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Differential regulation of protein tyrosine kinase signaling by Dock and the PTP61F variants

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Abbreviations: PTKs, protein tyrosine kinases; PTPs, protein tyrosine phosphatases; EGFR, Epidermal growth factor receptor; PVR, Platelet derived growth factor/vascular endothelial growth factor receptor; InR, Insulin receptor; Jak, Janus kinase; STAT, Signal transducer and activator of transcription protein; SFK, Src family kinases; MAPK, Mitogen-activated protein kinase; ERK, Extracellular signal-regulated kinase; MMP1, metalloproteinase 1; Hop, Hopscotch; Abl, Abelson tyrosine kinase; PI3K, Phospho-inositol-3 protein kinase.

ABSTRACT

Tyrosine phosphorylation-dependent signalling is coordinated by the opposing actions of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). There is a growing list of adaptor proteins that interact with PTPs and facilitate the dephosphorylation of substrates. The extent to which any given adaptor confers selectivity for any given substrate *in vivo* remains unclear. Here we have taken advantage of *Drosophila melanogaster* as a model organism to explore the influence of the SH3/SH2 adaptor protein Dock on the abilities of the membrane (PTP61Fm)- and nuclear (PTP61Fn)-targeted variants of PTP61F (the *Drosophila* othologue of the mammalian enzymes PTP1B and TCPTP respectively) to repress PTK signaling pathways *in vivo*. PTP61Fn effectively repressed the eye overgrowth associated with activation of the epidermal growth factor receptor (EGFR) PTK, or the expression of the platelet derived growth factor/vascular endothelial growth factor receptor (PVR) or insulin receptor (InR) PTKs. PTP61Fn repressed EGFR and PVR-induced mitogen-activated protein kinase signaling and attenuated PVR-induced STAT92E signaling. By contrast, PTP61Fm effectively repressed EGFR- and PVR-, but not InR-induced tissue overgrowth. Importantly, co-expression of Dock with PTP61F allowed for the efficient repression of the InR-induced eye overgrowth, but did not enhance the PTP61Fm-mediated inhibition of EGFR and PVR-induced signaling. Instead, Dock expression increased, and PTP61Fm co-expression further exacerbated the PVR-induced eye overgrowth. These results demonstrate that Dock selectively enhances the PTP61Fm attenuation of InR signaling and underscores the specificity of PTPs and the importance of adaptor proteins in regulating PTP function *in vivo*.

INTRODUCTION

Tyrosine phosphorylation is a reversible dynamic process controlled by the opposing activities of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) [1]. PTPs are a large and structurally diverse family of enzymes found in eukaryotes, prokaryotes and plants. Classical tyrosine-specific PTPs are integral to the control of PTK activation and downstream tyrosine-phosphorylation-dependent signaling, initiating or setting thresholds for PTK activation and influencing both the amplitude and duration of tyrosine phosphorylation-dependent signaling [1]. Multiple PTPs are involved in the regulation of any given PTK or PTK substrate, acting on distinct, as well as overlapping tyrosine phosphorylation sites [1]. However, such apparent overlapping regulation can occur in a temporally and spatially restricted manner [2-5].

The capacity for PTPs to act in a coordinated manner to regulate signaling is exemplified by the activities of two of the most closely related tyrosine-specific PTPs in the human genome, PTP1B (encoded by *PTPN1*) and TCPTP (encoded by *PTPN2*) [1]. The catalytic domains of these two phosphatases share a high degree of primary (72% identity, 86% similarity) and tertiary structure similarity [1, 6]. The two phosphatases have virtually identical active sites and have an adjacent phosphotyrosine-binding pocket that allows for the recognition of substrates with tandem phosphorylation sites [7, 8], such as the Y1162/Y1163 site present in the insulin receptor (InR) β-subunit PTK activation loop, or the corresponding tandem tyrosine phosphorylation site in Janus-activated kinase (JAK) family PTKs [1, 5].

Despite this similarity, PTP1B and TCPTP are not redundant, but rather function cooperatively to regulate PTK activation and signaling [1, 5]. For example both phosphatases dephosphorylate the InR Y1162/Y1163 site in a temporally distinct manner with PTP1B acting early to regulate the intensity of InR activation, and TCPTP controlling the duration of InR signaling [2, 5]. Similarly, PTP1B and TCPTP differentially regulate JAK signaling with PTP1B dephosphorylating JAK-2 and Tyk2 and TCPTP dephosphorylating JAK-1 and JAK-3 [9-11]. Additionally PTP1B and TCPTP act cooperatively to regulate JAK/signal transducer and activator of transcription (STAT) signaling [1, 12, 13], with PTP1B for example dephosphorylating JAK2 in the cytoplasm [10, 11] and TCPTP dephosphorylating the JAK-2 substrate STAT-3 in the nucleus, to suppress leptin-induced JAK-2/STAT-3 signaling in the hypothalamus [13]. Although in part, substrate selectivity is conferred by inherent catalytic domain specificity, the distinct subcellular distributions of the phosphatases also influence substrate access [1].

PTP1B is targeted to the cytoplasmic face of the endoplasmic reticulum (ER) by a non-catalytic hydrophobic C-terminus [14]. PTP1B dephosphorylates receptor PTKs, such as the InR, platelet-derived growth factor receptor (PDGFR) and the epidermal growth factor receptor (EGFR) at the cell surface and cell junctions and after receptor PTK endocytosis [1, 3, 4, 15, 16]. PTP1B also dephosphorylates cytosolic PTKs to either suppress or promote PTK signaling [1]. For example, PTP1B dephosphorylates the C-terminal Y527 inhibitory site on Src family kinases (SFKs) to activate SFKs and promote PTK signaling [17, 18], but dephosphorylates and inactivates JAK PTKs to attenuate cytokine signaling [9-11]. TCPTP is expressed as two variants with identical catalytic domains, but distinct C-termini arising from the alternative splicing of mRNA: a 48 kDa variant (TC48) with a hydrophobic C-terminus that is targeted to the ER, like PTP1B, and a 45 kDa variant (TC45) with a shorter hydrophilic C-terminal tail that is targeted to the nucleus by a bipartite nuclear localisation sequence (NLS) [19, 20]. Despite having an apparent exclusive nuclear localisation in resting mammalian cells, TC45 can shuttle between the nucleus and cytoplasm [21, 22] and access substrates such as the InR, JAK-1/3 [2, 9, 23, 24] and SFKs [25-27], and nuclear substrates such as STAT-1/3 [13, 28] to regulate growth factor- and cytokine-induced signaling. Recent studies suggest that TC45's capacity to dephosphorylate STAT-3 in the nucleus may be controlled by targeting proteins, such as GDX [29] and SIPAR [30], whereas the dephosphorylation of cytoplasmic substrates, such as c-Src or JAK-1/3, for the repression of TNF-induced MAPK signaling and interleukin (IL)-2 induced STAT-5 signaling may be orchestrated by the adaptor proteins TRAF2 [25] and TRAF3 [31], respectively. Similarly we have shown previously that the *Drosophila* SH3/SH2 adaptor protein Dock (Dreadlocks), or its mammalian counterpart Nck, facilitate PTP61F-mediated InR dephosphorylation in flies, or PTP1B-mediated InR dephoshorylation in mammalian cells [32]. However, a direct assessment of the contribution of any given adaptor protein to the regulation of defined PTKbiological processes *in vivo* by PTPs has not been assessed.

In *Drosophila* PTP1B and TCPTP have a single orthologue, PTP61F [33]. Alternative splicing of *PTP61F* mRNA gives rise to PTP61Fm, which is targeted to the ER by a hydrophobic C-terminus [33] like PTP1B/TC48 [1], and PTP61Fn, which lacks the hydrophobic C-terminus and is targeted to the nucleus by a NLS [33], like TC45 [19]. The PTP61F catalytic domain exhibits 69-72% similarity to that of PTP1B or TCPTP, and also contains the additional phosphotyrosine-binding pocket that allows for the recognition of tandem tyrosine phosphorylated substrates in PTP1B/TCPTP [1]. Indeed, like PTP1B/TCPTP, PTP61F dephosphorylates the *Drosophila* InR Y1553/Y1554 PTK activation loop autophosphorylation site to suppress insulin signaling in flies [34]. PTP61F also regulates JAK/STAT (Hopscotch/STAT92E) signaling in flies [34-37]. For example, we have shown previously that STAT92E Y711 phosphorylation is increased in the ovaries of *PTP61FΔ* mutant flies (null for PTP61Fm and PTP61Fn) contributing to defects in fecundity [34]. The PTP61F-mediated attenuation of JAK/STAT signaling is thought to occur at the level of Hopscotch or STAT92E [36, 37]. It remains unclear if PTP61F can also act directly on Hopscotch and whether the PTP61F ER and nuclear variants coordinately regulate Hopscotch/STAT92E signaling in flies as noted for PTP1B and TCPTP in mammals [1]. Here we have taken advantage of *Drosophila* as a model organism to explore the *in vivo* roles of the PTP61F variants in receptor PTK and JAK/STAT signaling and the role of the adaptor Dock in influencing PTP61F function in the regulation of defined receptor PTKs.

RESULTS

PTP61Fn negatively regulates EGFR signaling

We had shown previously that PTP61F and its mammalian orthologues PTP1B and TCPTP dephosphorylate the InR and attenuate InR signaling *in vivo* [32]. Although PTP1B and TCPTP have been implicated in the regulation of several receptor PTKs, including the EGFR and PDGFR [1], the role of PTP61F in the regulation of other receptor PTKs *in vivo* remains unclear. Accordingly, we examined whether PTP61F might regulate *Drosophila* EGFR signaling. To this end, we made use of the dominant EGFR *Ellipse* mutant, $EGFR^{ElpB1}$ ($EGFR^{E3}$). This mutant fly arose from an ethylmethylsulfonic acid mutagenesis screen and was mapped to the endogenous *EGFR* locus [38]. $EGFR^{ElpB1}$ has a single amino acid substitution in the EGFR PTK domain that is activating and results in a small rough eye phenotype, primarily due to reduced specification of the R8 photoreceptor cell and differentiation defects [39]. During pupal development, excess cells surrounding each photoreceptor cluster are eliminated by apoptosis, thereby resulting in a smaller and disorganised adult eye. To modulate PTP61F expression we used the *ey-GAL4* driver (which is expressed in the developing eye epithelium from the late embryo to late larval stages) to ectopically express either GFP as a control, or the PTP61F nuclear-targeted splice variant PTP61Fn [34], in the developing eye epithelium. Although expression of *PTP61Fn* in a wild-type background showed a small, but significant reduction in eye size, there was no effect on eye patterning in both males (Figure 1B relative to 1A, quantified in 1G) and females (Figure 1I relative to 1H, quantified in 1N). Importantly, expression of *PTP61Fn* in the $EGFR^{ElpB1}$ background significantly corrected the small rough eye phenotype in males (Figure 1E compared with 1D, quantified in 1G) and females (Figure 1L compared with 1K, quantified in 1N). Conversely, reducing the dosage of PTP61F by heterozygosity of the *PTP61FΔ* mutant null allele (*PTP61FΔ*/+) (Figure 1F compared with 1D, quantified in 1G) or using the *PTP61F-RNAi* transgene to knockdown PTP61F expression (Figure 1M compared with 1K, quantified in 1N) enhanced the small rough eye phenotype of $EGFR^{ElpB1}$ flies; *PTP61F-RNAi* expression alone resulted in an enlarged eye (Figure 1J, quantified in 1N), consistent with PTP61F knockdown driving endogenous RTK pathways that promote growth.

To reaffirm the role of PTP61F in EGFR signaling we also tested if PTP61F overexpression or deficiency could modulate the eye overgrowth phenotype associated with the ectopic expression of a constitutively active EGFR transgene ($UAS\text{-}EGFR^{\lambda top}$), which contains a extracellular dimerization domain from the lambda CI protein, and therefore is constitutively dimerized and active [40]. When this activated form of EGFR is expressed via the *ey-GAL4* driver throughout development in the proliferating cells of the eye epithelium, additional founder cells are produced leading to more R8 cells and additional photoreceptor clusters, resulting in a larger adult eye (Figure 2E). PTP61Fn overexpression robustly suppressed the *ey>EGFR*$^{\lambda top}$ overgrown eye phenotype (Figure 2F compared with 2E, quantified in 2M). Conversely, reducing PTP61F levels, via heterozygosity of the *PTP61F* null allele (*PTP61FΔ),* or using the *PTP61F-RNAi* transgene to knockdown PTP61F expression, resulted in the enhancement of the *ey>EGFR*$^{\lambda top}$ overgrown eye phenotype (Figure 2G, H compared with 2E, quantified in 2M). *PTP61F-RNAi* expression alone also resulted in an enlarged eye (Figure 2D), however this was significantly increased when *EGFR*$^{\lambda top}$ was co-expressed (Figure 2H, quantified in 2M). Thus, PTP61F represses the tissue overgrowth effects due to ectopic EGFR activation. Taken together these results identify PTP61F as a negative regulator of EGFR signaling in flies.

PTP61F negatively regulates PVR signaling

We next examined whether PTP61F could modulate signaling through PVR [41], the *Drosophila* homologue of the mammalian PDGFR and vascular endothelial growth factor receptor PTKs, both of which are dephosphorylated by PTP1B and TCPTP [16, 42-44]. To this end we assessed the influence of PTP61Fn overexpression or deficiency on the overgrowth associated with the expression of a ligand-independent activated allele of PVR (*UAS-PVR*$^{\lambda}$) in the *Drosophila* eye [45]. As expected, overexpression of *UAS-PVR*$^{\lambda}$ via the *ey-GAL4* driver resulted in overgrown adult eyes (Figure 2I, quantified in 2M). Importantly, expression of *PTP61Fn* in the *ey>PVR*$^{\lambda}$ background robustly suppressed the overgrown eye phenotype (Figure 2J compared with 2I, quantified in 2M), whereas reducing PTP61F levels by crossing flies onto the PTP61FΔ heterozygous background, or by RNAi-mediated knockdown, not only enhanced the *ey>PVR*$^{\lambda}$ overgrown eye phenotype (Figure 2K, 2L compared with 2I, quantified in 2M), but also resulted in overt morphological defects in every instance (arrowheads). These results are consistent with PTP61F being a negative regulator of PVR signaling.

PTP61F negatively regulates EGFR-MAPK signaling

Having established that PTP61F negatively regulates EGFR and PVR signaling, we next explored the pathways by which PTP61F may mediate its effects on receptor PTK signaling. Recent studies have shown that PTP61F negatively regulates mitogen-activated protein kinase (MAPK) signaling in flies [46]. In *Drosophila* the MAPK extracellular signal-regulated kinase (ERK) is a well-established downstream effector of EGFR signaling, promoting cellular proliferation, growth and survival [47]. To determine whether the PTP61F-mediated effects on the $EGFR^{ElpB1}$ rough eye phenotype were accompanied by alterations in Ras-MAPK signaling, we monitored for ERK phosphorylation (p-ERK) in third instar larval eye-antennal epithelial tissue by immunoblot analysis (Figure 3A). Reducing the dosage of PTP61F by using the heterozygous *PTP61FΔ* mutant null allele (*PTP61FΔ*/+) increased p-ERK in wild type flies and exacerbated the enhanced p-ERK associated with $EGFR^{ElpB1}$ expression (Figure 3A) in keeping with the effects on eye overgrowth. Thus, PTP61F negatively regulates EGFR signaling and the eye overgrowth at least in part by repressing EGFR-Ras-MAPK signaling.

PTP61F negatively regulates PVR-MAPK signaling

It is well established that PVR signals via the MAPKs ERK and c-Jun N-terminal kinase (JNK) [41, 45, 48]. We therefore examined the influence of PTP61F on PVR-induced ERK and JNK signaling. First, we used the *ey-FLP-out* system to express activated PVR^{λ} or PVR^{λ} plus $PTP61F^{RNAi}$ in the whole eye-antennal epithelial tissue and assessed p-ERK status by immunoblotting. As with EGFR$^{\mathrm{ElpB1}}$ signaling, *PTP61F* knockdown resulted in increased ERK phosphorylation and activation (Figure 3B). Next we examined whether PTP61F regulates PVR-induced Rac-JNK signaling. We used the *equatorial-GAL4* (*Eq1-GAL4,* termed *eq>* henceforth) driver, which is expressed in a patch of cells in the centre of the developing eye epithelia [49], to drive expression of PVR^{λ} , $PTP61F^{RNAi}$, or PVR^{λ} plus $PTP61F^{RNAi}$ and stained for the JNK reporter, metalloproteinase 1 (MMP1) [50] (Figure 4). In the Eq patch (marked by green fluorescent protein, GFP), MMP1 was not upregulated by *PTP61F* knockdown relative to the control (Figure 4B compared with 3A). PVR^{λ} expression resulted in the upregulation of MMP1 (Figure 4C), which was enhanced by knocking down *PTP61F* (Figure 4D compared with 4C). Interestingly, PTP61F knockdown alone or with PVR^{λ} also resulted in some non-cell autonomous upregulation of MMP1 adjacent to the Eq patch (Figures 4B, D). This is consistent with a ‘super-competitor’ phenotype being conferred by PTP61F deficiency leading to increased JNK signalling and cell death in surrounding wild-type cells [51]. The growth of the Eq patch in the eye epithelial tissue was also enhanced by PVR^{λ} expression, although *PTP61F* knockdown did not appear to noticeably enhance this overgrowth (Figure 4B compared with 4A, and see Figure 6E for quantification of Eq domain growth). However, in the third instar larval wing epithelial tissue, where the *eq*> driver is also expressed, PTP61F-deficiency resulted in robust enhancement of PVR^{λ}-induced tissue overgrowth with overt perturbations in cellular morphology (Figure 5B compared to 5A). Taken together these results are consistent with PTP61F negatively regulating PVR-driven tissue growth and inhibiting MAPK signaling.

PTP61F negatively regulates PVR- but not EGFR-induced STAT signaling

The morphological alterations associated with PVR overexpression and PTP61F-depletion are reminiscent of defects in tissue architecture associated with STAT92E hyperactivation during *Drosophila* eye development [52, 53]. Given that STAT92E can serve as PTP61F substrate [34-37], we next examined whether PTP61F deficiency might enhance PVR-induced STAT92E signaling using a *10XStat-GFP* (Stat-GFP) reporter for STAT92E transcriptional activity [54]. We used the *eq*> driver (marked by *UAS-RFP* expression) to express PVR^{λ}, $PTP61F^{RNAi}$, or PVR^{λ} plus $PTP61F^{RNAi}$ in the *Drosophila* developing eye and monitored GFP fluorescence as a read-out of STAT92E activity (Figure 6). PTP61F knockdown alone had no overt effect on Stat-GFP reporter expression (Figure 6A versus 6B, quantified in 6F). PVR^{λ} expression was associated with an expansion of the Eq domain (Figure 6C, quantified in 6E) and modest increases in STAT92E activity as assessed by GFP expression (Figure 6C, A, F). Strikingly, *PTP61F* knockdown in PVR^{λ}-expressing tissue resulted in robust increases in Stat-GFP reporter expression (Figure 6D, quantified in 6F). By contrast, PTP61F-deficiency did not alter STAT92E activity in the context of $EGFR^{\lambda top}$ expression (data not shown), consistent with the effects being specific to the PVR receptor PTK. Since STAT92E hyperactivation elicits both cell autonomous and non-cell autonomous effects in the developing *Drosophila* eye, driving tissue overgrowth [55, 56], ectopic organiser formation [57] and perturbing specification and omnatidial polarity [53, 58], it is likely that the heightened JAK/STAT signaling contributes to the tissue overgrowth and the morphological defects associated with PTP61F-deficiency and activated PVR.

PTP61Fm and PTP61Fn differentially regulate Hopscotch and STAT92E

The PTP61F orthologues, PTP1B and TCPTP, dephosphorylate JAK family PTKs and STATs to coordinately regulate JAK/STAT signaling [1]. Both PTP1B and TCPTP dephosphorylate and inactivate JAK PTKs [9-11], whereas TCPTP dephosphorylates STAT family members, such as STAT-3, in the nucleus [13, 28]. Given the influence of PTP61F-deficiency on PVR-induced STAT92E signaling, we assessed the influence of the endoplasmic reticulum (ER)- versus nuclear-targeted variants of PTP61F on Hopscotch and STAT92E signaling respectively. To this end, we co-expressed in *Drosophila* Schneider II (S2) cells the Myc-tagged constitutively active mutant version of Hopscotch, *Hop*$^{Tum\text{-}1}$ (Myc-Hop$^{Tum\text{-}1}$), with either the wild-type or the C237S 'substrate-trapping' mutant versions of PTP61Fm or PTP61Fn that can form stable complexes with substrates to prevent dephosphorylation by endogenous PTPs [1]. We assessed Hop$^{Tum\text{-}1}$ tyrosine phosphorylation in Myc immunoprecipitates and found that expression of HA-tagged PTP61Fm but not PTP61Fn tended to reduce Myc-Hop$^{Tum\text{-}1}$ tyrosine phosphorylation (Figure 7A). Moreover, the PTP61Fm-C237S 'substrate-trapping' mutant but not the PTP61Fn-C237S mutant enhanced Hop$^{Tum\text{-}1}$ tyrosine phosphorylation (Figure 7A), in keeping with Hop$^{Tum\text{-}1}$ serving as a substrate of PTP61Fm, but not PTP61Fn. The ability of the PTP61Fm 'substrate-trapping' mutant to form an enzyme-substrate complex with tyrosine phosphorylated Hop$^{Tum\text{-}1}$ was reaffirmed using another PTP 'substrate-trapping' mutant, PTP61Fm-D203A (Figure 7B). Co-expression of HA-tagged PTP61Fm-D203A with Myc-tagged wild type Hopscotch (Myc-Hop) or Myc-Hop$^{Tum\text{-}1}$ increased Hop and Hop$^{Tum\text{-}1}$ tyrosine phosphorylation (Figure 7B). In addition, Hop and even greater amounts of activated Hop$^{Tum\text{-}1}$ co-immunoprecipiated with the HA-PTP61Fm-D203A 'substrate-trapping' mutant, but not wild type HA-PTP61Fm, consistent with the formation of an active site-dependent PTP-substrate complex (Figure 7B). To determine whether the nuclear-targeted PTP61Fn attenuates Hop$^{Tum\text{-}1}$/STAT92E signaling by dephosphorylating STAT92E, we co-expressed wild-type PTP61Fn or PTP61Fn-C237S with Myc-Hop$^{Tum\text{-}1}$ and assessed STAT92E Y711-phosphorylation (p-STAT92E) using rabbit antibodies raised to the peptide ^{704}VLDPVTG-pY-VKST^{715} from Y711 phosphorylated STAT92E (Figure 8). We found that wild-type PTP61Fn repressed p-STAT92E whereas the PTP61Fn-C237S 'substrate-trapping' significantly enhanced p-STAT92E (Figure 7C). Since PTP61Fn or PTP61Fn-C237S did not overtly influence Hop$^{Tum\text{-}1}$ phosphorylation (Figure

7A), these results are consistent with the formation of a stable PTP active site-dependent complex between p-STAT92E and PTP61Fn-C237S. Finally we found that the specific knockdown of PTP61Fn in S2 cells enhanced STAT92E tyrosine phosphorylation induced by the secreted ligand Unpaired (Upd), which acts via the Domeless receptor to signal to Hopscotch and STAT92E (Figure 7D). Taken together, these results are consistent with both PTP61Fm and PTP61Fn contributing to the suppression of JAK/STAT signaling through the dephosphorylation of Hopscotch and STAT92E respectively.

Eyeless-mediated PTP61Fm expression represses EGFR and PVR but not InR signaling.

Given the differential regulation of JAK/STAT signaling by PTP61Fm and PTP61Fn, we also examined the influence of PTP61Fm on the EGFR- versus PVR-induced *Drosophila* eye overgrowth. Previously we compared the effects of PTP61Fm versus PTP61Fn on InR-mediated eye overgrowth using the *GMR-GAL4* driver, which is expressed in the posterior differentiating cells of the developing eye during third instar larval development [32, 34]. *GMR-InR* results in a deformed rough eye phenotype due to defects in cell cycle exit and patterning; overexpression of *PTP61Fn* corrected this phenotype, whereas *PTP61Fm* overexpression only moderately improved the phenotype despite being expressed at higher levels than *PTP61Fn* [32, 34]. Here, to assess the effects of PTP61Fm versus PTP61Fn on RTK signaling, we used the *ey>* driver, which is a weaker driver expressed throughout the developing eye epithelium from embryonic development. *ey>InR* results in hyperplasia without overt effects on differentiation (Figure 9B compared with the wild type control, 9A). We found that *PTP61Fn* robustly suppressed the *ey>InR* overgrown eye phenotype (Figure 9C, quantified in Figure 9G) but *PTP61Fm* did not (Figure 9D, quantified in 9G), consistent with the differential effects on InR signaling by the PTP61F variants observed previously [32, 34]. By contrast, we found that *PTP61Fm* was just as effective as *PTP61Fn* in repressing the eye overgrowth associated with *EGFR*$^{\lambda top}$ or *PVR*$^{\lambda}$ overexpression (Figures 2F compared with 10C, and 2J compared with 10G). Therefore, PTP61Fm and PTP61Fn differentially contribute to the regulation of receptor PTKs.

Dock is required for the PTP61Fm repression of InR but not EGFR or PVR signaling.

We have shown previously that PTP61Fm requires the adaptor protein Dock to effectively repress InR signaling [32]. Dock, or it mammalian counterpart Nck, form stable complexes with PTP61F/PTP1B and the InR to facilitate receptor dephosphorylation and inactivation. We have shown that Dock co-expression, using the *GMR-GAL4* driver, enhances the *PTP61Fm*-mediated repression of the *GMR>InR* phenotype [32]. Accordingly, we next determined whether the effects of *PTP61Fm* on the *ey>InR* versus *ey>EGFR*$^{\lambda top}$ and *ey>PVR*$^{\lambda}$ phenotypes may be ascribed to a differential reliance on Dock. Although *ey>PTP61Fm* or *ey>dock* had no effect on the *ey>InR* phenotype (Figure 9D and 9E, compared with 9B, quantified in 9G), the co-expression of *dock* together with *PTP61Fm* repressed the eye overgrowth as effectively as *PTP61Fn* (Figure 9F, compared with Figure 9C, quantified in 9G). By contrast, the overgrown eye phenotype of *ey>EGFR*$^{\lambda top}$ was attenuated by *PTP61Fm* in the absence of *dock* overexpression (Figure 10C compared with 10A, B, quantified in 10J). *Dock* overexpression alone had no effect and did not enhance the effects of *PTP61Fm* on the *ey>EGFR*$^{\lambda top}$ overgrown eye phenotype (Figure 10D, E compared with 10A-C, quantified in 10J). Similarly, *dock* overexpression did not enhance the ability of *PTP61Fm* to suppress the overgrown eye phenotype of *ey>PVR*$^{\lambda}$ flies (Figure 10G compared with 10A, F, quantified in 10J). Surprisingly, *dock* expression alone enhanced the *ey>PVR*$^{\lambda}$ overgrowth phenotype (Figure 10H compared with 10A, F, quantified in 10J) and whilst the co-expression with *PTP61Fm* seemingly partially repressed the overgrown *ey>PVR*$^{\lambda}$ *+ dock* phenotype in side-views (Figure 10I compared with 10A, F, G, quantified in J), in top-views the eye tissue was bulgy, folded and overgrown relative to *ey>PVR*$^{\lambda}$, *ey>PVR*$^{\lambda}$ + dock and *ey>PVR*$^{\lambda}$ + *PTP61Fm* Figure 10N compared with 10K-M). Thus, rather than suppressing the *ey>PVR*$^{\lambda}$ phenotype, the co-expression of *dock* with *PTP61Fm* exacerbated the *ey>PVR*$^{\lambda}$ phenotype. Nonetheless, taken together these results point towards Dock selectively influencing PTP61Fm function by promoting the inhibition of InR, but not EGFR or PVR signaling.

DISCUSSION

The role of adaptor proteins in influencing the spatial and temporal control of signaling is widely appreciated [59] but their influence on PTP function is not well understood. Although PTPs exhibit inherent catalytic domain substrate specificity [1], as highlighted for example by catalytic domain swapping experiments between the highly conserved tyrosine phosphatases PTP1B and TCPTP [21], or SHP-1 and SHP-2 [60], different adaptors have been shown to enhance the PTP-mediated dephosphorylation of PTKs and their target substrates [25, 29-32]. It remains unclear if any given adaptor can confer specificity for select substrates and biological pathways. Here we have used *Drosophila* as a model organism to demonstrate that the adaptor protein Dock is required for the PTP61Fm-mediated repression of InR, but importantly, not EGFR- or PVR-induced *Drosophila* eye overgrowth (Figure 11). These results highlight the capacity of adaptor proteins to selectively influence PTK-mediated biological processes through the targeted recruitment of PTPs.

In contrast to the requirement for Dock in the PTP61Fm-mediated repression of InR signaling, the nuclear-targeted PTP61Fn repressed the InR-induced eye overgrowth in the absence of Dock. Similarly, PTP61Fn also effectively suppressed EGFR- and PVR-induced hypertrophy in the absence of Dock. Genetic analyses of *Drosophila InR*, *EGFR* and *PVR* have contributed significantly to our understanding of the corresponding receptor PTK pathways in mammals and have highlighted the high degree of pathway conservation between mammals and flies [61]. We have established previously that the mammalian PTP61Fn orthologue TC45 shuttles in and out of the nucleus and is targeted to the cell periphery where it desphosphorylates the EGFR family member ErbB1 to repress phosphatidylinositol 3-kinase (PI3K)-mediated AKT and JNK signaling [21, 22, 62]. Similarly, TC45 exhibits specificity for PDGFR tyrosine phosphorylation sites to selectively regulate phospholipase Cγ1 signaling [44]. *PTP61Fn* overexpression in the *Drosophila* eye represses InR Y1553/Y1554 phosphorylation and the *InR*-induced hypertrophy, but has no effect on overgrowth induced by *PI3K* overexpression [32], in keeping with PTP61Fn acting upstream of PI3K to dephosphorylate the InR. In addition, recent studies have shown that PVR can serve as a direct substrate for PTP61F [63]. Although in this study we have not specifically examined EGFR or PVR tyrosine phosphorylation after *PTP61Fn* overexpression or deletion, we propose that PTP61Fn represses the eye overgrowth through the dephosphorylation and inactivation of the respective receptor PTKs at the plasma membrane and/or cytoplasm. In keeping with this, we found that downstream MAPK signaling was attenuated by the *PTP61Fn* variant. However, we cannot exclude the possibility that PTP61Fn may in part elicit its effects by acting on downstream of receptor PTKs, or indeed through direct effects on the Ras/MAPK pathway, since recent studies suggest that PTP61F may act on MAPK itself [46]. Downstream PTKs that may be dephosphorylated by PTP61F include the Abl PTK, with previous studies suggesting that Abl may mediate EGFR signaling in flies, and that Abl can serve as a direct substrate of PTP61F to regulate actin organisation [46, 64]. Yet another potential downstream tyrosine phosphorylation-dependent pathway by which PTP61Fn may mediate its effects on receptor PTK signaling is the JAK/STAT pathway. Indeed, we found that JAK/STAT signaling, as assessed by monitoring STAT92E transcriptional activity, was elevated in the absence of PTP61F, in keeping with previous studies showing that *PTP61F* deficiency is associated with enhanced STAT92E Y711 phosphorylation [34-37]. However, this only occurred when PVR^{λ} was overexpressed. *PTP61F* deficiency did not enhance $EGFR^{\lambda top}$-induced STAT92E transcriptional activity, pointing towards context-dependent regulation of the JAK/STAT pathway. Importantly, we also found that the PTP61F variants differentially contributed to the regulation of JAK/STAT pathway, at least in S2 cells, with PTP61Fm acting on Hopscotch and PTP61Fn acting on STAT92E. These results suggest that the ER localisation of PTP61Fm may be essential for the dephosphorylation of Hopscotch, whereas PTP61Fn may dephosphorylate STAT92E in the nucleus, as noted previously for TC45 and STAT3 [13]. Together, these results provide evidence for PTPs acting in a context-dependent-manner to regulate PTK signaling and reinforce the importance of localisation in dictating substrate selectivity.

Despite PTP61Fm being reliant on Dock for the repression of InR-induced eye overgrowth, PTP61Fm was capable of effectively repressing the EGFR or PVR-induced eye overgrowth without Dock. We have argued previously that the differential effects of PTP61Fm versus PTP61Fn on InR signaling may be ascribed to their differential localisation with PTP61Fn having unfettered access by virtue of its ability to shuttle between the nucleus and cytoplasm, and PTP61Fm being restricted because of its ER localisation [32]. We were able to demonstrate that PTP61Fm, Dock and InR formed a ligand-dependent complex and that the PTP61F-mediated interaction with Dock was required for InR dephosphorylation *in vitro* and *in vivo* [32]. Accordingly, we reasoned that Dock was required to recruit the ER-restricted PTP61Fm to the InR at the plasma membrane. Dock interacts with PTP61F via the Dock SH3 domains and the PTP61F non-catalytic C-terminus that contains five proline-rich SH3-interaction (PxxP) motifs [65, 66]. Dock forms a complex with the InR and PTP61Fm and facilitates the PTP61Fm-mediated dephosphorylation of the InR [32]. InR contains a 400 amino acid C-terminal extension that is not found in mammalian IR and contains PxxP motifs that can interact with the Dock SH3 domain in a yeast 2-hybrid screen [66-68]. Other studies point towards InR tyrosine phosphorylation and the Dock SH2 phosphotyrosine-binding domain being important for the interaction, whereas genetic studies point towards a redundancy between the Dock SH2 and SH3 domains during the process of InR-mediated R-cell axon guidance [32, 34, 66, 69]. Therefore, Dock's specific contributions to InR regulation by PTP61Fm may be attributed to sequence-specific interactions between the Dock SH2/SH3 domains and the InR. However, this does not explain how PTP61Fm can function independently of Dock in the regulation of EGFR and PVR signaling. One possible reason may be that PTP61Fm also acts downstream of the EGFR and PVR receptor PTKs. However, another possibility is that the differential requirement for Dock may relate to differences in receptor PTK trafficking. Although this has not been studied extensively in flies, in mammalian cells the InR undergoes rapid recycling to the plasma membrane [70], whereas EGF-bound EGFR (ErbB1) undergoes endosomal sorting and lysosomal degradation [71, 72]. Similarly, the PDGFR is reported not to recycle [73]. Interestingly, TCPTP deficiency induces recycling of PDGFR β homodimers and αβ heterodimers [74]. Therefore, one possibility is that PTP61Fm may have access to the EGFR and PVR receptor PTKs after endocytosis, where Dock or other adaptor proteins may not be necessary for their dephosphorylation.

Although PTP61F expression repressed the PVR-induced eye hypertrophy, the expression of Dock with PVR exacerbated tissue growth. Paradoxically, the co-expression of PTP61F with PVR and Dock further exacerbated the Dock-induced overgrowth and resulted in morphological defects. Precisely how PTP61F might cooperate with Dock to worsen the Dock-mediated PVR-dependent overgrowth remains unclear. It is possible that the PTP61F-mediated repression of PVR-, but promotion of the Dock-induced overgrowth may be elicited via independent pathways. Dock and PTP61F may have opposing roles in actin polymerisation [63, 64, 75];[76]. Perhaps in the activated PVR background the co-expression of PTP61Fm with Dock, which itself is tyrosine phosphorylated by Src42A and dephosphorylated by PTP61F [77], perturbs actin polymerisation to influence tissue growth [78].

In summary, our studies provide insight into potential approaches for selectively manipulating receptor PTK signaling in biology and disease. PTP1B negatively regulates InR signaling in muscle and liver and PTP1B knockout mice have improved glucose metabolism [79-83]. Moreover, increased PTP1B expression and/or activity may contribute to the development of muscle insulin resistance in rodents and humans [84-89]. The inhibition of PTP1B may provide an important means by which to selectively promote insulin sensitivity and alleviate insulin resistance in type 2 diabetes. We have shown previously that the mammalian Dock homolog Nck similarly facilitates InR dephosphorylation [32]. Moreover Nck deletion in high fat fed obese mice improves glucose tolerance and insulin sensitivity [90], whereas Nck-deficiency in hepatocytes promotes insulin-induced PI3K/AKT signaling [91]. Our studies suggest that the specific targeting of PTP61F/PTP1B interactions with Dock/Nck might allow for the selective enhancement of InR signaling without affecting other PTKs and provide an alternative means by which to enhance insulin sensitivity in type 2 diabetes.

MATERIALS AND METHODS

Fly stocks

Fly stocks used in this study were: *UAS-PTP61F*^{n}, *UAS-PTP61F*^{m}, *PTP61FΔ* [32, 34], *UAS-PTP61F*^{RNAi} (2nd chromosome, v37436, Vienna *Drosophila* Resource Centre), *UAS-Dock* [92], *EGFR*^{ElpB1} (*EGFR*^{E3}) [39], *UAS-EGFR*$^{\lambda top}$ [40], *UAS-PVR*$^{\lambda}$ [41], *UAS-InR* [93], *Eq1-GAL4* [49], *Stat-10xGFP* ([54], and *ey-GAL4, UAS-GFP, UAS-RFP* (Bloomington stock centre). The *ey-FLP-out* stock used was, *ey-FLP;; act>CD2>GAL4,UAS-GFP* generated from component stocks from the Bloomington stock centre. All flies were raised on a standard semolina agar food at 25°C, or 29^{o}C when using the PTP61F-RNAi line. At least 50 progeny of the relevant genotype were analysed per cross and images were taken from at least 5 representative fly eyes per genotype for analysis.

Immunofluorescence microscopy

Third-instar larval eye-antennal or wing discs, were dissected in phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde for 30 min, washed in PBS + 0.1 or 0.3% Triton X-100 (PBT), and blocked in PBT + 1% BSA. To stain for MMP1, the mouse monoclonal anti-MMP1 antibody was used at 1:20 (Developmental Studies Hybridoma Bank, 5H7B11, 3B8D12 & 3A6B4). The secondary antibody, anti-mouse Alexa 568 was used. DNA was stained with 2-(4-amidinophenyl)-1H-indole-6-carboxamidine (DAPI, 1µM). F-actin was detected using phalloidin-tetramethylrhodamine isothiocyanate-Rhodamine (Sigma, 0.3mM). Labelled samples were mounted in 80% glycerol and analysed by confocal microscopy (LEICA TCS SP5, Zeiss Confocal LSM 780 PicoQuant FLIM).

Imaging adult fly eyes

Adult male eyes were imaged using a light dissection microscope or a Scitec Infinity1 camera, or a fluorescence dissection microscope and a CCD black-white camera. Male flies were collected after anaesthetising with CO_2 and stored at -20^{o}C until ready for imaging. Flies were mounted on a device that enabled consistent positioning of each fly eye at the same distance from the microscope. Images from at least 5 adult male eyes were taken per genotype using identical microscopy settings.

Cell culture and transfections

Drosophila S2 cells were maintained in 1X Schneider medium (Invitrogen) plus 10% (v/v) FBS at 24°C. Transient transfections were performed with Lipofectamine^{2000} (Invitrogen) as described previously [32, 34]. For RNA interference (RNAi)-mediated knockdown in S2 cells, double stranded RNAs (dsRNAs) corresponding to the PTP61Fn gene were added to S2 cells cultured in serum free medium and processed as described previously [32, 34].

Generation of p-STAT92E antibodies

The synthetic peptide $C^{703}VLDPVTGpYVKST^{715}–NH_2$ corresponding to Y711 phosphorylated STAT92E from *Drosophila melanogaster* was conjugated to keyhole limpet hemocyanin (KLH) and New Zealand White rabbits injected with 200 μg conjugate in 50% (v/v) Freund's incomplete adjuvant bi-weekly. Serum was collected ten days after the sixth boost and frozen at -80°C.

Immunoblotting

Drosophila eye-antennal discs (~20) were homogenized in 100 mM M Tris-HCl pH 6.8, 2% (w/v) SDS, 5 mM EDTA, 5 mM DTT, complete protease inhibitor cocktail (Roche), 1 mM $Na_3(VO)_4$ and 5 mM NaF and processed for immunoblotting with α-phospho-ERK (Sigma), α-ERK (Cell Signaling) and α-tubulin (Sigma).

S2 cells were processed for immunoprecipitation with anti-Myc (9E10; Abcam) and/or immunoblotting as described previously [32, 34] with antibodies to phosphotyrosine (4G10; Merck Millipore), Myc (9E10), HA (12CA5; Abcam), Y704 phosphorylated STAT92E and STAT92E (Santa Cruz).

Statistical analysis

Adult eye areas were measured (from n>5 flies per genotype) using the magnetic lasso tool to highlight the eye perimeter and the Measurement plugin of Adobe Photoshop CS5.1 or CC Premium. The data was plotted using Microsoft Excel or Graphpad Prism, and the data analysed using the Student's t-test with significance set at $p<0.05$.

Relative *Stat-GFP* staining within eye epithelium was determined from images taken at the same confocal settings. Average pixel intensity of Stat-GFP was measured using the measurement log tool from Photoshop 5.1 in the Eq domain (marked by RFP) in the eye epithelium. Average pixel intensity was measured in the Eq domain relative to the adjacent wild-type tissue of the same area (n~10 for each sample) and data was normalized to the *eq>RFP* control. Data was compared and plotted using Microsoft Excel and the data analysed using the Student's t-test with significance set at $p<0.05$.

Relative Eq domain area (marked by RFP) was measured in each sample relative to the total eye disc area using the measurement log tool from Photoshop 5.1. Data was compared and plotted as a percentage of total eye size for each genotype using Microsoft Excel and the data analysed using the Student's t-test with significance was set at $p<0.05$.

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AUTHOR’S CONTRIBUTIONS

Planned experiments: LFW, JM, KA, HL, T-C M, TT, HER
Performed experiments: LFW, JM, KA, HL
Analysed data: LFW, JM, KA, HL, MP, FW, T-C M, TT, HER
Contributed reagents/materials: CW
Wrote the paper: TT, HER
Other – Prepared Figures: LFW, JM, KA, HL, MP, FW, T-C M, TT, HER

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FIGURES LEGENDS

Figure 1. PTP61F modulates EGFR signaling. (A-F) Representative male adult male eye images showing genetic interactions of Ptp61F with activated EGFR and controls. Male fly eyes from the following genotypes are shown: (A) *ey-GAL4 UAS-GFP (wild-type),* (B) *ey-GAL4 UAS-PTP61F*^{n} (PTP61Fn expression alone) (C) *ey-GAL4 PTP61FΔ/+* (PTP61F heterozygosity alone), (D) *EGFR*^{ElpB1} *ey-GAL4,* (E) *EGFR*^{ElpB1} *ey-GAL4 + UAS-PTP61F*^{n}, (F) *EGFR*^{ElpB1} *ey-GAL4 PTP61FΔ/+*. (A-C) were imaged using a dissecting light microscope with a Scitec Infinity1 camera and (D-F) were imaged using a fluorescent microscope and a CCD black-white camera. *EGFR*^{ElpB1} results in a small rough eye, which is suppressed by expression of *PTP61F*^{n} and enhanced by heterozygosity for *PTP61FΔ*. (G) Quantification (means ± SEM) of eye sizes from the indicated genotypes; **** p<0.00001. (H-M) Representative female fly eye images showing genetic interactions of Ptp61F with activated EGFR and controls, imaged using a Scitec Infinity1 camera. Female fly eyes from the following genotypes are shown: (H) *ey-GAL4 UAS-GFP (wild-type),* (I) *ey-GAL4 UAS-PTP61F*^{n} (PTP61Fn expression alone) (J) *ey-GAL4 UAS-PTP61F*^{RNAi} (PTP61F knockdown alone), (K) *EGFR*^{ElpB1} *ey-GAL4,* (L) *EGFR*^{ElpB1} *ey-GAL4 + UAS-PTP61F*^{n}, (M) *EGFR*^{ElpB1} *ey-GAL4 UAS-PTP61F*^{RNAi}. *EGFR*^{ElpB1} small rough eyes are suppressed by *PTP61Fn* expression and enhanced by RNAi-mediated knockdown of PTP61F. (N) Quantification (means ± SEM) of eye sizes from the indicated genotypes; **** p<0.00001.

Figure 2. PTP61F modulates EGFR and PVR eye overgrowth phenotypes. (A-L) Representative adult male eye images showing genetic interaction of PTP61F with activated EGFR and PVR, and controls. Male fly eyes from the following genotypes are shown: (A) *ey-GAL4 UAS-GFP*, (B) *ey-GAL4 UAS-PTP61F*^{n}, (C) *ey-GAL4 PTP61FΔ/+*, (D) *ey-GAL4 UAS-PTP61F-RNAi*, (E) *ey-GAL4 UAS-EGFR*$^{\lambda top}$, (F) *ey-GAL4 UAS-EGFR*$^{\lambda top}$ + *UAS-PTP61F*^{n}, (G) *ey-GAL4 UAS-EGFR*$^{\lambda top}$ + *PTP61FΔ/+*, (H) *ey-GAL4 UAS-EGFR*$^{\lambda top}$ + *UAS-PTP61F*^{RNAi}, (I) *ey-GAL4 UAS-PVR*$^{\lambda}$, (J) *ey-GAL4 UAS- PVR*$^{\lambda}$ + *UAS-PTP61F*^{n}, (K) *ey-GAL4 UAS-PVR*$^{\lambda}$+ *PTP61FΔ/+*, (L) *ey-GAL4 UAS-PVR*$^{\lambda}$ + *UAS-PTP61F*^{RNAi}. Expression of *EGFR*$^{\lambda top}$ (E) or *PVR*$^{\lambda}$ (I) in the developing eye epithelium results in overgrown adult eye phenotypes, which are suppressed by *PTP61F*^{n} (F, J) and enhanced by knockdown of PTP61F

(G, H, K, L). (K, L) Arrowheads point to morphological defects observed in the eyes. (M) Quantification (mean ± SEM) of eye sizes from the indicated genotypes; **** $p<0.0001$.

Figure 3. PTP61F knockdown enhances MAPK signaling by activated EGFR or PVR

(A) Immunoblot of p-ERK, ERK and tubulin from wild-type, *PTP61F*Δ*/+*, *EGFR*^{ElpB1} and *EGFR*^{ElpB1} + *PTP61F*Δ*/+* eye-antennal discs. p-ERK was increased by PTP61F heterozygosity in the *wild-type* and *EGFR*^{ElpB1} background. (B) Western analysis of p-ERK and tubulin from wild type and *ey-FLP-out* (EAG) *PVR*$^{\lambda}$ and *PVR*$^{\lambda}$ + *PTP61F*^{RNAi} showing that p-ERK is increased upon knockdown of PTP61F.

Figure 4. PTP61F-deficiency promotes PVR-induced JNK signaling. A-D) Confocal images of eye-antennal discs stained for MMP1 (grey scale and red in merges). GFP marks the Eq expression domain and the tissue is counterstained with DAPI. Genotypes: (A) *eq-GAL4 + UAS-GFP*, (B) *eq-GAL4 + UAS-PTP61F*^{RNAi}, (C) *eq-GAL4 UAS-PVR*$^{\lambda}$, (D) *eq-GAL4 UAS-PVR*$^{\lambda}$ + *UAS-PTP61F*^{RNAi}. The Eq expression domain is outlined by dotted lines and indicated by the arrows. MMP1 is upregulated slightly in *PVR*$^{\lambda}$ expressing tissue and more robustly increased upon PTP61F knockdown. PTP61F knockdown alone resulted in some increased MMP1 expression adjacent to the Eq expression domain, consistent with PTP61F depletion resulting in super-competitor cell behaviour.

Figure 5. Knockdown of PTP61F enhances the overgrowth of PVR$^{\lambda}$-expressing wing disc tissue. (A, B) Confocal images of wing discs showing expressing PVR$^{\lambda}$ or *PVR*$^{\lambda}$ *PTP61F*^{RNAi} in the Eq domain. GFP marks the Eq domain in the wing disc hinge region. The discs are counterstained for F-actin and with DAPI. *PVR*$^{\lambda}$ expression results in tissue overgrowth (A), which is robustly enhanced by *PTP61F* knockdown (B). F-actin staining shows that *PTP61F* knockdown in the *PVR*$^{\lambda}$ background alters cell morphology and increases F-actin accumulation.

Figure 6. PTP61F-deficiency promotes PVR-induced STAT signaling. (A-D) Confocal images of eye-antennal discs showing Stat-reporter-GFP expression. RFP marks the Eq

expression domain and the tissue is counterstained with DAPI. Genotypes: (A) *eq-GAL4 UAS-RFP Stat-GFP,* (B) *eq-GAL4 UAS-RFP Stat-GFP + UAS-PTP61F*^{RNAi}, (C) *eq-GAL4 UAS-RFP Stat-GFP + UAS-PVR*$^{\lambda}$, (D) *eq-GAL4 + UAS-RFP + Stat92E–GFP + UAS-PVR*$^{\lambda}$ *+ UAS-PTP61F*^{RNAi}. The Eq expression domain is outlined with dotted lines and indicated with arrows. (E) Quantification (mean ± SEM) of Eq domain area relative to the total eye epithelial area for the genotypes indicted. The Eq domain area was increased for *eq>PVR*$^{\lambda}$ and *eq>PVR*$^{\lambda}$ *PTP61F*^{RNAi} relative to the control (** $p<0.01$). There was no significant change in the area between *eq>PVR*$^{\lambda}$ and *eq>PVR*$^{\lambda}$ *PTP61F*^{RNAi}. (F) Quantification of Stat-GFP pixel intensity in the Eq domain relative to the surrounding wild-type tissue of the same area, and normalised to the *eq>RFP* control for the genotypes indicated. Stat-GFP was increased per unit area in *eq>PVR*$^{\lambda}$ *PTP61F*^{RNAi} relative to the control (* $p<0.05$). There was no significant change in *Stat-GFP* levels between *eq>RFP* and *eq>PTP61F*^{RNAi} or *eq>PVR*$^{\lambda}$.

Figure 7. Differential regulation of JAK-STAT signaling by PTP61Fm and PTP61Fn. (A-B) Wild-type (WT) or the C237S (C/S) or D203A (D/A) 'substrate-trapping' mutant forms of HA-tagged PTP61Fm or PTP61Fn were co-expressed with the Myc-tagged Hop or Hop^{Tum1} in S2 cells as indicated. (A-B) Immunoprecipitated Myc-Hop, Myc-Hop^{Tum1} or HA-tagged PTP61F (B) and aliquots of S2 cell lysates were analysed by immunoblotting with the indicated antibodies. (C) WT or the C237S 'substrate-trapping' mutant of HA-PTP61Fn were co-expressed with the Myc-Hop^{Tum1} in S2 cells as indicated and STAT92E Y711 phosphorylation (p-STAT92E) assessed. (D) Upd-expressing S2 cells were added to a five-fold excess of S2 cells treated with increasing doses of dsRNA targeting specifically endogenous *PTP61Fn*. After 30 min of incubation, cells were harvested and processed for immunoblotting with the indicated antibodies. The dsRNA-mediated knockdown of PTP61Fn mRNA was assessed by RT-PCR. Representative and quantified (means ± SEM) results from three independent experiments are shown in A, C and D; * $p<0.05$; ** $p<0.01$. Results from two independent experiments are shown in B.

Figure 8. Specificity of p-STAT92E antibodies. S2 cells were left untreated or treated with *Stat92E* dsRNA to knockdown STAT92E expression as described previously [32]. A) STAT92E knockdown was verified by RT-PCR. B) Control or *Stat92E* dsRNA-treated S2 cells were co-cultured with control or Upd-expressing S2 cells for 12 h to stimulate

Hop/STAT92E signaling and processed for immunoblotting with polyclonal rabbit antibodies to Y711 phosphorylated STAT92E (p-STAT92E) followed by the indicated loading controls. STAT92E knockdown decreased STAT92E Y711 phosphorylation validating the specificity of the p-STAT92E antibodies.

Figure 9. InR overgrowth phenotype is suppressed by co-expression of PTP61Fm and Dock. (A-F) Representative adult male eye images showing genetic interactions of Ptp61F with the InR and Dock, when overexpressed using the *ey-GAL4* driver. Male fly eyes of the following genotypes are shown: (A) *ey-GAL4 UAS-GFP* (control), (B) *ey-GAL4 UAS-InR,* (C) *ey-GAL4 UAS-InR + UAS-PTP61F*^{n}, (D) *ey-GAL4 UAS-InR + UAS-PTP61F*^{m}, (E) *ey-GAL4 UAS-InR + UAS-Dock*, (F) *ey-GAL4 UAS-InR + UAS-PTP61F*^{m} *+ UAS-Dock.* InR expression results in an overgrown eye phenotype (B), which is suppressed by expression of PTP61F^{n} (C), but not by PTP61F^{m} (D). Coexpression of Dock with PTP61F^{m} results in suppression of the InR overgrown eye phenotype (F). (G) Quantification (mean ± SEM) of eye sizes of the indicated genotypes. The data was compared using t-test analysis. The significance for *ey>GFP* versus *ey>InR* and *ey>InR* versus *ey>InR PTP61F*^{n} were both $p<0.0001$ (****), and *ey>InR* versus *ey>InR PTP61F*^{m}*Dock* was $p<0.001$ (***). Comparisons between *ey>InR* versus *ey>InR PTP61F*^{m} or *ey>InR PTP61F*^{m} *Dock* were not significant.

Figure 10. Dock is not required for PTP61F^{m} ***mediated inhibition of EGFR or PVR signaling.*** (A-I) Representative adult male eye images showing genetic interactions between PTP61F and activated EGFR and PVR. Male fly eyes from the following genotypes are shown: (A) *ey-GAL4 UAS*-GFP (*wild-type*), (B) *ey-GAL4 UAS-EGFR*$^{\lambda top}$, (C) *ey-GAL4 UAS-EGFR*$^{\lambda top}$ *+ UAS-PTP61F*^{m}, (D) *ey-GAL4 UAS-EGFR*$^{\lambda top}$ *+ UAS-Dock,* (E) *ey-GAL4 UAS-EGFR*$^{\lambda top}$ *+ UAS-PTP61F*^{m} *UAS-Dock,* (F) *ey-GAL4 UAS-PVR*$^{\lambda}$, (G) *ey-GAL4 UAS-PVR*$^{\lambda}$ *+ UAS-PTP61F*^{m}, (H) *ey-GAL4 UAS-PVR*$^{\lambda}$ *+ UAS-Dock, (I) ey-GAL4 UAS-PVR*$^{\lambda}$*+ UAS-PTP61F*^{m} *+ UAS-Dock.* The overgrown eye phenotype of *EGFR*$^{\lambda top}$ is suppressed by *PTP61F*^{m} and the addition of Dock expression does not influence this rescue. The *PVR*$^{\lambda}$ overgrown eye phenotype is suppressed by *PTP61F*^{m} but enhanced by *Dock* and by

PTP61F^{m} *Dock* coexpression. (J) Quantification (mean ± SEM) of eye sizes of the indicated genotypes. The data was compared using t-tests. The significance for *ey>EGFR*$^{\lambda top}$ versus *ey>EGFR*$^{\lambda top}$ *PTP61F*^{m} and *ey>EGFR*$^{\lambda top}$ versus *ey>EGFR*$^{\lambda top}$ *PTP61F*^{m}*Dock* were both significant, p<0.0001 (****). *ey>EGFR*$^{\lambda top}$ versus *ey>EGFR*$^{\lambda top}$ *Dock* and *ey>EGFR*$^{\lambda top}$ *PTP61F*^{m} versus *ey>EGFR*$^{\lambda top}$ *PTP61F*^{m}*Dock* were not significant. The significance for *ey>PVR*$^{\lambda}$versus *ey>PVR*$^{\lambda}$ *PTP61F*^{m}, *ey>PVR*$^{\lambda}$ versus *ey>Dock* and *ey>PVR*$^{\lambda}$ *PTP61F*^{m} versus *ey>PVR*$^{\lambda}$ *PTP61F*^{m} *Dock* were all p<0.0001 (****). *ey>PVR*$^{\lambda}$ versus *ey>PVR*$^{\lambda}$ *PTP61F*^{m} *Dock* was not significant from the side measurements of eye area, however top views the *ey>PVR*$^{\lambda}$ *PTP61F*^{m}*Dock* eyes were clearly more bulgy (M). (K-N) Representative adult male eye images (top views) showing genetic interactions between PTP61Fm and activated PVR and Dock. Male fly eyes of the indicated genotypes are shown: (K) *ey-GAL4 UAS-PVR*$^{\lambda}$, (B) *ey-GAL4 UAS-PVR*$^{\lambda}$ + *UAS-PTP61F*^{m}, (L) *ey-GAL4 UAS-PVR*$^{\lambda}$+ *UAS-Dock*, (D) *ey-GAL4 UAS-PVR*$^{\lambda\lambda}$+ *UAS-PTP61F*^{m} + *UAS-Dock*. Whilst expression of PTP61Fm suppressed the *PVR*$^{\lambda}$ overgrown eye phenotype (M), coexpression of Dock with PTP61F^{m} enhanced the phenotype (N), resulting in a bulgy, folded eye.

Figure 11. Model for PTP61F Dock-dependency in RTK signalling

In InR signaling, PTP61Fm requires Dock to dephosphorylate and restrain InR activation, whilst PTP61Fn acts in a Dock-independent manner. In EGFR and PVR signaling, PTP61Fn and PTP61Fm act independently of Dock in their dephosphorylation of the receptor PTKs and suppression of signalling.

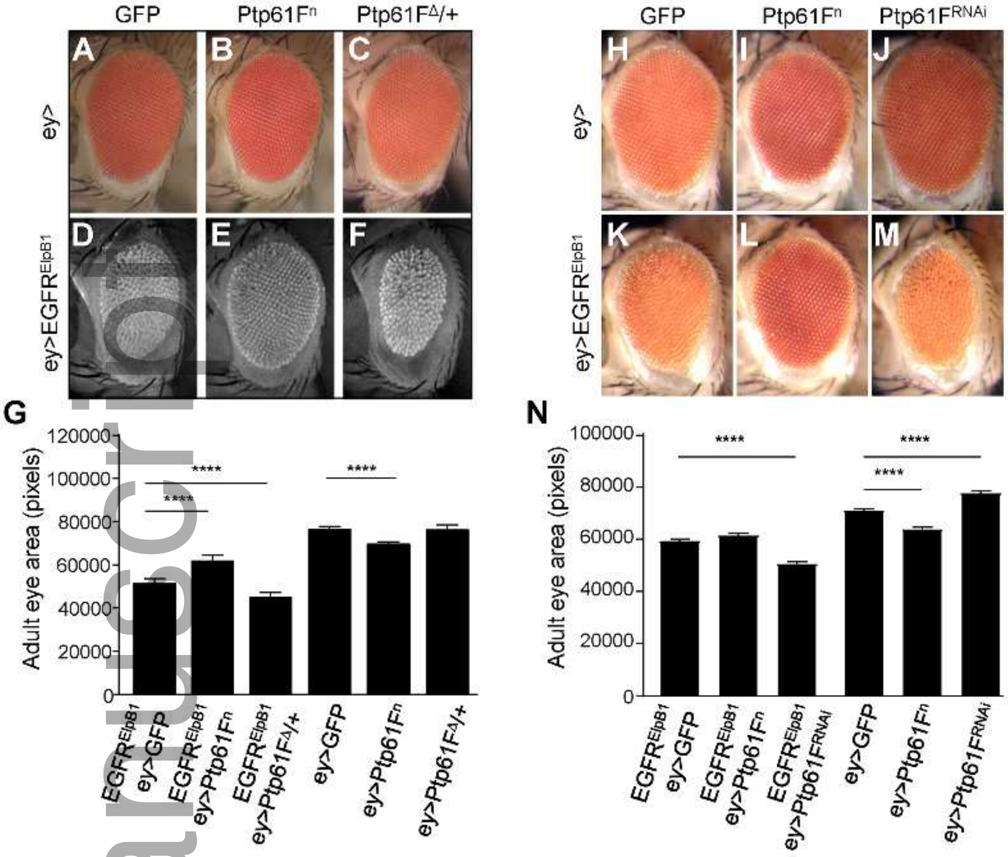

Figure 1

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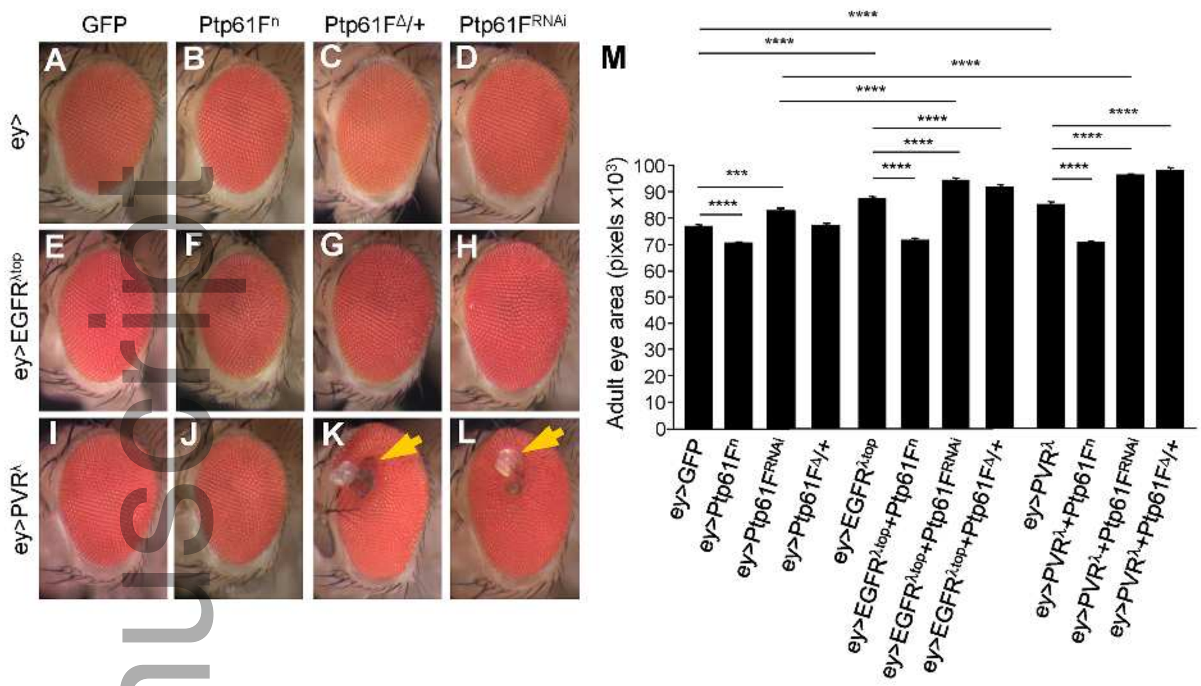
Figure 2



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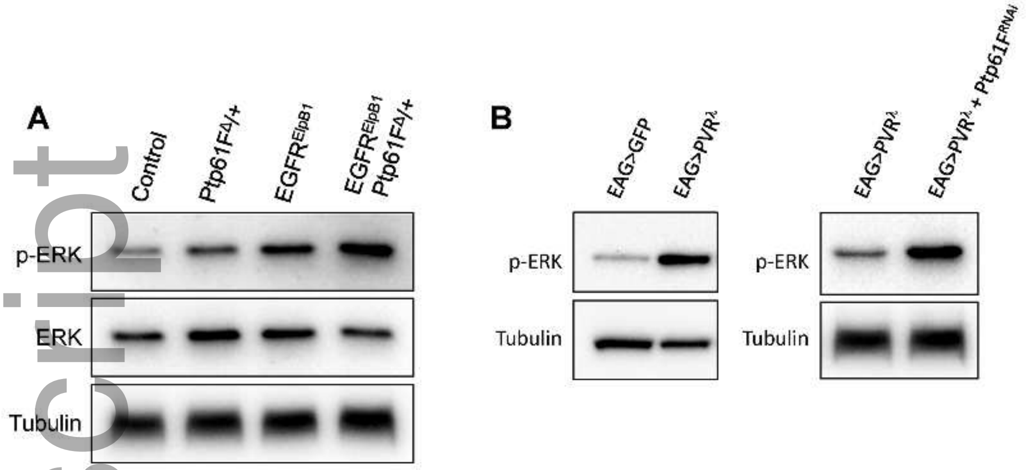

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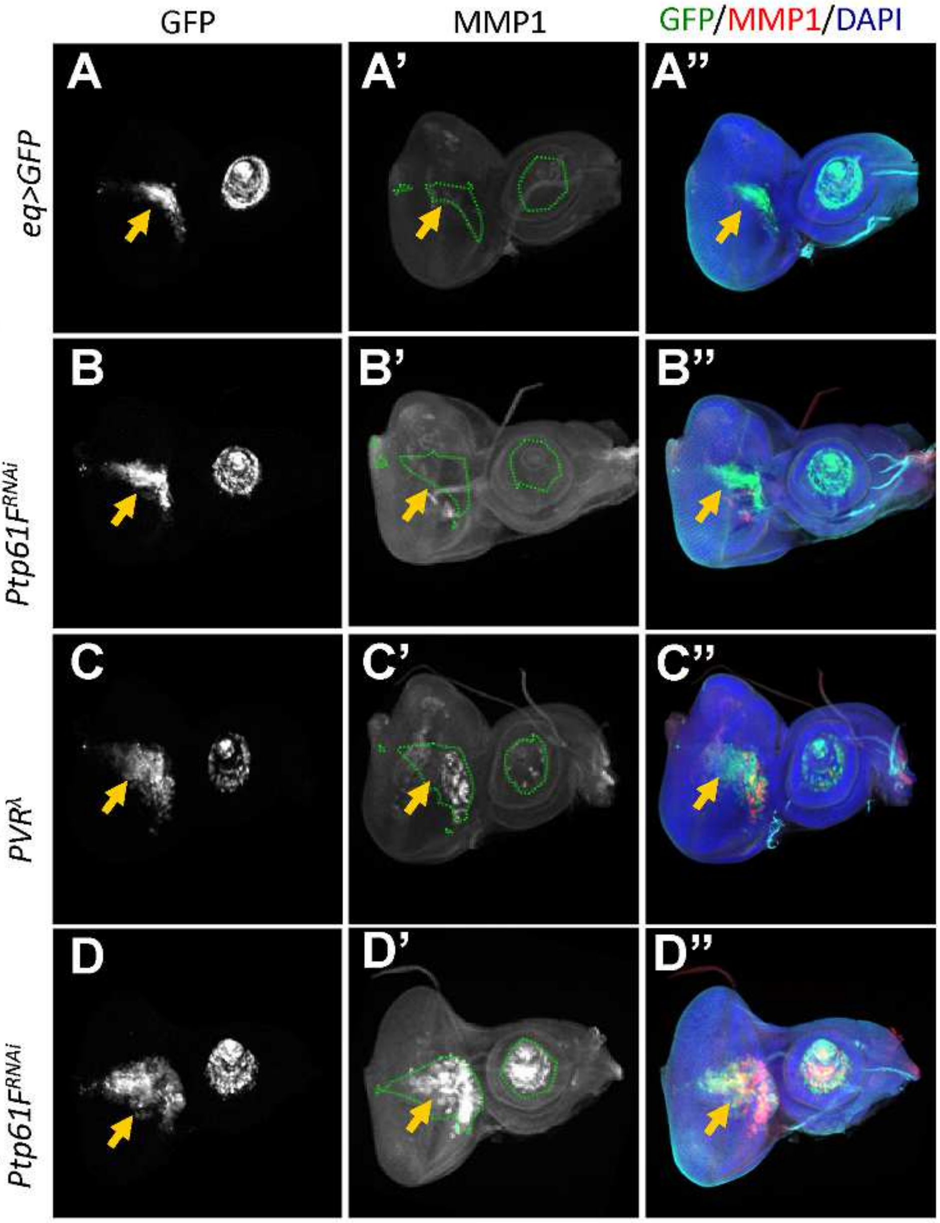

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Figure 5

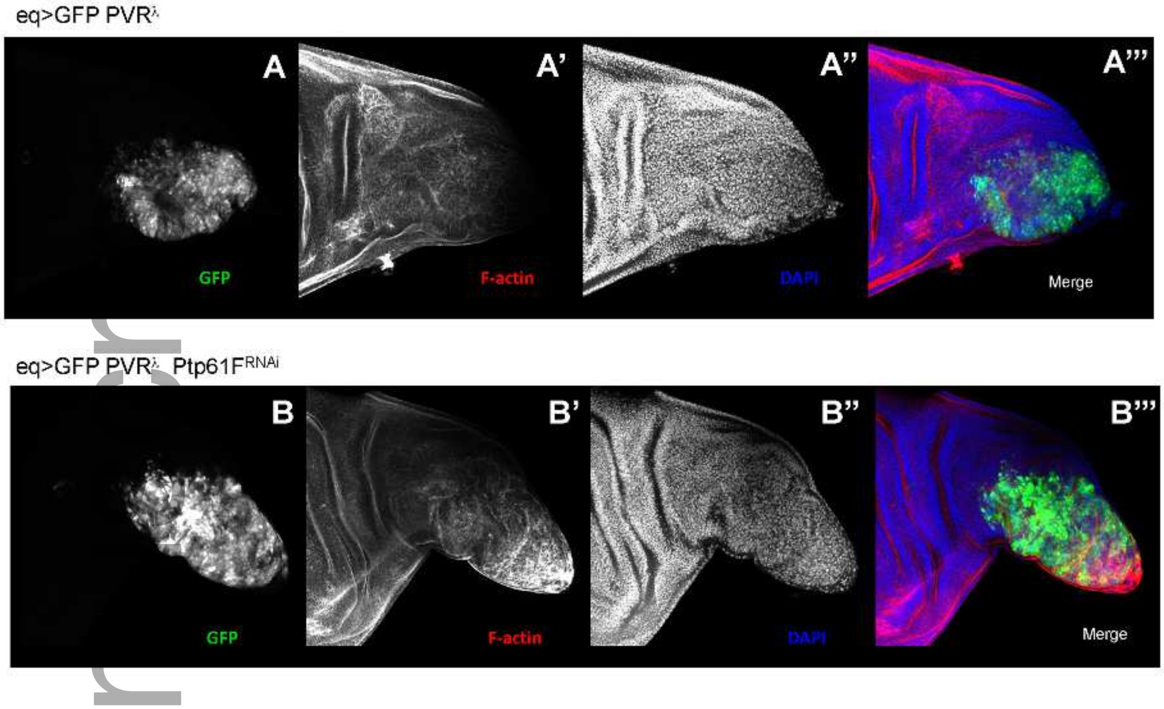

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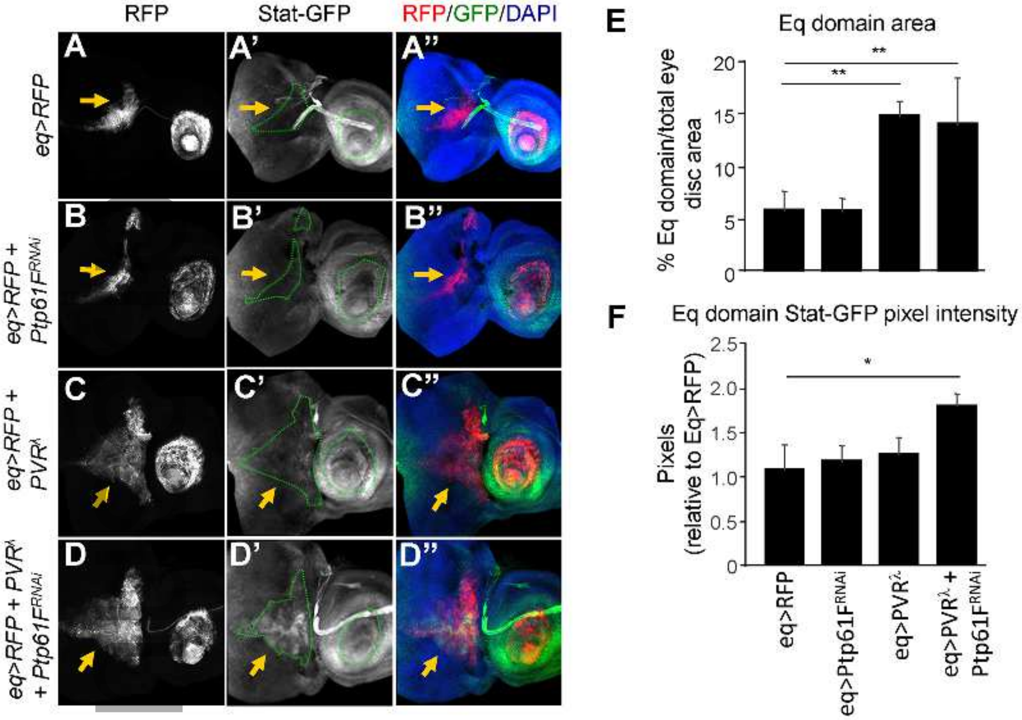
Figure 6



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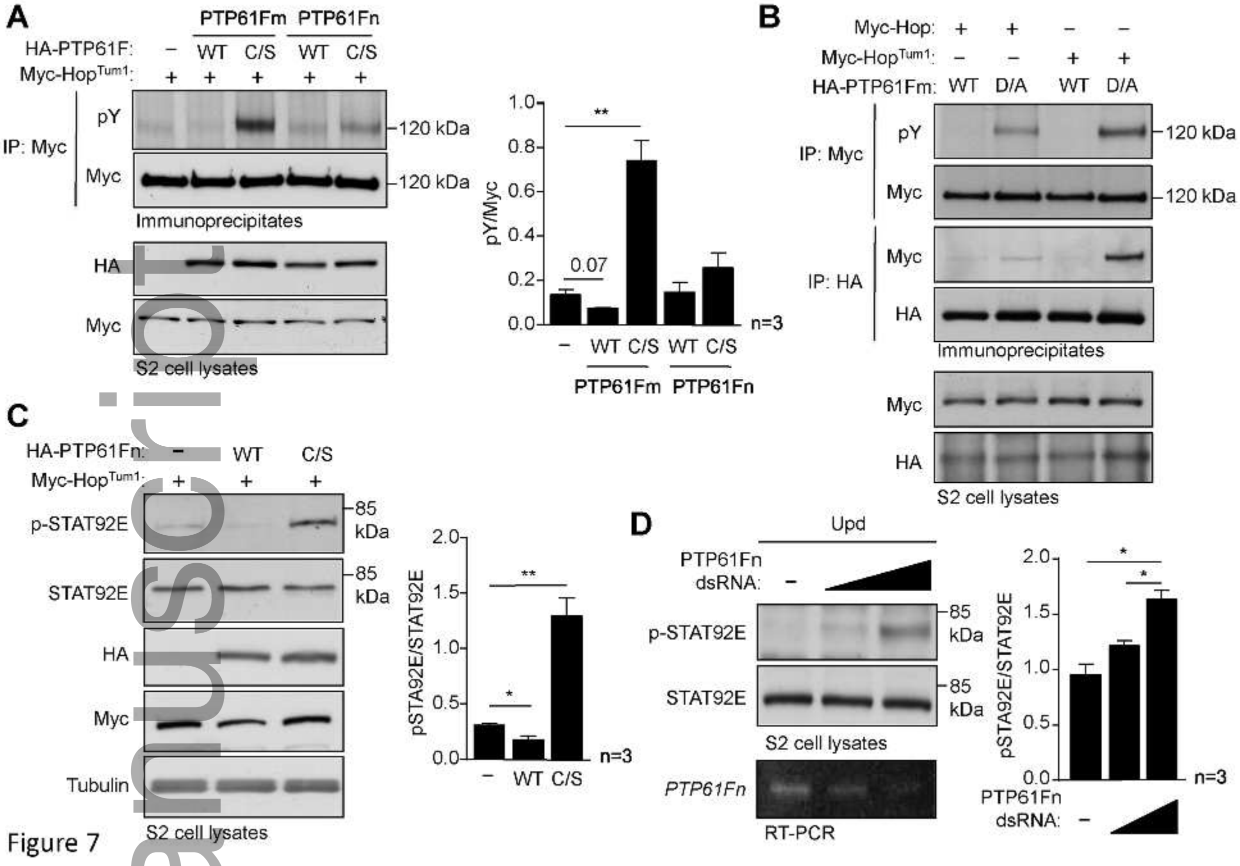

Figure 7

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Figure 8

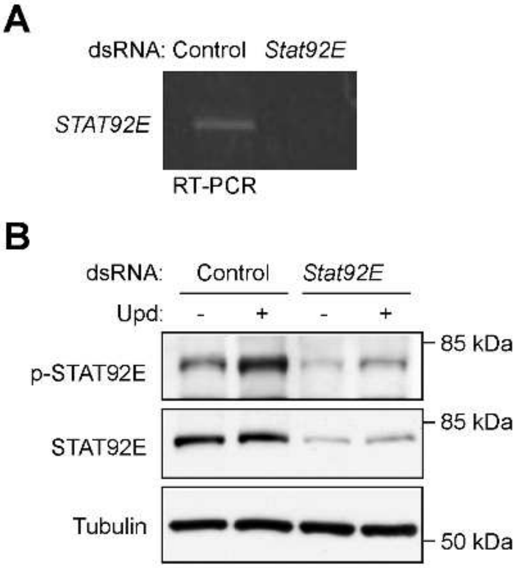

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Figure 9

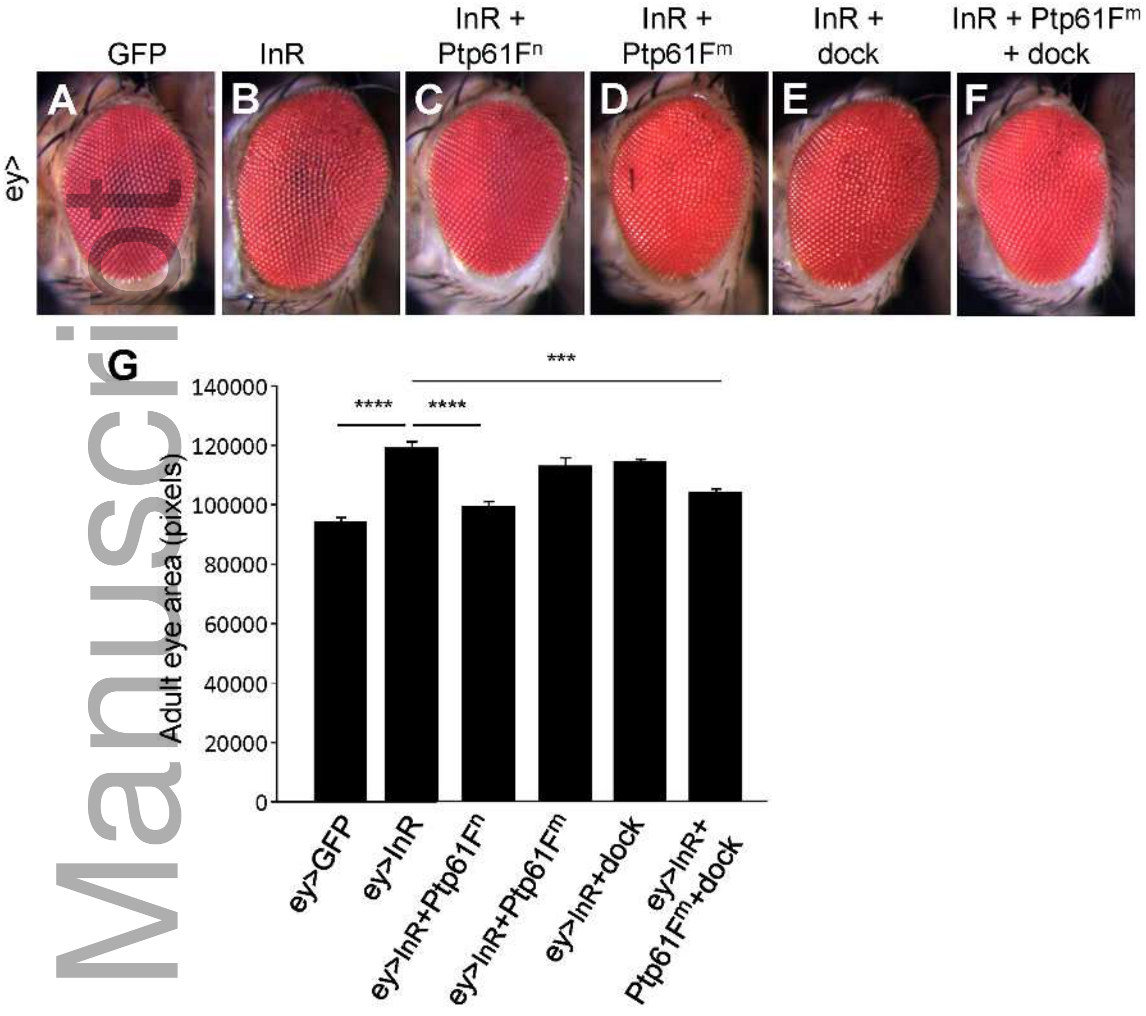

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Figure 10

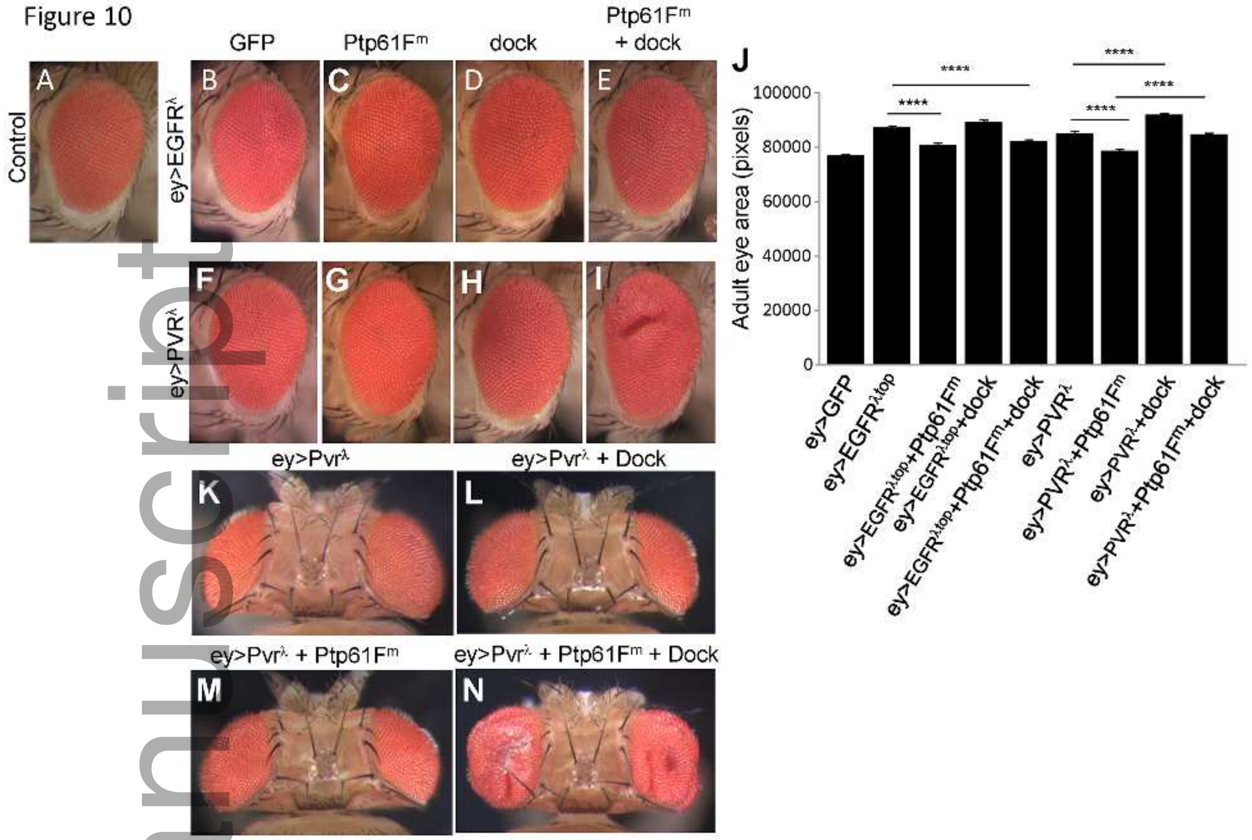

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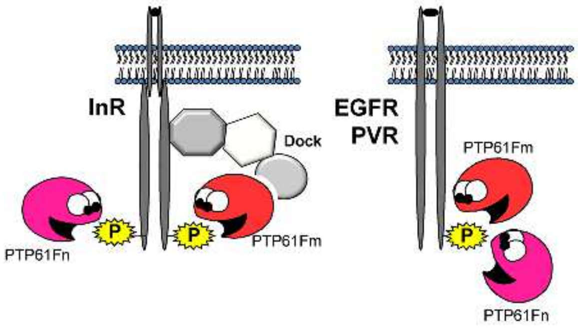
Figure 11



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