A., and B. A. Edgar, 2002 Interactions between Ras1, dMyc, and dPI3K signaling in the developing Drosophila wing. Genes Dev. **16:** 2286–2299.

Prokopenko, S. N., A. Brumby, L. O'Keefe, L. Prior, Y. He *et al.*, 1999 A putative exchange factor for Rho1 GTPase is required for initiation of cytokinesis in Drosophila. Genes Dev. **13:** 2301–2314.

Rhiner, C., J. M. Lopez-Gay, D. Soldini, S. Casas-Tinto, F. A. Martin *et al.*, 2010 Flower forms an extracellular code that reveals the fitness of a cell to its neighbors in Drosophila. Dev. Cell **18:** 985–998.

Richardson, H., L. V. O'Keefe, T. Marty and R. Saint, 1995 Ectopic cyclin E expression induces premature entry into S phase and disrupts pattern formation in the Drosophila eye imaginal disc. Development **121:** 3371–3379.

Richardson, H. E., L. V. O'Keefe, S. I. Reed and R. Saint, 1993 A Drosophila G1-specific cyclin E homolog exhibits different modes of expression during embryogenesis. Development **119:** 673–690.

Ridley, A. J., 2004 Rho proteins and cancer. Breast Cancer Res. Treat. **84:** 13–19.

Robinson, B. S., J. Huang, Y. Hong and K. H. Moberg, 2010 Crumbs regulates Salvador/Warts/Hippo signaling in Drosophila via the FERM-domain protein Expanded. Curr. Biol. **20:** 582–590.

Sahai, E., and C. J. Marshall, 2002 Rho-GTPases and cancer. Nat. Rev. Cancer **2:** 133–142.

Schiff, R., P. Reddy, M. Ahotupa, E. Coronado-Heinsohn, M. Grim *et al.*, 2000 Oxidative stress and AP-1 activity in tamoxifen-resistant breast tumors in vivo. J. Natl. Cancer Inst. **92:** 1926–1934.

Settleman, J., 2001 Rac 'n Rho: the music that shapes a developing embryo. Dev. Cell **1:** 321–331.

Shim, K., K. J. Blake, J. Jack and M. A. Krasnow, 2001 The Drosophila ribbon gene encodes a nuclear BTB domain protein that promotes epithelial migration and morphogenesis. Development **128:** 4923–4933.

Somers, W. G., and R. Saint, 2003 A RhoGEF and Rho family GTPase-activating protein complex links the contractile ring to cortical microtubules at the onset of cytokinesis. Dev. Cell **4:** 29–39.

Sorlie, T., C. M. Perou, R. Tibshirani, T. Aas, S. Geisler *et al.*, 2001 Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc. Natl. Acad. Sci. USA **98:** 10869–10874.

Sorlie, T., R. Tibshirani, J. Parker, T. Hastie, J. S. Marron *et al.*, 2003 Repeated observation of breast tumor subtypes in independent gene expression data sets. Proc. Natl. Acad. Sci. USA **100:** 8418–8423.

Sotillos, S., M. T. Diaz-Meco, E. Caminero, J. Moscat and S. Campuzano, 2004 DaPKC-dependent phosphorylation of Crumbs is required for epithelial cell polarity in Drosophila. J. Cell Biol. **166:** 549–557.

Sotiriou, C., S. Y. Neo, L. M. McShane, E. L. Korn, P. M. Long *et al.*, 2003 Breast cancer classification and prognosis based on gene expression profiles from a population-based study. Proc. Natl. Acad. Sci. USA **100:** 10393–10398.

Speck, O., S. C. Hughes, N. K. Noren, R. M. Kulikauskas and R. G. Fehon, 2003 Moesin functions antagonistically to the Rho pathway to maintain epithelial integrity. Nature **421:** 83–87.

Srivastava, A., J. C. Pastor-Pareja, T. Igaki, R. Pagliarini and T. Xu, 2007 Basement membrane remodeling is essential for Drosophila disc eversion and tumor invasion. Proc. Natl. Acad. Sci. USA **104:** 2721–2726.

Stronach, B., 2005 Dissecting JNK signaling, one KKKinase at a time. Dev. Dyn. **232:** 575–584.

Sun, G., and K. D. Irvine, 2011 Regulation of Hippo signaling by Jun kinase signaling during compensatory cell proliferation and regeneration, and in neoplastic tumors. Dev. Biol. **350:** 139–151.

Titus, B., M. A. Schwartz and D. Theodorescu, 2005 Rho proteins in cell migration and metastasis. Crit. Rev. Eukaryot. Gene Expr. **15:** 103–114.

Toba, G., T. Ohsako, N. Miyata, T. Ohtsuka, K. H. Seong *et al.*, 1999 The gene search system: a method for efficient detection and rapid molecular identification of genes in *Drosophila melanogaster*. Genetics **151:** 725–737.

Uhlirova, M., and D. Bohmann, 2006 JNK- and Fos-regulated Mmp1 expression cooperates with Ras to induce invasive tumors in Drosophila. EMBO J. **25:** 5294–5304.

Vidal, M., S. Warner, R. Read and R. L. Cagan, 2007 Differing Src signaling levels have distinct outcomes in Drosophila. Cancer Res. **67:** 10278–10285.

Wagner, E. F., and A. R. Nebreda, 2009 Signal integration by JNK and p38 MAPK pathways in cancer development. Nat. Rev. Cancer **9:** 537–549.

Warner, S. J., H. Yashiro and G. D. Longmore, 2010 The Cdc42/Par6/aPKC polarity complex regulates apoptosis-induced compensatory proliferation in epithelia. Curr. Biol. **20:** 677–686.

Wasser, M., and W. Chia, 2000 The EAST protein of Drosophila controls an expandable nuclear endoskeleton. Nat. Cell. Biol. **2:** 268–275.

Weber, U., N. Paricio and M. Mlodzik, 2000 Jun mediates Frizzled-induced R3/R4 cell fate distinction and planar polarity determination in the Drosophila eye. Development **127:** 3619–3629.

Weston, C. R., and R. J. Davis, 2007 The JNK signal transduction pathway. Curr. Opin. Cell. Biol. **19:** 142–149.

Whitmarsh, A. J., and R. J. Davis, 2007 Role of mitogen-activated protein kinase kinase 4 in cancer. Oncogene **26:** 3172–3184.

Whyte, J., O. Bergin, A. Bianchi, S. McNally and F. Martin, 2009 Key signalling nodes in mammary gland development and cancer: mitogen-activated protein kinase signalling in experimental models of breast cancer progression and in mammary gland development. Breast Cancer Res. **11:** 209.

Widmann, T. J., and C. Dahmann, 2009 Dpp signaling promotes the cuboidal-to-columnar shape transition of Drosophila wing disc epithelia by regulating Rho1. J. Cell Sci. **122:** 1362–1373.

Woodhouse, E., E. Hersperger and A. Shearn, 1998 Growth, metastasis, and invasiveness of Drosophila tumors caused by mutations in specific tumor suppressor genes. Dev. Genes Evol. **207:** 542–550.

Wu, M., J. C. Pastor-Pareja and T. Xu, 2010 Interaction between Ras(V12) and scribbled clones induces tumour growth and invasion. Nature **463:** 545–548.

Yan, J., Q. Lu, X. Fang and P. N. Adler, 2009 Rho1 has multiple functions in Drosophila wing planar polarity. Dev. Biol. **333:** 186–199.

Zhang, D., W. Zhou, C. Yin, W. Chen, R. Ozawa *et al.*, 2006 Misexpression screen for genes altering the olfactory map in Drosophila. Genesis **44:** 189–201.

Zhang, J. Y., A. E. Adams, T. W. Ridky, S. Tao and P. A. Khavari, 2007 Tumor necrosis factor receptor 1/c-Jun-NH2-kinase signaling promotes human neoplasia. Cancer Res. **67:** 3827–3834.

Zhu, M., T. Xin, S. Weng, Y. Gao, Y. Zhang *et al.*, 2010 Activation of JNK signaling links lgl mutations to disruption of the cell polarity and epithelial organization in Drosophila imaginal discs. Cell. Res. **20:** 242–245.

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