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Interaction between *Ras*^{V12} and *scribble* clones induces tumour growth and invasion

Ming Wu[*], **José Carlos Pastor-Pareja**[*], and **Tian Xu**

Howard Hughes Medical Institute, Department of Genetics, Yale University School of Medicine, 295 Congress Avenue, New Haven CT 06519, USA

Abstract

Human tumours exhibit a large degree of cellular and genetic heterogeneity 1. Complex cell interactions in the tumour and its microenvironment are thought to play a significant role in tumourigenesis and cancer progression 2. It is also known that cooperation between oncogenic genetic lesions is required for tumour development 3. However, it is not known how cell interactions contribute to oncogenic cooperation. The genetic techniques available in the fruit fly *Drosophila melanogaster* allow analysis of the behavior of cells with distinct mutations 4, giving this model organism a privileged position to study cell interactions and oncogenic cooperation. In *Drosophila* eye-antennal discs, cooperation between the oncogenic protein *Ras*^{V12} 5 and loss-of-function mutations in the conserved tumour suppressor *scribble (scrib)* 6,7 gives rise to metastatic tumours that display many characteristics observed in human cancers 8-11. Here we show that clones of cells bearing different mutations can cooperate to promote tumour growth and invasion in *Drosophila*. We found that the *Ras*^{V12} and *scrib*$^{-}$ mutations can also cause tumours when they affect different adjacent epithelial cells. We show that this interaction between *Ras*^{V12} and *scrib*$^{-}$ clones involves JNK signaling propagation and JNK-induced upregulation of JAK/STAT-activating cytokines, a compensatory growth mechanism for tissue homeostasis. The development of *Ras*^{V12} tumours can also be triggered by tissue damage, a stress condition that activates JNK signaling. Given the conservation of the pathways examined here, similar cooperative mechanisms could play a role in the development of human cancers.

Keywords

Interclonal oncogenic cooperation; Ras; Cell Polarity Mutants; JNK-induced Cytokines; JAK/STAT

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Correspondence and requests for materials should be addressed to T.X. (tian.xu@yale.edu)..

[*]Equal contribution, in random order

Tian Xu Yale University School of Medicine Department of Genetics 295 Congress Ave New Haven, CT 06519 USA tian.xu@yale.edu Telephone: (203)737-2623 Fax: (203)737-1762

Author contributions M.W., J.C.P.-P. and T.X designed research, M.W. and J.C.P.-P. performed experiments and analyzed the data. M.W., J.C.P.-P. and T.X wrote the manuscript.

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Clones of mutant cells marked with green fluorescent protein (GFP) can be generated in the eye-antenna imaginal discs of *Drosophila* larvae by mitotic recombination. Clones expressing the oncogenic protein Ras^{V12} moderately overgrow 12 (Fig. 1a, b). Clones mutant for *scrib* lose apico-basal polarity and die 6,13 (Fig. 1c). In contrast, *scrib* clones simultaneously expressing Ras^{V12} grow into large metastatic tumours (Fig. 1d) 8. To better understand cooperation between these two mutations, we produced animals in which cell division after a mitotic recombination event creates two daughter cells, one expressing Ras^{V12} and the other mutant for *scrib*. Discs containing adjacent Ras^{V12} (GFP-positive) and $scrib^{-}$ clones developed into large tumours, capable of invading the ventral nerve cord (VNC) (Fig. 1e). This shows that Ras^{V12} and *scrib* cooperate for tumour induction also when they occur in different cells. We will refer to these tumours as $Ras^{V12}//scrib^{-}$ tumours, to denote interclonal oncogenic cooperation and distinguish them from $Ras^{V12}scrib^{-}$ tumours, in which cooperation occurs in the same cells intraclonally.

In the late stages of the development of $Ras^{V12}//scrib^{-}$ tumours, most cells in the tumour mass are Ras^{V12} cells (Fig. 1h, i). $scrib^{-}$ cells, as well as residual wild-type cells, are almost completely absent from the tissue, similar to the absence of wild-type cells in late $Ras^{V12}scrib^{-}$ tumours (Fig. 1f, g). To test the possibility that $Ras^{V12}//scrib^{-}$ tumours are caused by unopposed growth of Ras^{V12} cells, we examined eye-antennal discs where all cells expressed Ras^{V12}. Dramatic overgrowth or invasion did not occur (Supplementary Fig. 1), showing that interaction between Ras^{V12} and $scrib^{-}$ cells is required for tumour development. Interclonal cooperation between Ras^{V12} and *lethal giant larvae* (*lgl*) also produced tumours (Supplementary Fig. 2), suggesting that other polarity mutations can cooperate interclonally with Ras^{V12}. Intrigued by these findings, we decided to investigate the mechanisms underlying non-autonomous oncogenic cooperation and sustained growth in $Ras^{V12}//scrib^{-}$ tumours.

JAK/STAT signaling promotes cell proliferation in different contexts in mammals and flies 14, including the overgrowth caused by mutation of several tumour suppressors 15. In a cDNA microarray analysis of $Ras^{V12}scrib^{-}$ tumours, we discovered upregulation of the *unpaired* genes (*upd, upd2* and *upd3*; data not shown), which encode JAK/STAT-activating cytokines related to Interleukin 6 16-18. We confirmed the upregulation of the *unpaired* genes in $Ras^{V12}scrib^{-}$ and $Ras^{V12}//scrib^{-}$ tumours by real-time RT-PCR (Fig. 2a). Furthermore, we observed elevated expression of the JAK/STAT reporter STAT-GFP 19 in both $Ras^{V12}scrib^{-}$ and $Ras^{V12}//scrib^{-}$ tumours (Fig. 2b-e), thus correlating high expression of Upd cytokines with increased JAK/STAT activity.

To test the involvement of JAK/STAT signaling in the growth of $Ras^{V12}scrib^{-}$ and $Ras^{V12}//scrib^{-}$ tumours, we used a dominant negative form of the JAK/STAT receptor Domeless ($Dome^{DN}$) 20. Expression of $Dome^{DN}$ achieved suppression of overgrowth and invasion of the VNC in $Ras^{V12}scrib^{-}$ tumours (Fig. 2f). Also $Ras^{V12}//scrib^{-}$ tumours were suppressed by expression of $Dome^{DN}$ in Ras^{V12} cells (Fig. 2g). A loss-of-function mutation in s*tat92E*, encoding the *Drosophila* STAT transcriptional activator, reduced growth and invasiveness of $Ras^{V12}scrib^{-}$ and $Ras^{V12}//scrib^{-}$ tumours (Supplementary Fig. 3). From these experiments, we conclude that JAK/STAT signaling is required for the development of $Ras^{V12}scrib^{-}$ and $Ras^{V12}//scrib^{-}$ tumours.

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The suppression of *Ras^{V12}scrib*$^{-}$ and *Ras^{V12}//scrib*$^{-}$ tumours by reducing JAK/STAT activity in *Ras^{V12}* cells points to cooperation between Ras and JAK/STAT signaling as a cause of tumour growth. To confirm this, we generated clones of cells co-expressing Upd cytokines and Ras^{V12}. While Upd overexpression in wild-type cells (Fig. 2h), in *scrib*$^{-}$ cells or in wild-type cells adjacent to *scrib*$^{-}$ cells (Supplementary Fig. 4) did not cause tumours, co-expression of Ras^{V12} and Upd produced large invasive tumours (Fig. 2i). Similar results were obtained co-expressing *Ras^{V12}* and Upd2 (Fig. 2j), whereas co-expression of Ras^{V12} and Upd3 caused smaller, non-invasive tumours (Fig. 2k). Finally, *Ras^{V12}*Upd Upd2 tumours were larger than *Ras^{V12}*Upd and *Ras^{V12}*Upd2 tumours (Fig. 2l), suggesting an additive effect of the expression of different Upd cytokines (see also Supplementary Fig. 5).

Prevention of actual cell death in cells apoptotically stimulated has been shown to potently promote overgrowth 21. To assess a possible involvement of apoptosis prevention in the synergy between Ras and JAK/STAT signaling, we coexpressed the apoptosis inhibitor p35 with Ras^{V12} or Upd. Neither conditions produced tumours (Supplementary Fig. 6), suggesting that cooperation between Ras and JAK/STAT involves a mechanism other than apoptosis prevention. *Ras^{V12}*Upd tumours were suppressed by expression of $Dome^{DN}$ (Fig. 2m), thus confirming that their development requires JAK/STAT activity. In all, both loss- and gain-of-function experiments lead us to conclude that Ras and JAK/STAT signaling exhibit a strong synergistic tumour-promoting interaction, responsible for the development of *Ras^{V12}scrib*$^{-}$ and *Ras^{V12}//scrib−* tumours.

Having established the involvement of JAK/STAT signaling in the growth of *Ras^{V12}scrib−* and *Ras^{V12}//scrib*$^{-}$ tumours, we decided to investigate how expression of the Upd cytokines is upregulated. We previously showed that expression of the *unpaired* genes is elevated in wounds in a JNK-dependent manner 22. It has been shown as well that JNK signaling can induce non-autonomous overgrowth 23,24 and that JNK signaling is upregulated in *scrib*$^{-}$ clones 13,25 and *scrib*$^{-}$ discs 22, which develop as tumours in *scrib*$^{-}$ larvae 6. To test the possibility that JNK activation causes ectopic JAK/STAT signaling in *scrib*$^{-}$ cells, we monitored STAT-GFP expression in discs double mutant for *scrib* and *hep*, coding for the *Drosophila* JNK-kinase Hemipterous. In these discs, STAT-GFP expression was reduced and overgrowth suppressed (Fig. 3a-c), showing that JAK/STAT elevation in *scrib*$^{-}$ cells depends on JNK activity.

The induction of Upd cytokines by JNK in *scrib*$^{-}$ cells can explain the growth of *Ras^{V12}scrib*$^{-}$ tumours, placing JAK-STAT signaling downstream of JNK. In support of this, a dominant negative form of the Jun-kinase Basket (Bsk^{DN}) suppressed *Ras^{V12}scrib−* tumours 9 (Fig. 3d, e), but not Ras^{V12}Upd tumours (Fig. 3f, g). In the case of *Ras^{V12}//scrib*$^{-}$ tumours, few *scrib*$^{-}$ cells remain in the tissue at late stages (Fig. 1i). Therefore, Upd induction in *scrib*$^{-}$ cells cannot fully account for tumour development. Indeed, expression of Bsk^{DN} in *Ras^{V12}* cells partially suppressed the growth of *Ras^{V12}//scrib*$^{-}$ tumours (Fig. 3h, i) and expression of the *unpaired* genes (Fig. 3j). This shows that in *Ras^{V12}//scrib*$^{-}$ tumours expression of Upd cytokines downstream of JNK signaling also occurs in *Ras^{V12}* cells.

scrib^{-} clones cause JNK activation both autonomously and non-autonomously 25. Furthermore, in wing discs, wounding induces JNK activation away from the site of

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wounding 22,26, suggesting that JNK activity can propagate. To investigate this, we wounded wing discs and examined the expression of *puckered* (*puc*), a JNK downstream gene encoding a JNK-phosphatase that negatively regulates the pathway 27. When wounds were induced in the anterior or posterior wing regions, JNK activation, revealed by *puc*-lacZ expression, was observed across the disc in the opposite compartment (Fig. 3k). In contrast, overexpression of Puc in a central stripe of cells prevented expansion of JNK to the opposite compartment. (Fig. 3l, Supplemental Fig. 7). This indicates that JNK activity propagates through a feed-forward loop and, together with previous findings, suggests that in Ras^{V12}//*scrib*$^{-}$ tumours, *scrib*$^{-}$ cells trigger JNK activation and that this activation propagates to adjacent Ras^{V12} cells. JNK-dependent upregulation of Upd cytokines in Ras^{V12} cells, thus, can sustain tumour growth when the original source of JNK activity, the *scrib*$^{-}$ cells, is no longer present.

The previous experiments reveal a central role for JNK in the cooperation of Ras^{V12} and *scrib*$^{-}$. Since both wounds and *scrib*$^{-}$ induce JNK activation, we tested the possibility that tissue damage could cooperate with Ras^{V12} to promote tumour overgrowth. We wounded larval right wing discs and examined them 48 hours later. In wild-type discs, compared to the unwounded left disc, wounding resulted in size reduction (Fig 4a, b; Supplementary Fig. 8; wounded/unwounded size ratio ±SD=0.70±0.18). In contrast, wounding of $\mathrm{Ras}^{\mathrm{V12}}$-expressing discs caused a marked increase in $\mathrm{Ras}^{\mathrm{V12}}$-induced overgrowth (Fig. 4c, d; Supplementary Fig. 8; 1.46±0.31). No metastasis was detected in this experiment (not shown). Finally, the wounded/unwounded ratio in p35-expressing discs (1.09±0.14, Supplementary Fig. 8) shows that apoptosis prevention by $\mathrm{Ras}^{\mathrm{V12}}$ cannot completely account for its cooperation with mechanically-induced damage.

The fact that both *scrib*$^{-}$ clones and tissue damage induce overgrowth of Ras^{V12} tissue suggests that compensatory proliferation in response to *scrib*$^{-}$ cells could underlie cooperation in Ras^{V12}//*scrib*$^{-}$ tumours. To test this, we studied the effect of confronting *scrib*$^{-}$ cells with cells mutant for *stat92E*. When *scrib*$^{-}$ clones are generated in eye-antennal discs, *scrib*$^{-}$ cells in the adult eye are mostly absent 13 and the eye appears normal in size (Fig. 4e, f). When *stat92E*$^{-}$ cells confront *scrib*$^{-}$ cells, in contrast, the eye is greatly reduced (Fig. 4g, h; Supplemental Fig. 9), showing that *stat92E*$^{-}$ cells cannot compensate for the loss of *scrib*$^{-}$ cells. These data indicate a role for JAK/STAT signaling in tissue homeostasis through compensatory proliferation (see also Supplemental Figs. 9 and 10). Therefore, a mechanism to ensure recovery after damage explains the development of Ras^{V12}*scrib*$^{-}$ and Ras^{V12}//*scrib*$^{-}$ tumours and can mediate interclonal oncogenic cooperation (Fig. 4i).

We have used *Drosophila* to investigate how oncogenic cooperation between different cells can promote tumour growth and invasion. Our experiments, addressed to understanding interclonal cooperation in Ras^{V12}//*scrib*$^{-}$ tumours, uncovered a two-tier mechanism by which *scrib*$^{-}$ cells promote neoplastic development of Ras^{V12} cells: (1) propagation of stress-induced JNK activity from *scrib*$^{-}$ cells to Ras^{V12} cells and (2) expression of the JAK/STAT-activating Unpaired cytokines downstream of JNK. Our findings, therefore, highlight the importance of cell interactions in oncogenic cooperation and tumour development. We also show that stress-induced JNK signaling and epigenetic factors such as tissue damage can contribute to tumour development in flies. Interestingly, tissue damage caused by

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conditions such as chronic inflammation has been linked to tumourigenesis in humans 28,29. Furthermore, expression of the Unpaired cytokines promotes tumour growth (this study) as well as an antitumoural immune response 22, which parallels the situation in mice and humans 30. Future research into phenomena such as compensatory growth and interclonal cooperation in *Drosophila* will provide valuable insights into the biology of cancer.

METHODS SUMMARY

Clones of mutant cells in the eye-antennal discs were generated as previously described 8. Detailed genotypes of the experimental individuals are described in Supplemental Information. The following antibodies and dyes were used: mouse monoclonal anti-βgal (1:500, Sigma), goat Alexa-488-conjugated anti-mouse IgG (1:200, Molecular Probes), phalloidin Texas Red (Molecular Probes). Wounds were performed with forceps in mid third-instar larvae as described previously 22.

METHODS

Strains and culture

Cultures were maintained at 25°C on standard medium. Whenever staging of larvae was required, parental flies were placed in a fresh culture vial and left there to lay eggs for 1 day; we considered the time of removing the flies from the vial 12h (±12) AEL (after egg laying). The following strains were used in this study:

y w; FRT82B

y w; FRT82B,scrib^{1}/TM6B

w; UAS-Ras^{V12} (II)

w; UAS-Ras^{V12} (III)

y w,ey-Flp1;act>y+>Gal4,UAS-GFP.S65T;FRT82B,tub-Gal80

w; FRT82B, tub-Gal80,scrib^{1}/TM6B

y w,ey-Flp1;act>y+>Gal4,UAS-myrRFP;FRT82B,tub-Gal80

tub-Gal80,FRT19A;eyFLP5,act>y^{+}>Gal4,UAS-GFP

y w,ey-Flp1;tub-Gal80,FRT40A;act>y+>Gal4,UAS-GFP.S65T

y w,ey-Flp1;FRT82B,ubi-GFP

w;10XSTAT-GFP.1 (II)

w; ey-Flp6 (III)

w; UAS-upd (II)

w; UAS-upd2 (III)

w; UAS-upd3 (II)

w; UAS-upd-IR(R-1) (III)

w,UAS-dome $^{cyt1.1}$

w; UAS-dome $^{cyt2.1}$ (II)

y w upd2 $^{3\text{-}62}$

w,UAS-bsk^{DN}

w; UAS-bsk^{DN} (III)

y w hep^{r75}/FM7i,act-GFP

w; ptc-GAL

w; UAS-myr-RFP (II)

puc^{E69}-lacZ,ry/TM3,Sb

w; UAS-puc (III)

w; nub-GAL4.K

w; UAS-p35 (II)

w; UAS-p35 (III)

w; FRT82B,stat92E^{06346}/TM6B

w; FRT82B,stat92E^{397}/TM3,Sb

w; FRT82B,stat92E^{85C9}/TM3,Sb

w; FRT82B,ubi-GFP,RpS3^{Plac92}/TM6C, Sb

y w; ey-GAL4,UAS-Flp; FRT82B,GMR-hid,CL3R/TM6B.

Real-time RT-PCR

Total RNA from wild-type and tumour discs was isolated using Trizol (Invitrogen). cDNA was synthesized from 2 μg of RNA with the SuperScriptIII First-Strand Synthesis System (Invitrogen). Resulting DNA was subjected to real-time PCR with SYBR green fast kit (Applied Biosystems) according to manufacturers instructions. Relative gene expression was compared to *rp49* as an internal control. Three experiments for each condition were averaged. The following primers were used: *upd*: 5′ TCCACACGCACAACTACAAGTTC 3′ and 5′ CCAGCGCTTTAGGGCAATC 3′; *upd2*: 5′ AGTGCGGTGAAGCTAAAGACTTG 3′ and 5′ GCCCGTCCCAGATATGAGAA 3′; *upd3*: 5′ TGCCCCGTCTGAATCTCACT 3′ and 5′ GTGAAGGCGCCCACGTAA 3′; *rp49*: 5′ GGCCCAAGATCGTGAAGAAG 3′ and 5′ ATTTGTGCGACAGCTTAGCATATC 3′.

Stainings and imaging

Images documenting tumour size and VNC invasion were taken in a Leica MZ FLIII fluorescence stereomicroscope with an Optronics Magnafire camera. Antibody staining was performed according to standard procedures for imaginal discs. The following antibodies and dyes were used: mouse monoclonal anti-βgal (1:500, Sigma), goat Alexa-488-conjugated anti-mouse IgG (1:200, Molecular Probes), phalloidin Texas Red (Molecular

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Probes). Samples imaged through confocal microscopy were mounted in DAPI-Vectashield (Vector Labs). Confocal images were taken in a Zeiss LSM510 Meta confocal microscope. Adult eyes were imaged with a Leica DFC300FX camera in a Leica MZ FLIII stereomicroscope. Measurements of wing blade size were performed from confocal pictures using NIH Image-J software. Adult eye size measurements were performed for each genotype from pictures of at least ten female flies collected 1-3 days after hatching using NIH Image-J software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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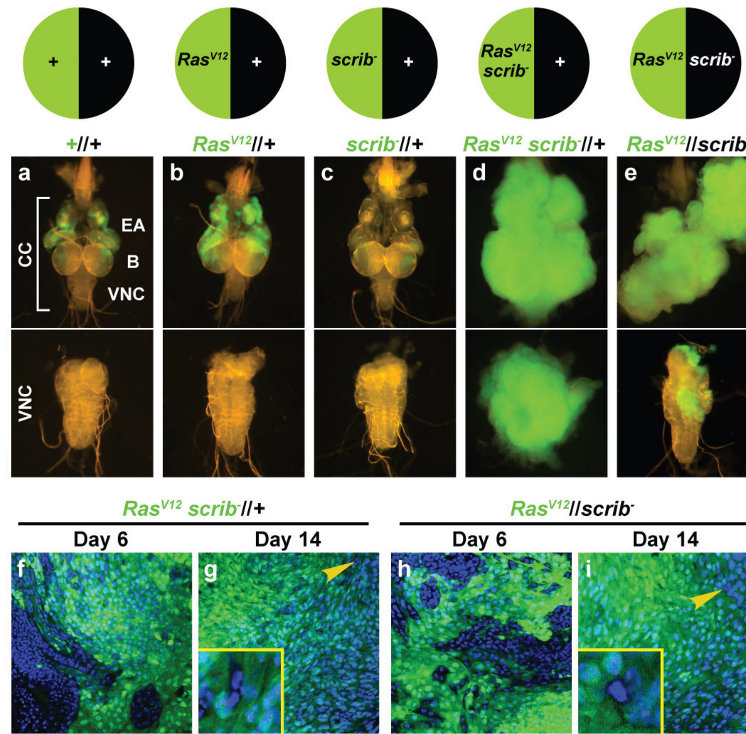

Figure 1. Interclonal cooperation between *Ras*^{V12} and *scrib*$^{-}$ causes tumours

a-e, Clones of cells marked with GFP in the eye-antennal discs of third-instar larvae. Upper subpanels show the cephalic complex (CC), consisting of eye-antennal discs (EA), brain (B) and ventral nerve cord (VNC). Lower subpanels show the dissected VNC. Compared to wild-type clones (**a**), *Ras*^{V12} clones overgrow moderately (**b**). *scrib*$^{-}$ clones are eliminated from the tissue (**c**). Double mutant *Ras*^{V12}*scrib*$^{-}$ clones (**d**, intraclonal cooperation), as well as *Ras*^{V12} clones confronted with *scrib*$^{-}$ clones (**e**, interclonal cooperation), cause tumours that overgrow and invade the VNC. **f-i**, Confocal sections of the inner tumour mass in *Ras*^{V12}*scrib*$^{-}$ (**f, g**) and *Ras*^{V12}*//scrib*$^{-}$ tumours (**h, i**) at day 6 and 14 after egg laying (AEL). Non-*Ras*^{V12} cells (absence of GFP) are progressively eliminated from the tissue. Cell nuclei labeled with DAPI. Yellow arrowheads point to scattered remaining GFP-negative cells (insets in **g** and **i**).

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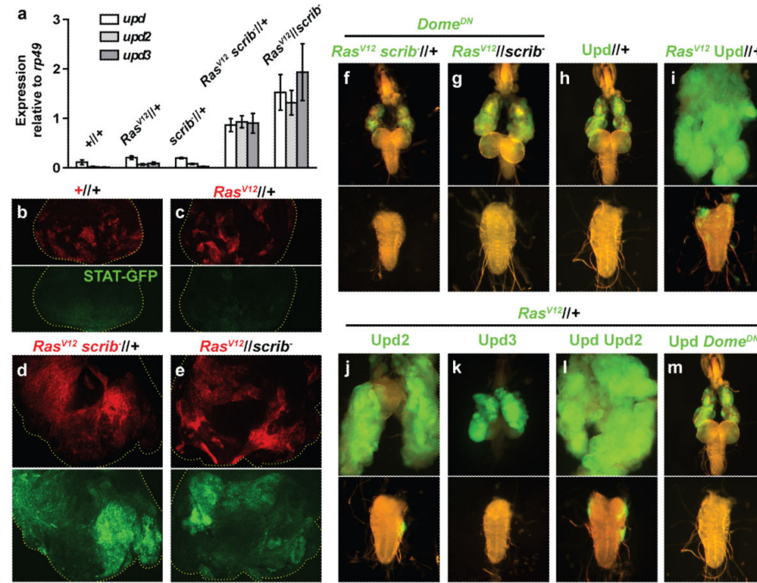

Figure 2. Synergy between Ras and JAK/STAT signaling promotes growth and invasion in *Ras^{V12}scrib^{-}* and *Ras^{V12}//scrib^{-} tumours*

a, Quantification by real-time RT-PCR of expression of the *upd* genes, encoding the JAK/STAT-activating cytokines Upd, Upd2 and Upd3, in eye-antennal discs containing wild-type clones, *Ras^{V12}*-expressing clones, *scrib^{-}* clones (day 6 AEL), *Ras^{V12}scrib−* tumours and *Ras^{V12}//scrib^{-}* tumours (day 10 AEL). Expression is normalized to the housekeeping gene *rp49*. Error bars depict 95% confidence intervals (1.96 × s.e.m., n=3). **b-e**, Expression of the JAK/STAT reporter STAT-GFP (green) in day 6 AEL eye-antennal discs containing wild-type clones (**b**, red), *Ras^{V12}* clones (**c**), *Ras^{V12}scrib^{-}* (**d**) and *Ras^{V12}//scrib^{-}* tumours (**e**). **f, g**, Suppression of *Ras^{V12}scrib^{-}* (**f**) and *Ras^{V12}//scrib^{-}* (**g**) tumours by expression of a dominant negative form of the JAK/STAT receptor Domeless (Dome^{DN}). **h**, Clones overexpressing Upd. **i-l**, Tumours caused by *Ras^{V12}* clones co-overexpressing Upd (**i**), Upd2 (**j**), Upd3 (**k**) and both Upd and Upd2 (**l**). **m**, *Ras^{V12}*Upd tumours are suppressed by Dome^{DN}.

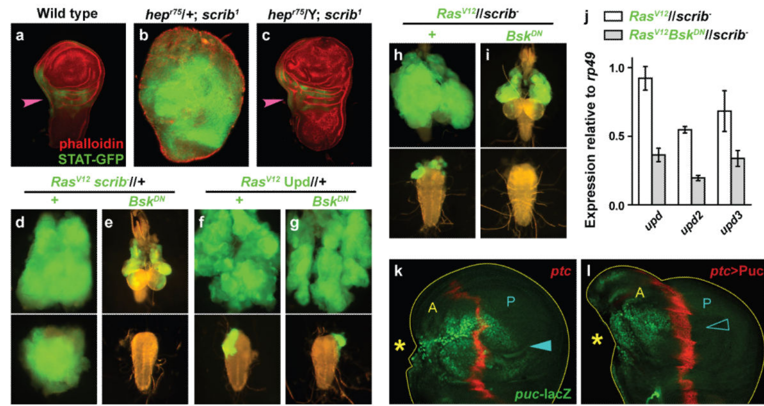

Figure 3. JNK signaling drives oncogenic cooperation upstream of JAK/STAT

a-c, STAT-GFP expression in wing discs of wild-type larvae (**a**) and *scrib*$^{-}$ larvae heterozygous (**b**) or hemizygous (male, **c**) for the JNK-kinase loss-of-function mutation *hep*^{r75}. Overgrowth and STAT-GFP upregulation are suppressed by *hep*^{r75}. Arrowheads point to normal STAT-GFP expression in the wing hinge. Discs stained with phalloidin (red). **d-g**, Expression of a dominant negative form of the Jun-kinase Basket (*Bsk*^{DN}) suppresses *Ras*^{V12}*scrib*$^{-}$ tumours (**d, e**), but not *Ras*^{V12}Upd tumours (**f, g**). **h, i**, Expression of *Bsk*^{DN} in *Ras*^{V12} cells partially suppresses *Ras*^{V12}*//scrib*$^{-}$ tumours. **j**, Quantification by real-time RT-PCR of expression of the *upd* genes in *Ras*^{V12}*//scrib*$^{-}$ and *Ras*^{V12}*Bsk*^{DN}*// scrib*$^{-}$ tumours (day 6 AEL). Error bars represent 95% confidence intervals (n=3). **k**, Propagation of JNK activity (*puc*-lacZ reporter, green) into the posterior (P) compartment (arrowhead) in wing discs wounded in the anterior (A) compartment (asterisk) 24 hours after wounding. **l**, *puc*-lacZ expression in discs expressing the JNK-phosphatase Puc under control of *ptc*-GAL4 (red cells, expressing RFP), wounded as in **k**. Puc is both a downstream target and a negative regulator of JNK. Propagation of *puc*-lacZ expression into the P compartment is not observed (hollow arrowhead).

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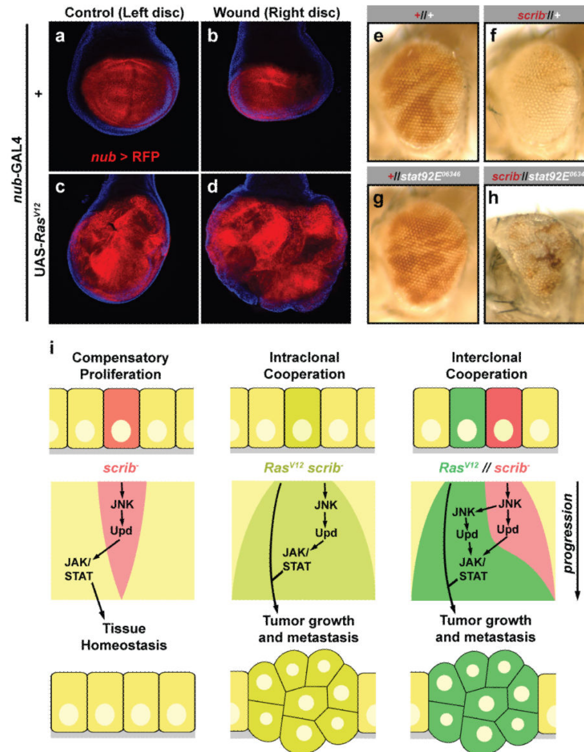

Figure 4. Tissue damage, compensatory growth and a model for interclonal oncogenic cooperation

a-d, Cooperation between *Ras*^{V12} and tissue damage. Right (wounded) and left (unwounded) wing discs of a wild-type larva (**a, b**) and a larva expressing Ras^{V12} under control of *nub*-GAL4 (**c, d**). Discs were wounded by repeated pinching and dissected 48h later. Expression of RFP driven by *nub*-GAL4 marks the wing blade region (red). Cell nuclei stained with DAPI (blue). **e-h**, Requirement of JAK/STAT signaling in compensatory proliferation. Wild-type (**e**) and *stat92E*$^{-}$ (**g**) clones in adult eyes, marked by absence of red pigment. In eyes containing *scrib*$^{-}$ clones confronted with wild-type cells (**f**), *scrib*$^{-}$ cells (red) are mostly absent and size of the eye is largely normal. In eyes containing *scrib*$^{-}$ clones (red) confronted with *stat92E*$^{-}$ cells (**h**), the size of the eye is reduced. **i**, Model for the involvement of JNK and JAK/STAT signaling in intraclonal and interclonal cooperation between *Ras*^{V12} and *scrib*$^{-}$. See text for details.

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