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Drosophila as Model System to Study Ras-Mediated Oncogenesis: The Case of the Tensin Family of Proteins

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Abstract: Oncogenic mutations in the small GTPase Ras contributes to ~30% of human cancers. However, tissue growth induced by oncogenic Ras is restrained by induction of cellular senescence and additional mutations are required to induce tumor progression. Therefore, it is paramount to identify cooperating cancer genes. Recently, the tensin family of focal adhesion proteins, TNS1-4, have emerged as regulators of carcinogenesis, yet their role in cancer appears somewhat controversial. Around 90% of human cancers are of epithelial origin. We have used the *Drosophila* wing imaginal disc epithelium as model system to gain insight into the roles of two orthologs of human TNS2 and 4, *blistery* (*by*) and *PVRAP*, in epithelial cancer progression. We have generated null mutations in *PVRAP* and found that, as it is the case for *by* and mammalian tensins, *PVRAP* mutants are viable. We have also found that elimination of either *PVRAP* or *by* potentiates Ras^{V12}-mediated wing disc hyperplasia. Furthermore, our results have unravelled a mechanism by which tensins may limit Ras oncogenic capacity, the regulation of cell shape and growth. These results demonstrate that *Drosophila* tensins behave as suppressors of Ras-driven tissue hyperplasia, suggesting that the roles of tensins as modulators of cancer progression might be evolutionary conserved.

Keywords: oncogenic Ras; overgrowth; tensins; *Drosophila*

Introduction

Cancer is the second leading cause of death worldwide {Ferlay, 2021 #1727}. Cancer is characterized by the uncontrolled growth and spread of cells leading to invasion of normal tissues. Extensive research over the last years have revealed that cancer is a genetically complex and heterogenous disease with each tumour carrying mutations not in a single gene but in several genes {Stratton, 2011 #1742; Vogelstein, 2013 #1743}. Thus, the discovery of genes cooperating with tumour progression is crucial to understand tumour growth and malignancy.

The *Drosophila* genome is 60% homologous to that of humans and about 75% of genes responsible for human diseases have homologs in flies {Ugur, 2016 #1740}. This combined with a short generation time, low maintenance costs and powerful genetic tools, make the fly an ideal model system to study cancer. About 90% of human cancers are of epithelial origin {Hanahan, 2000 #466}. Epithelial tissues are composed of cells with an apico-basal polarity held together in sheets by specialized junctions. The apical face of the epithelial tissue is exposed to either the external environment or the body fluid, while the basal face is attached to a specialized extracellular matrix (ECM), called the basement membrane (BM). In this context, the primordium of the *Drosophila* wing, the larval wing imaginal disc epithelia has been successfully used to study epithelial tumour progression and oncogenic cooperation (reviewed in {Gonzalez, 2013 #1363}. The wing imaginal disc (henceforth, wing disc) is a monolayer epithelium that is limited apically by a squamous epithelium, the peripodial epithelium (PE), and basally by a BM. The formation of the wing starts during early embryonic development when about 30 cells are allocated to form the wing disc primordium. During

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larval life, the number of cells in the disc increases to about 50.000 {Garcia-Bellido, 1971 #940}. The wing disc epithelium is often divided into four broad domains, the pouch, the hinge, the notum and the PE. The pouch and the hinge give rise to the wing and wing hinge, while the notum gives rise to the dorsal half of the body wall in the second thoracic segment, which include the notum, the back of the thorax and the pleura (reviewed in {Tripathi, 2022 #1747}. The induction of groups of cells over-expressing mutated forms of *Ras* (Ras^{V12}), present in 30% of human cancers {Samatar, 2014 #1741}, in the wing disc gives rise to hyperplastic growth {Prober, 2000 #1251}. This has been exploited, by several labs including ours, in genetic screens to identify additional genes that can either suppress or enhance the growth of cells overexpressing Ras^{V12} {Mirzoyan, 2019 #1333}.

To isolate new modulators of oncogenic Ras activity, we performed an RNAi screen and searched for genes that when knockdown enhanced the wing disc Ras^{V12} overgrowth phenotype. One of the genes identified has been proposed to be the fly ortholog of human tensins 2 and 4 (TNS2, TNS4, FlyBase), which we have named *EGFRAP* {Soler Beatty, 2021 #1561}. Likewise, our preliminary results showed that downregulation of another fly ortholog of human TNS2 and 4 (FlyBase), *PVRAP*, a gene that lies adjacent to *EGFRAP*, enhanced the Ras^{V12} overgrowth and folding wing disc phenotype in a similar way to *EGFRAP*. Tensins are a family of focal adhesion proteins. The mammalian tensin family comprises four members (tensin 1-4, TNS1-4), which link the cell membrane to the actin cytoskeleton and are lost in most cancer cell lines {Liao, 2021 #1461; Mouneimne, 2007 #1464}. Knockdown of human TNS2 and TNS4 increase tumorigenicity in several cancer lines {Sakashita, 2008 #1467}. Interestingly, the role of tensins as tumor suppressors has been linked to their ability to bind and regulate the main cell-ECM receptors, the integrins, (reviewed in {Mouneimne, 2007 #1464} which are themselves involved in almost every step of cancer progression (reviewed in {Hamidi, 2018 #1555}. In this context, it is worth mentioning here that we have recently found that downregulation of integrins also enhanced the Ras^{V12} overgrowth and folding wing disc phenotype {Valencia-Exposito, 2022 #1718}. However, even though *blistery* (*by*) is the clearest *Drosophila* ortholog of vertebrate TNS4 {Adams, 2000 #1744} and has been proposed to provide a final strengthening of the integrin adhesion complex {Torgler, 2004 #1745}, it has not yet been implicated in tumorigenesis {Adams, 2000 #1744}.

In this work, we have used the wing disc to analyze the role of *PVRAP* and *by* on the progression of Ras^{V12} epithelial tumour cells. We have generated mutants in *PVRAP* and found that, as it is the case for mutations in *by*, *PVRAP* mutants are viable, strongly suggesting that *PVRAP* function is not required for normal development. In addition, we have found that downregulation of either *PVRAP* or *by* enhances Ras^{V12}-mediated tissue hyperplasia. Our results also unravel a new mechanism by which tensins could modulate the oncogenic capacity of Ras^{V12}, the regulation of cell shape and growth. These results demonstrate that in *Drosophila*, as it is the case in some human cancer cell lines, tensins modulate the metastatic capacity of Ras^{V12}-dependent epithelial tumour cells, suggesting that the role of the tensin family of proteins as suppressor of tumour metastasis might be evolutionary conserved.

Materials and methods

Fly strains

The following stocks were used: *UAS-by*^{RNAi}, *UAS-PVRAP*^{RNAi} (Vienna Stock Center); *UAS-*Ras^{V12} {Lee, 1996 #195}; *apGal4-UASGFP* (Bloomington Drosophila Stock Center). The two *PVRAP* mutants, $PVRAP^{1}$ and $PVRAP^{2}$ were generated by CRISPR in this study (see below). Flies were raised at 25°C.

Generation of *PVRAP* mutants with CRISPR/Cas9

Two sgRNAs were designed against sequences located in the first exon close to the ATG to generate null alleles We used the following sequences:

sgRNA1: GTCGCCAGCAGCACAATAATAGC
sgRNA2: AAACGCTATTATTGTGCTGCTGG
sgRNA3: GTCGGAATTGGAATTGTCCGCCG

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sgRNA4: AAACCGGCGGACAATTCCAATTC

The sgRNAs were cloned into the PCFD3 vector as previously described {Port, 2014 #1173} and http://www.crisprflydesign.org/plasmids/. Transgenic gRNA flies were generated by the Best Gene Company (Chino Hills, USA) using either *y sc v P{nos-phiC31\int.NLS}X; P{CaryP}attP2* (BDSC 25710) or *y v P{nos-phiC31\int.NLS}X; P{CaryP}attP40* (BDSC 25709) flies. Transgenic lines were verified by sequencing (Biomedal, Armilla, Spain). Males carrying the sgRNA were crossed to nos-Cas9 females and the progeny was screened for the v+ch- eye marker. To identify CRISPR/Cas9-induced mutations, genomic DNA was isolated from flies and sequenced using the following primers (5'-3'):

PVRAP primer Forward: GTCCTGGTGGTGACTGGAAC

PVRAP primer Reverse: AATCGCATAGCTGCCAACTT

Two *PVRAP* null mutant alleles were generated (Figure 2B), $PVRAP^{1}$ and $PVRAP^{2}$. $PVRAP^{1}$ was a deletion of X base pairs in exon 1, which resulted in a frame-shift generating a stop codon after amino acid 29. $PVRAP^{2}$ carry an insertion of 10 base pairs, which resulted in a frame-shift generating a stop codon after amino acid 80 (Figure 2B).

Immunocytochemistry, *in situ* hybridization, adult wing mounting and imaging

Wing imaginal discs were stained using standard procedures and mounted in Vectashield (Vector Laboratories, Burlingame, California). The following primary antibodies were used: goat anti-GFP^{FICT} (Abcam, 1:500), rabbit anti-aPKC (Santa Cruz Biotechnology, 1:300), mouse anti-RFP (Proteintech, 1:500), rabbit anti-PH3 (EMD Millipore Corporation; 1:250), rabbit anti-caspase Dcp1 (Cell Signaling; 1:100) and rabbit anti-pJNK (Promega, 1:200). The secondary antibodies used were: goat anti-mouse Alexa-488, Cy3 and Cy5 (Life Technologies, 1:200) goat anti-rabbit Cy3 and Cy5 (Life Technologies, 1:200) and goat anti-rat Cy3 (Life Technologies, 1:200). F-actin was visualized using Rhodamine Phalloidin (Molecular Probes, 1:50). DNA was marked using Hoechst (Molecular Probes, 1:1000).

Confocal images were obtained using a Leica SP5-MP-AOBS or a Zeiss LSM 880 microscope, equipped with a Plan-Apochromat 20x oil objective (NA 0.7), 40x oil objective (NA 1.4) and 63x oil objective (NA 1.4).

In situ hybridization was performed using standard procedures. A digoxygenin-UTP (Boerhringer-Mannheim) labelled PVRAP anti-sense RNA probe was generated using the plasmid cDNA EST RE08107 (Drosophila Genomics Resource Center).

Quantification of fluorescence intensity

For quantification of fluorescent intensity of different markers, fluorescent signaling was measured on several confocal images per genotype using the square tool in FIJI-Image J.

For calculation of cell areas, the Huang threshold algorithm was applied to maximum projections of confocal sections. Cell volumes were calculated considering wing disc cells as truncated prisms and applying the formula Volume=Height/3 (Basal Area + Apical Area + √ Basal Area x Apical Area).

To measure cell height, a vertical line was drawn from the apical to the basal surface in a region of interest of a wind disc stained for F-actin to visualize cell limits. The total length of the resulting line was measured using FIJI-ImageJ software

To quantify cell proliferation, the Trainable Weka 2D Segmentation plug-in, which transforms 8-bit images into a binary system, was used to quantify dots of fluorescent intensity of wing discs stained with an anti-PH3 antibody using the FIJI-Image J tool analyze particles.

We used welch-test and xi-square test for statistical comparisons.

Results

The knockdown of *PVRAP* or *by* enhances Ras^{V12} hyperplastic phenotype

Ectopic expression of activated *Ras* (Ras^{V12}) in *Drosophila* wing discs produces hyperplasia due to increased cell growth, accelerated G1-S transition and cell shape changes {Prober, 2000 #1251; Soler

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Beatty, 2021 #1561} (Figure 1A, A', B, B'). To test the role of *PVRAP* and *by* in Ras^{V12}-mediated transformation, we reduced their levels in Ras^{V12} wing disc tumour cells by expressing specific RNAis against these two genes. Ectopic expression of Ras^{V12} in the dorsal compartment of wing discs, by means of *apterous*-Gal4 (*ap*>GFP; Ras^{V12}, n=50), induces overgrowth of the tissue and the formation of ectopic folds {Prober, 2000 #1251; Soler Beatty, 2021 #1561} (Figure 1A-B', G). We found that although *PVRAP* or *by* RNAis had no detectable effect in wild-type wing discs (*ap*>GFP; $PVRAP^{RNAi}$, Figure 1C, C', G, n=40 and *ap*>GFP; by^{RNAi}, Figure 1E, E', G, n=40), it enhanced the overgrowth and ectopic fold phenotype of Ras^{V12} wing discs (*ap*>GFP; Ras^{V12}; $PVRAP^{RNAi}$, Figure 1D, D', G, n=38 and *ap*>GFP; Ras^{V12}; by^{RNAi}, Figure 1F, F', G, n=36).

Figure 1. *by* and *PVRAP* knock down enhances Ras^{V12} hyperplasia in *Drosophila* wing discs. (A-F') Maximal projection of confocal views of 3rd instar larvae wing discs, expressing GFP (green) and the indicated UAS transgenes under the control of apterous Gal4 (*apGal4*) driver, stained with anti-GFP (green in A, B, C, D, E and F) and Rhodamine Phalloidin (RhPh) to detect F-actin (red) (A' in A'-F' and white in A', B', C', D', E' and F'). Scale bars 50 μm.

CRISPR/Cas9 mediated generation of *PVRAP* mutant alleles

The *by* mRNA is expressed in wing discs, being highly enriched in the wing pouch {Lee, 2003 #1463}. To analyze PVRAP expression, we performed in situ hybridization in wing discs (see Material and Methods). We found that the PVRAP transcript was abundantly expressed in the wing pouch of wild-type flies (Figure 2A).

Figure 2. Generation of *PVRAP* mutant alleles by CRISPR-Cas9. (A, A') In situ hybridization of 3rd instar wild-type (A) and *PVRAP* mutant (A') wing discs, using a probe for the *PVRAP* mRNA. (B) Schematic representation of the *PVRAP* locus, *PVRAP* mutants generated and sgRNAs used for the generation of the mutants (green and purple). (C-D) Maximal projections of confocal images of third-instar wing discs of the indicated genotypes stained with RhPh to detect F-actin (grey). Scale bars 50 μm.

While there are available null mutations for *by*, mutations in *PVRAP* have not yet been isolated. Thus, to further characterize the role of *PVRAP* as a modulator of Ras^{V12}-mediated hyperplasia, we used CRISPR/Cas9 to generate specific *PVRAP* alleles (see Materials and Methods). The *PVRAP* gene encodes for only one transcript (*PVRAP*-RA, Flybase), whose transcription start site maps to the beginning of exon 1 (Figure 2B). We generated two *PVRAP* mutant alleles, $PVRAP^{1}$ and $PVRAP^{2}$, which truncate 97% and 96% of the PVRAP protein, respectively, and can be considered therefore as null mutations by molecular criteria (Figure 2C). In addition, no *PVRAP* mRNA expression was detected in wing discs from these mutants (Figure 2B). As it is the case for *by* {Torgler, 2004 #1745; Lee, 2003 #1463}, *PVRAP* mutant alleles were homozygous viable. However, while *by* mutant flies show a fully penetrant wing blister phenotype {Torgler, 2004 #1745; Lee, 2003 #1463}, PVRAP mutant flies did not display any obvious morphological abnormalities, indicating that *PVRAP* is dispensable for development in *Drosophila*.

To confirm the role of *PVRAP* and *by* as modulatosr of Ras^{V12}-mediated hyperplasia, we tested for synergetic interactions between *PVRAP* or *by* mutations and Ras^{V12} in wing discs (Figure 3). We found that expression of Ras^{V12} in the posterior compartment of *PVRAP* (*ap*>Ras^{V12}; $PVRAP^{1}$, n=20), or *by* (*ap*>Ras^{V12}; by^{33c}, n=16) mutant discs resulted in an enhanced folding phenotype, similar to that observed in Ras^{V12}; $PVRAP^{RNAi}$ and Ras^{V12}; by^{RNAi} wing discs (Figure 3 and Figure 1).

Figure 3. Hyperplasia due to Ras^{V12} over expression in wing discs is enhanced in *by* and *PVRAP* mutant backgrounds. (A-F) Confocal views of 3rd instar larvae wing discs, expressing GFP (green), the indicated UAS transgenes driven by *apGal4*, in wild-type (A) and mutant genotypes (B-F''), stained with anti-GFP (green in A-A', B-B', C-C', D-D' E-E' F.F' and white in A''-A''', B''-B''', C''-C''',

D''-D''', E''-E''', F''-F'''), RhPh to detect F-actin (red). (A''-F''') and Hoechst (DNA, blue). (A', A'''- F'-F'') Confocal sections of wing discs of the specified genotypes along the white dotted lines shown in A-F, respectively. (G) Violin plot of the percentage of GFP area per disc of the indicated genotypes. The statistical significance of differences was assessed with a welch-test, ****P value<0.0001. Scale bars 50 μm (A-F, A''-F'') and 20 μm (A'-F', A'''-F''').

PVRAP and *by* restrain Ras^{V12} hyperplastic phenotype by regulating cell shape changes and growth

The formation of additional folds could be due to changes in cell polarity, proliferation, shape or a combination of some or all of these cellular properties. The role of human tensins in cell proliferation in normal and cancer cells is complex and tensin type-specific. Thus, while knockdown of TNS1, 3 and 4 reduces proliferation in several normal and cancer cell lines, overexpression of TNS2 reduces the proliferation of several cancer cell lines (reviewed in {Liao, 2021 #1461}. Thus, we next decided to test whether removal of *PVRAP* or *by* would affect the proliferative state of Ras^{V12} cells. Previous results have shown that overexpression of Ras^{V12} results in a reduction in the number of wing disc cells in mitosis, as revealed with an antibody against phosphorylated Histone H3 (PH3) (Figure S1A, A', B, B' and G, {Karim, 1998 #1256; Prober, 2000 #1251; Soler Beatty, 2021 #1561}. Here, we found that while removal of *PVRAP* did not affect the proliferation of wing imaginal disc cells (Figure S1A, A', C, C' and G, n=25), elimination of *by* led to an increase in cell proliferation (Figure S1A, A', E, E' and G, n=20). In addition, elimination of either *PVRAP* (*ap>Ras^{V12}; PVRAP^{1}*, Figure S1D, D', G, n=13) or *by* (*ap>Ras^{V12}; by^{33c}*, Figure S1F, F', G, n=20) enhanced the proliferative capacity of Ras^{V12} wing imaginal discs.

Although ectopic *Ras^{V12}* expression in wing disc cells alone does not affect cell polarity ({Genevet, 2009 #1338}; Figure S2A, A', B and B'), the removal of polarity genes enhances the hyperplastic phenotype of *Ras^{V12}* {Brumby, 2003 #702}. Thus, we tested whether cell polarity was affected in *Ras^{V12}; PVRAP1* or *Ras^{V12}; by^{33c}* disc cells. In order to do this, we analyzed the localization of the apical polarity marker atypical protein kinase C (aPKC, {Tepass, 2001 #1751} in control and experimental conditions (Figure S2). We found that elimination of either *PVRAP* or *by* did not alter the localization of aPKC in normal (Figure S2A, A', C, C', E, E') and *Ras^{V12}* cells (Figure S2D, D', F, and F'), suggesting that polarity was not affected.

To analyze possible cell shape changes, we visualized cell limits using Rhodamine-Phalloidin (Rh-Ph) that labels F-actin and therefore the cell cortex (Figure 4). The wing pouch cells in late third-instar control discs are columnar, with a mean height of 25 μm, an apical area of 8.93 μm^2 and a basal area of 9.41 μm^2 (Figure 4A-A''', G, H, I). In contrast, *Ras^{V12}* expressing cells have been found to be shorter and more cuboidal, with a mean height of 19 μm, an apical area of 10.72 μm^2 and a basal area of 15.86 μm^2 (Figure 4D-D''', G, H, I {Soler Beatty, 2021 #1561}. Considering disc cells as truncated prisms, this has been shown to result in an increase in cell volume {Soler Beatty, 2021 #1561}. This is in agreement with previous results showing that *Ras^{V12}* cells show increased cellular growth {Karim, 1998 #1256; Prober, 2000 #1251}. Here, we found that elimination of either PVRAP or *by* in normal cells (n=109 and 105, respectively) did not cause any effect on cell size (Figure 4B-B''', C-C''', G, H, I). In contrast, the knock down of *PVRAP* or *by* in *Ras^{V12}* cells enhanced the expansion of the apical area around 30% and 27%, respectively (Figure 4E-E', F-F', G) but did not cause any effect on either the basal area (Figure 4E'', F'', H) or the height (Figure 4E''', F''', I). This besides causing a change in cell shape, if we consider disc cells as truncated prisms, resulted in a further increased in cell volume of 15% and 13% when removal *PVRAP* or *by*, respectively.

Figure 4. increase *Ras^{V12}*-dependent cell shape changes and growth of ***Drosophila*** wing discs is increased in *by* and *PVRAP* mutant backgrounds. (A-F) Maximal projections of confocal images of wing imaginal discs from third-instar larvae of the indicated genotypes expressing GFP (green) and the designated UAS transgenes under the control of *apGal4*, stained with anti-GFP (green), RhPh to detect F-actin (red in A-F, white in A'-F'''). (A'-F') Apical and (A"-F") basal surface views of the indicated genotypes. (A'''-F''') Confocal *xz* sections along the white dotted lines of wing discs shown

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in A-F. The apical side of wing discs is at the top. The red dotted lines indicate cell height. (G-I) Violin plots of the apical (G) and basal (H) cell areas and cell height (I) of the indicated genotypes. The statistical significance of differences was assessed with a welch-test, ****, *** and ** P values <0.0001, <0.001 and <0.01 respectively. Scale bars 50 μm (A-F), 5 μm (A'-F', A"-F") and 10 μm (A'"- F'").

Besides an increase in hyperplastic growth, the expression of Ras^{V12} in wing disc cells have also been shown to promote transition from G1 to S phase {Prober, 2000 #1251}. Furthermore, the use of Fly-Fucci, which relies on fluorochrome-tagged degrons from the cyclin B -degraded during mitosis- and E2F1 -degraded at the onset of S phase (Figure 5A), has confirmed the role of Ras^{V12} in driving transition from G1 to S phase {Murcia, 2019 #1336}, showing that Ras^{V12} expressing wing discs exhibited an increase in the G2 population, 68,39% versus 42.05% in control wing discs, and a reduction in the G1 population, 22,64% vs 33.61% of controls (Figure 5B, B', C, C', number of Ras^{V12} cells=10965 cells, number of control cells=35768 cells). Using Fly-Fucci, we found that expression of an RNAi against either *PVRAP* or *by* did not affect progression through the cell cycle (Figure 5D, D', F, F'). In addition, reducing the levels of *PVRAP* did not seem to substantially change the behaviour of Ras^{V12} cells, with populations of G2 and G1 of 64,75% and 26,92%, respectively (Figure 5E, E', n= 27211 cells). Unfortunately, for unknown reasons, flies carrying the Fly-Fucci transgenes and co-expressing Ras^{V12} and an RNAi against *by* under the control of the *apGal4* driver were not viable. Thus, we could not asses the effect of removal *by* in the progression of the cell cycle of Ras^{V12} cells.

Figure 5. Elimination of *by* or *PVRAP* enhances the changes in cell cycle progression due by to Ras^{V12} over expression in wing discs. (A) Scheme showing the expression of CycB-GFP and E2F1-RFP during the cell cycle. (B-F) Maximal projection of confocal images of 3rd instar wing imaginal discs of the designated genotypes expressing the indicated UAS transgenes under the control of *apGal4-flyfucci*, stained for anti-GFP (green) and anti-RFP (red). (B', C', D', E', F') Scatter plots representing the fluorescence intensity of both proteins in each cell. The statistical significance of differences was assessed with Xi-square test, having all comparisons a ****P value<0.0001. Scale bars 50 μm (B-F).

All together, these results suggest that *PVRAP* and *by* modulate Ras^{V12}-mediated tissue hyperplasia by enhancing cell shape changes and cellular growth. Given the constraints imposed by the peripodial membrane, we propose that the observed cell shape changes and the increase in cell size could explain the formation of extra folds.

Consequences of *PVRAP* or *by* elimination in the non-autonomous effects of Ras^{V12} tumor cells.

Preceding observations have shown that JNK activity was elevated in wild type cells surrounding Ras^{V12} expressing cells (Figure 6A, B, G; {Chabu, 2014 #1380; Soler Beatty, 2021 #1561}. This has been shown to non-autonomously activate the JAK-STAT pathway in the tumor cells promoting their growth {Chabu, 2014 #1380}. As we show here that elimination of either *PVRAP* or *by* enhanced the growth of Ras^{V12} tumor cells, we tested whether activity of the JNK pathway was affected in these tumor conditions.

Figure 6. *by* and *PVRAP* do not affect the ability of Ras^{V12} tumor cells to induce JNK activation in nearby wild type tissue. (A-F) Maximal projections of confocal views of 3rd instar wing discs expressing the indicated UAS transgenes under the control of *ap*Gal4, stained with anti-GFP (green), anti-pJNK (red in A-F, white in A', B', C', D', E', F'), and Hoechst (DNA, blue). (G) Violin plots of mean fluorescent pJNK intensity in the dorsal-ventral boundary of wing discs of the designated genotypes. The statistical significance of differences was assessed with a welch-test, **** and *** P values <0.0001 and <0.001, respectively. Scale bars, 50 μm (A-F').

Previous analysis has shown that *by* genetically interacts with the JNK pathway and that activity of this pathway, measured by examining the extent of JNK phosphorylation using an anti-phosphospecific JNK antibody (pJNK), is dramatically increased or reduced upon *by* overexpression

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or downregulation, respectively {Lee, 2003 #1463}. However, here we found that pJNK levels did not change in either *PVRAP*^{1} (n= 17) or *by*^{33c} (n=17) mutant wing discs compared to controls (n=14) (Figure 6A, C, E and G). In addition, we found that the levels of JNK activity in wild type cells next to *Ras*^{V12} tumor cells mutant for *PVRAP* (Figure 6D and G, n=12) or *by* (Figure 6F and G, n=17) were not significantly different from those found in wild type cells adjacent to *Ras*^{V12} (Figure 6B and G, n=20) tumor cells. These results suggest that proteins of the tensin family modulate the growth of tumour cells independently of the JNK pathway.

Overexpression of activated Ras has also been shown to promote the death of nearby wild-type tissue in *Drosophila* imaginal tissues {Karim, 1998 #1256; Soler Beatty, 2021 #1561}. In agreement with this, a clear enrichment of apoptosis was detected in wild-type (GFP negative) ventral cells located at the D/V boundary in *ap*>GFP; *Ras*^{V12} discs (n=24) compared to controls (n=23) (Figure S3A, A', B, B' and G; {Soler Beatty, 2021 #1561}. Here, we found that while removal of *by* did not affect apoptosis in wing imaginal discs (n= 19, Sup. Figure 3E, E' and G), elimination of *PVRAP* led to a general increase in cell death in the wing disc, with no clear concentration at the D/V boundary (n=21, Figure S3A, A', E, E' and G). In addition, we found that elimination of either *PVRAP*^{1} (*ap*>*Ras*^{V12}; *PVRAP*^{1}, Figure S3D, D', G, n=20) or *by*^{33c} (*ap*>*Ras*^{V12}; *by*^{33c}, Figure S3F, F', G, n=20) enhanced the cell death of wild type cells at the D/V boundary in *Ras*^{V12} expressing wing discs.

Apoptosis of wild-type cells near *Ras*^{V12} cells has also been explained by an increase in tissue compaction due to the enhanced growth of mutant cells {Moreno, 2019 #1339}. In fact, we have previously found that the wild-type ventral region of *ap*>GFP; *Ras*^{V12} discs (n=40) was more compressed than in *ap*>GFP discs (n=34) (Figure 3A'' and B'', {Soler Beatty, 2021 #1561}. This *Ras*^{V12} phenotype was further enhanced in *PVRAP* (n=20) and *by* (n=16) mutant backgrounds (Figure 3D'' and F'').

Effector caspases are active in tumors and have been associated with metastasis {Wild-Bode, 2001 #1378}. In fact, caspase activity has been suggested to drive the migration of transformed cells in wing imaginal discs {Rudrapatna, 2013 #1377}. In agreement with this, we reported in a previous study the presence of *Ras*^{V12} cells positive for Dcp1 in the ventral domain of *ap*>*GFP*; *Ras*^{V12} {Soler Beatty, 2021 #1561}, Figure S4). Tensins have been reported to play a role in cell migration and invasion. However, experiments in cell culture analyzing the roles of the different tensins in cell invasion have yielded contradictory results and appeared to be cell context dependent (review in {Liao, 2021 #1461}. Thus, we tested whether elimination of either *PVRAP* or *by* would affect the migration of normal or *Ras*^{V12} tumor cells. In order to do this, we analyzed the presence of GFP+ cells outside of the dorsal domain in control and experimental wing discs. We found that in either *ap*>GFP; *PVRAP*^{1} (n=25) or *ap*>*GFP; by* mutant wing discs (n=28), the cells did not invade the ventral compartment (Figure S4A, B, C, G, H). In addition, we found that the removal of either *PVRAP* (n=36) or *by* (n=36) did not affect the number or the migration distance of *Ras*^{V12} GFP+ tumor cells into the ventral compartment (Figure S4D, E, F, G, H).

Genetic interactions between proteins of the tensin family

The absence of a loss-of-function phenotype for *PVRAP* could be due to compensation by other proteins of the tensin family performing similar functions, such as *by*. Thus, we examined whether *PVRAP* would genetically interact with *by*. As *PVRAP* and *by* are both on the second chromosome, we analyzed the effects of reducing the levels of both *PVRAP* and *by*, by expressing a *PVRAP*^{RNAi} in the posterior compartment of *by* mutant wing discs. While flies homozygous for *by*^{33c} are viable and show blisters in the wing {Lee, 2003 #1463; Torgler, 2004 #1745}; Figure 7B), flies heterozygous for *by*^{33c} (*by*^{33c}/+) or flies expressing an RNAi against *PVRAP* in the posterior compartment of control wing discs did not show any visible adult phenotype (*ap*>GFP; *PVRAP*^{RNAi}, Figure 7C). In contrast, expression of *PVRAP*^{RNAi} using the *apGal4* driver in a homozygous *by*^{33c} genetic background (*by*^{33c}; *ap*>GFP; *PVRAP*^{RNAi}) was lethal, with a very small fraction of flies (2%, n=100) reaching adulthood. These escapers showed strong defects in the wing and the notum and died soon after hatching (Figure 7D). In addition, expression of a *PVRAP*^{RNAi} in the posterior compartment of the wing imaginal disc of heterozygous *by*^{33c} resulted in a smaller scutellum and reduced number of bristles (*by*^{33c}/+; *ap*>GFP;

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PVRAP^{RNAi}; Figure7E, F). Interestingly, this phenotype was also observed when an RNAi against *EGFRAP*, the ortholog of human TNS2 (Flybase), is expressed in the dorsal compartment in a heterozygous *by*^{33c}/+ genetic background (*by*^{33c}/+; *ap*>GFP; *EGFRAP*^{RNAi}; Figure 7G).

Figure 7. *PVRAP* downregulation enhances *by* loss of function phenotypes. (A-D') Dorsal views of adult *Drosophila* flies expressing the indicated UAS transgenes under the control of *ap*Gal4. (E-G) Dorsal views of the *Drosophila* notum of the specified genotypes.

Mammalian tensins are known to participate in integrin signalling {Zamir, 2001 #1750}. Similarly, as mutations in *by* genetically interact with integrin viable alleles, the *Drosophila* tensin has also been proposed to functionally interact with integrins during wing development {Lee, 2003 #1463}. Here, we show that *by* also interacts with *PVRAP*. Thus, we next tested whether *PVRAP* would also interact with integrins. As mentioned above, the total elimination of *PVRAP* or its downregulation in the dorsal domain of wing imaginal discs (*ap>PVRAP*^{RNAi}) on its own did not cause any visible phenotype in the adult (Figure 7C). Integrins are heterodimers composed of a α and a β subunit. Two integrins, which share the same β subunit, are expressed in the *Drosophila* wing disc, the αPS1βPS (PS1) on the dorsal side and αPS2βPS (PS2) in the ventral domain. Eliminating integrin activity in the wing imaginal disc results in blisters in the adult appendage (reviewed in {Brower, 2003 #374; Brown, 2000 #404}. Here, we found that the co-expression of a *mys*^{RNAi} and a *PVRAP*^{RNAi} in the dorsal region of wing discs (*ap>PVRAP*^{RNAi}; *mys*^{RNAi}) led to lethality. This result suggests that PVRAP also interact with integrins.

Discussion

Cancer is a devastating disease that threatens human health worldwide {Siegel, 2020 #1330}. One of the most frequently affected genes in cancer is the proto-oncogene Ras. In fact, mutations leading to its overactivation are present in ~30% of human cancers {Forbes, 2015 #1319}. However, hyperactivation of Ras signaling alone is not sufficient to produce malignancy, additional mutations in other genes are required to drive Ras-dependent tumorigenesis (reviewed in {Dimauro, 2010 #1331}. Thus, identifying genes that modulate the oncogenic capacity of Ras is vital in our fight against cancer. The tensin family of focal adhesion proteins has emerged as regulators of tumor progression in many cancer types {Wang, 2022 #1757}. However, the role of tensins in cancer is not fully established, since they can serve either as cancer-promoting or as cancer-inhibitory factors. Most experimental studies have mainly explored the positive or negative effects of tensins in *in vitro* cell culture models of different cancer cell lines (reviewed in {Liao, 2021 #1461}. Thus, a better understanding of the role of tensins in cancer development in the context of a whole organism is still missing. Here, we have used the *Drosophila* model to analyze the mechanism by which tensins modulate the progression of epithelial tumors. We show that *by*, the clearest homolog of human TNS4, and *PVRAP*, an ortholog of human TNS2 (FlyBase), act as tumor suppressors of Ras-mediated tumorigenesis, as their elimination enhances the overgrowth due to overexpression of oncogenic Ras in wing disc epithelial cells. In addition, we find that tensins regulate tumor progression by restraining cell proliferation, cell cycle progression and cellular growth. Our results suggest that the role of tensins as cancer-inhibitory factors has been conserved across evolution and unravel possible mechanisms of action.

Tensins belong to the family of adhesion proteins that form focal adhesions and serve as a bridge between the extracellular matrix and the intracellular actin cytoskeleton. The mammalian tensin family, which comprises four members, are multidomain proteins that contain, on its N-terminal region, an actin-binding domain, which overlaps with a focal-adhesion-binding site and PTEN-like protein tyrosine phosphatase and C2 domains, and, on its C-terminal region, an Src homology 2 (SH2) and a phosphotyrosine binding domains. These domains allow tensins to transduce several signaling pathways, such as PI3/Akt and β-integrin/Fax pathways, regulating a variety of physiological processes, including cell proliferation, survival, adhesion, migration and mechanical sensing. Analysis of the role of mammalian tensins in mice has revealed that while individual tensins are not

essential for embryonic or tissue development, they are required to maintain the structure and function of kidney and heart and for regeneration processes (reviewed in {Liao, 2021 #1461}. As for their role in tumorigenesis this appears to be quite controversial, and tensin and cell type specific, acting sometimes as tumor suppressors and others as tumor promoters (reviewed in {Pryczynicz, 2020 #1758}. Unlike mammals, *Drosophila melanogaster* and *Caenorhabditis elegans* have been proposed to possess one tensin each. In addition, while the worm tensin is more similar to TNS1, TNS2 and TNS3 and contains all domains present in these tensins, the fly one (*by*) is more similar to TNS4 and contains only the SH2 and PTB domains {Lee, 2003 #1463; Torgler, 2004 #1745; Bruns, 2020 #1759}. As it is the case in mammals, tensin knockout in flies and worms has no impact on development and survival and their role on tumor progression in these model systems had not been studied {Lee, 2003 #1463; Torgler, 2004 #1745; Bruns, 2020 #1759}. Besides *by*, two other genes, *EGFRAP* and *PVRAP*, have SH2 domains that are similar to the ones present in mammalian tensins and have been proposed to be orthologs of human TNS2 and TNS4, respectively (FlyBase). Similar to *by*, elimination of either *EGFRAP* {Soler Beatty, 2021 #1561} or *PVRAP* (this work) has no consequences for development and survival. In addition, elimination of any of these three *Drosophila* tensins enhances the overgrowth due to overexpression of oncogenic Ras {Soler Beatty, 2021 #156} and this work). These results suggest that in *Drosophila* all tensins seem to behave similarly with respect to their ability to suppress tumor progression, at least in epithelial wing imaginal disc cells. In the future, it will be interesting to analyze their role in other cancer cell types, such as those produced in the gut endoderm or the brain. Finally, our results also suggest that, as it could be the case in mammals, these *Drosophila* tensins may have redundant functions, since the elimination of either two of them enhances the phenotype of eliminating each of them individually {Soler Beatty, 2021 #1561} and this work).

As mentioned above, and similar to what we have found in *Drosophila*, mammalian tensins are not cancer driver molecules. Instead, they seem to act as modulators of tumor progression and they do so by regulating various cellular events, including cell polarization, proliferation, apoptosis and migration (reviewed in {Liao, 2021 #1461; Pryczynicz, 2020 #1758}. TNS1, TNS3 and TNS4 knockdown reduces the proliferation of several cancer cell lines, such as colon cancer and acute myeloid leukemia cell lines {Zhou, 2018 #1760; Hong, 2016 #1761; Sun, 2020 #1762}. In contrast, TNS2 overexpression reduces cell proliferation and survival of some cervical and lung cancer cells {Cheng, 2018 #1763}. In *Drosophila*, the overexpression of oncogenic Ras^{V12} in the wing disc results in a reduction in the number of cells in mitosis {Karim, 1998 #1256; Prober, 2000 #1251; Soler Beatty, 2021 #1561}. In contrast to our previous results showing that *EGFRAP* does not affect the proliferation of Ras^{V12} wing disc cells {Soler Beatty, 2021 #1561}, here we find that elimination of either *PVRAP* or *by* slightly, but significantly, increases the number of Ras^{V12} undergoing mitosis, suggesting that as it is the case for the mammalian tensins, the different *Drosophila* tensins may regulate the behaviour of tumor cells in a tensin type specific manner. However, even though the number of cells in mitosis in tumorigenic wing discs mutant for either *PVRAP* or *by* is higher than that found in tumorigenic wing discs, it is still lower than that found in controls. Thus, this increase on its own cannot account for the increase in tissue overgrowth found in Ras^{V12} wing discs mutant for either *PVRAP* or *by*, suggesting that these two tensins modulate Ras^{V12}-dependent tumorigenesis by regulating additional cellular events rather than just cell proliferation. Previous analysis has demonstrated that Ras^{V12} cells show increased cellular growth {Karim, 1998 #1256; Prober, 2000 #1251; Soler Beatty, 2021 #1561}. Here, we find that elimination of either *PVRAP* or *by* in Ras^{V12} cells results in a cell shape change, which leads to an increase in cell volume. This result unravels a new mechanism by which tensins could modulate tumor progression, the regulation of cell shape and growth.

Analysis of the roles of tensins in cell migration and invasion have shown that depending on the cellular context, tensins can either promote or inhibit cell migration. Thus, while knockdown of TNS1 reduces the migration of mouse fibroblasts and endothelial cells, overexpression of TNS1 or TNS2 promotes the migration of human embryonic kidney of cells {Chen, 2002 #1752; Shih, 2015 #1753}. In addition, downregulation of either TNS1 or TNS2 or TNS3 reduces the invasiveness of ovarian and breast cancer cell lines, by impairing integrins internalization and focal adhesion turnover {Rainero, 2012 #1101; Shinchi, 2015 #1755; Vess, 2017 #1756}. However, here we show that elimination of either

PVRAP or *by* does not affect the migration of normal or Ras^{V12} tumor epithelial wing disc cells. One possible explanation for this result is that the function of tensins in *Drosophila* might be different from that in mammals. In fact, while tensins have been shown to affect integrin internalization in mammalian cells (see above), integrin localization is not affected in *by* mutant wing discs {Torgler, 2004 #1745}. Furthermore, *Drosophila by* only affects a subset of integrin-mediated adhesion {Torgler, 2004 #1745}. An alternative explanation is redundancy among the *Drosophila* tensin family of proteins. In the future, it will be interesting to analyze the consequences of reducing simultaneously *PVRAP* and *by* in normal and Ras^{V12} tumor wing disc cells in cell migration.

Finally, our results also show that fly tensins do not seem to regulate the polarization or survival of tumor cells. However, they seem to influence the ability of tumor cells to induce the apoptosis of nearby wild type tissue. As removal of either *PVRAP* or *by* enhances the formation of extra folds due to overexpression of Ras^{V12}, we propose that the increased in cell death in nearby wild type tissue could be a direct consequence of an increase in its compaction due to the enhanced growth of Ras^{V12}cells in *PVRAP* or *by* mutant backgrounds.

Through their different domains, mammalian tensins can bind pathway signalling molecules, including β1-integrin, PI3K/Akt/mTOR, FAK, Rho GAP, p130Cas, TGF-β and the Ras/Raf pathways, thus regulating a myriad of different cellular responses in normal cells (reviewed in {Liao, 2021 #1461; Pryczynicz, 2020 #1758; Mouneimne, 2007 #1464}. Even though most studies analysing the role of mammalian tensins in cancer have been dedicated to assess the expression of tensins in different cancer types, there have been studies attempting to identify the role of tensins in carcinogenesis. Thus, TNS1 has been proposed to regulate tumor cell proliferation affecting Rho GAP through regulation of the hippo signalling pathway (reviewed in {Wang, 2022 #1757}. Other studies indicated that TNS4 could promote cancer progression via regulating the Akt/GSK-3β and TGF-β1 signalling pathways {Qi, 2020 #1765; Asiri, 2018 #1766}. In addition, a reciprocal TNS3-TNS4 switch regulates the invasive capacity of breast tumors downstream of the EGFR via direct interaction with β1 integrins {Katz, 2007 #1768}. In *Drosophila*, *by* interacts with integrins and the JNK pathways {Lee, 2003 #1463; Torgler, 2004 #1745}, while PVRAP physically interacts with PVR {Tran, 2013 #1474} and EGFRAP interacts and regulates the EGFR pathway {Soler Beatty, 2021 #1561}, interactions that regulate adhesion and fate in normal cells. In *Drosophila* wing disc tumor cells, we have recently shown that EGFRAP restrain the oncogenic capacity of EGFR/Ras hyperactivation {Soler Beatty, 2021 #1561}. Here, we show that *by* also acts as a tumor suppressor in Ras-mediated oncogenesis. As *by* has been shown to interact with integrins {Lee, 2003 #1463; Torgler, 2004 #1745} and we have recently shown that β1 integrins also behave as suppressors or Ras^{V12}-dependent tumorigenesis in *Drosophila* wing discs {Valencia-Exposito, 2022 #1718}, we propose that *by* may act as tumor suppressors via its ability to bind and regulate integrins. This suggests that the function of tensins as tumor modulators via regulating integrin function might have been conserved throughout evolution. Finally, overactivation of PVR has also been shown to produce overgrowth of the *Drosophila* wing disc {Rosin, 2004 #770}. As *PVRAP* interacts with PVR {Tran, 2013 #1474}, we propose that *PVRAP* could act as tumor suppressors by regulating the activity of PVR, similar to the relationship between EGFRAP and EGFR. Altogether, these results suggest that, similar to what happens with mammalian tensins, the *Drosophila* tensins orthologs could modulate tumorigenesis by regulating different signaling pathways. Finally, our results showing that downregulation of *PVRAP* and *EGFRAP* led to defects consistent with downregulation of integrin function {Soler Beatty, 2021 #1561}, suggest the existence of cross-talks between the different *Dosophila* tensins and the pathways they can modulate.

Even though tensins have been widely implicated in different types of cancers, to date, there is no clinical trial targeting them, as their impact in carcinogenesis is not fully established. Our results demonstrate that *Drosophila* tensins act as suppressors of Ras^{V12} tumor progression in wing disc epithelial cells. We can now use the advantages of the *Drosophila* system to increase our understanding of the mechanisms by which tensins modulate carcinogenesis and to identify new therapeutic drugs targeting malignancy, a top priority in cancer research.

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