Characterization of a Unique Basolateral Targeting Domain in the *Drosophila* TGF-β Type II Receptor Punt

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Abstract

In polarized epithelial cells, differential localization of receptors and ligands can control signaling. *Drosophila* wing imaginal discs are a polarized epithelial layer in which the TGF-β superfamily ligand Dpp is expressed both apically and basolaterally, yet requires a graded distribution throughout the disc to signal properly. We found that Punt, the type II TGF-β receptor that Dpp signals through, is localized specifically at the basolateral membrane, which limits Dpp signaling to the basolateral surface. In characterizing the sequence of Punt, our lab found a unique basolateral targeting domain, the Punt targeting domain (PTD). The PTD is both necessary and sufficient for basolateral localization. Mutation of the insect-conserved portion and whole PTD results in apical mislocalization but characterization of the PTD shows that there is no minimal sequence within the PTD responsible for function. Furthermore, changes in localization of Punt and the other type II receptor Wit affect fly viability.

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Introduction

Epithelial cell polarity

Many tissues that make up organisms are composed of sheets of polarized epithelial cells. These cells have distinct membrane compartments that allow each side of the sheet to have different properties and respond accordingly to their different environments. A polarized epithelial cell is divided into apical and basolateral compartments by tight junctions in mammals and adherens junctions in insects (Schock & Perrimon 2002, Laprise & Tepass 2011). The apical side of the epithelium contains microvilli that face the external environment or lumen, while the basolateral side is attached to the basement membrane and extracellular matrix (Bryant & Mostov 2008). Adjacent cells are connected at the junctions. Apical and basolateral membranes contain different lipid and protein microenvironments that are physically separated (Cao et al. 2012). The physical separation between the apical and basolateral compartments endows each domain with unique properties, enabling specialized interactions in the different environments found on each side of the epithelium face.

In *Drosophila,* the basolateral domain starts at the septate junction, found just below the adherens junction (Schock & Perrimon 2002). The basolateral polarity complex is composed of Discs large, Lethal giant larvae, and Scribble, along with a newly identified basolateral complex composed of Yrt, Coracle (Cora), the Na+K+ATPase and Neurexin IV (Nrx-IV) (Laprise & Tepass 2011). The protein Par1 is another basolateral protein crucial for cell polarity.

The apical domain in *Drosophila* starts above the adherens junction and is formed by two different apical polarity complexes, Par and Crumbs. The Par complex contains Par6, atypical protein kinase C (aPKC), Cdc42, and Bazooka (Baz), while the Crumbs complex contains Crumbs (Crb), Stardust (Sdt), Patj, Lin7, Moesin, Yurt, and β_{heavy}-Spectrin (Laprise & Tepass 2011). Early during the polarization process, Baz acts with the adherens junction proteins actin, E-cadherin, and Armadillo (Arm, mammalian β-catenin) to initiate the apical domain formation. To maintain proper apical/basolateral boundaries, many members of the apical and basolateral complexes are mutually antagonistic (Laprise & Tepass 2011).

When an epithelial layer undergoes morphogenesis, an epithelial-mesenchymal transition (EMT) occurs. Junctions are broken down and apical/basolateral polarity is lost in favor of cell motility (Bryant & Mostov 2008). Transforming growth factor- β (TGF-β) signaling is a known regulator of EMT and inappropriate signaling leads to cancer (Heldin et al. 2012). My work examines how controlling TGF-β signaling in *Drosophila* is regulated by selectively localizing TGF-β receptors to specific subcellular compartments.

TGF-β signaling

The TGF-β superfamily of signaling proteins is conserved from worms to flies to mammals (Massague 1998). Through its many family members, TGF-β signaling controls cell differentiation, proliferation, migration, adhesion, and death (Masssague & Gomis 2006). Because the components of the TGF-β signaling pathway are conserved, we can use *Drosophila* as a model system to uncover general principles of signal regulation that likely will apply to mammalian systems. Canonical TGF-β signaling is initiated when an extracellular ligand (which varies depending on the tissue) binds to the

type II receptor. This complex then recruits a type I receptor, which is activated by type II-mediated phosphorylation. The activated type I receptor then phosphorylates and activates the transducer protein R-Smad. Once activated, R-Smads pair with a co-Smad and are translocated to the nucleus, where the R-Smad/co-Smad complex works as a transcription factor (Massague 2012).

There are two main branches within the TGF-β superfamily: the bone morphogenetic protein (BMP) and TGF-β/Activin family. In *Drosophila,* BMP type ligands include Decapentaplegic (Dpp), Glass bottom boat (Gbb), Screw (Scw), and Maverick (Mav), while Activin ligands include Activinβ (Actβ), Dawdle (Daw), and Myoglianin (Myo) (Brummel et al. 1999, O'Connor et al. 2006, Awasaki et al. 2011). Both BMP and Activin share the type II receptors Punt (Put) or Wishful Thinking (Wit). Signal specificity is supplied by the type I receptor with Thickveins (Tkv) and Saxophone (Sax) used for BMP signaling and Baboon (Babo) used for Activin signaling.

Wing disc architecture

The *Drosophila* wing disc is comprised primarily of a single cell thick polarized epithelium that undergoes patterning, proliferation, and morphogenesis to become the adult wing. The wing disc is comprised of a columnar epithelium whose apical surface faces a lumen enclosed by a peripodial membrane, comprised of squamous epithelium (Figure 1). The columnar epithelium is a folded structure. At the folds two apical surfaces come together, resulting in a stripe at the apical fold upon staining with an apical marker, which we utilize for our receptor localization assays.

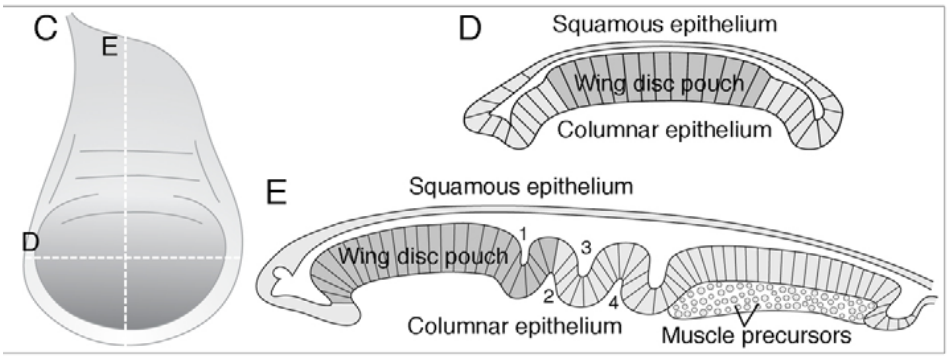

Figure 1. Wing disc architecture. C.) Cartoon of larval third instar wing disc. Sections through lines D and E are shown D.) Cross section through line D shows columnar epithelium in wing disc pouch, with the squamous epithelium lying above. E.) Section through line E shows the folded structure of the columnar wing disc. Apical surfaces come together at labels 1 and 3. Modified from Widmann & Dahmann J Cell Sci 2009.

Dpp localization in the wing disc

Decapentaplegic (Dpp) is BMP family member. Dpp is expressed at the midline of the developing wing disc along the anterior and posterior compartment border and is responsible for stimulating growth and patterning (Affolter & Basler 2007). Dpp protein acts as a morphogen. It diffuses from the producing cells and forms a gradient on either side of the compartment border. Wing disc cells differentially activate Dpp target genes depending on the extracellular concentration. High concentration of Dpp close to the midline promote expression of the transcription factor Spalt major (Salm), while lower concentrations more distal from the source facilitate Optomotor blind (Omb) in a broader domain. The activation of these genes is complex and involves relief of repression by the transcription factor Brinker (Brk) (Lecuit et al. 1996, Nellen et al. 1996) which is a direct target of Dpp signaling. Where Dpp signaling levels are low, Brk expression remains high, repressing Spalt and Omb.

Because Dpp signaling is crucial for proper wing disc development, Dpp signaling needs to be tightly controlled. In *Drosophila* wing discs, there is a uniform distribution of Dpp within the apical lumen and a graded distribution within the basolateral domain (Gibson et al. 2002, Teleman & Cohen 2000). One potential way of controlling Dpp signaling is by separating the ligand from its receptors. As described, Dpp signals through the type II receptor Punt and the type I receptors Tkv and Sax (Letsou et al. 1995, Ruberte et al. 1995, Haerry et al. 1998). Our lab discovered that the type II TGF-β receptor Punt is localized only within the basolateral compartment of wing disc cells (Figure 2). This suggests that only basolaterally distributed Dpp is able to signal since apically localized Dpp will not have access to the type II receptor.

Canonical apical/basolateral sorting and trafficking

Newly synthesized proteins need to be sorted and transported to the proper subcellular domain. Sorting of protein cargo occurs at the trans Golgi network (TGN), and the transport to the proper membrane that follows sorting can be direct or indirect. For direct sorting at the TGN, transport signals within the protein sequence are recognized, proteins are sorted according to their intended destination, and proteins are packaged into vesicles that will be transported to the proper membrane (Carmosino et al. 2010). For indirect sorting, cargo is transported to the plasma membrane, where it can be re-internalized into sorting endosomes, of which there are both apical and basolateral-specific types that will direct the protein to the intended membrane. Additionally, re-internalized cargo can be targeted to common recycling endosomes, where sorting occurs, followed by transport to the proper membrane (Carmosino et al. 2010, Cao et al. 2012) Once proteins arrive at the proper membrane, retention of the

proteins occurs through direct or indirect interactions with the cytoskeleton (Carmosino et al. 2010).

Apical sorting signals are highly variable and can be found intracellularly, extracellularly, or within the transmembrane domain of the protein. The main apical sorting signals are GPI-anchors, N and O-glycosylation, and various protein motifs, although no canonical apical motif has been established. (Cao et al. 2012, Carmosino et al. 2010, Rodriguez-Boulan et al. 2005). Examples of apical motifs include a transmembrane signal within hemagglutinin (HA) from the influenza virus and a cytoplasmic domain within the apical protein rhodopsin, although many more apical protein motifs have yet to be characterized (Rodriguez-Boulan & Musch 2005). Because of the variety of apical localization signals, there is likely to be a wide range of mechanisms for transporting proteins to the apical membrane. The most well studied mechanism of apical transport relies on clustering of apical proteins based on their affinity for lipid rafts (Carmosino et al. 2010, Cao et al. 2012). The previously mentioned HA protein is transported to the apical membrane in this manner (Rodriguez-Boulan et al. 2005). Additionally, trafficking to the apical membrane is dynein and kinesin dependent (Carmosino et al. 2010).

Canonical basolateral sorting and transport is direct and mediated by distinct intracellular protein sequences, either dileucine (LL) or tyrosine (YxxΦ) motifs (where Φ is a large hydrophobic acid). The cytoplasmic dileucine or tyrosine motif is recognized by the adaptor proteins AP-1B, AP-3 and AP-4 (Rodriguez-Boulan & Musch 2005, Harada 2010). Adaptor proteins AP-1 and AP-3 bind to clathrin, and clathrin is necessary for basolateral transport, although the exact mechanism is not yet understood (Harada 2010, Cao et al. 2012). Additionally, Rab proteins and the exocyst complex have been

associated with basolateral trafficking (Carmosino et al. 2010, Cao et al. 2012). Kinesin is also crucial to basolateral transport (Carmosino et al. 2010).

TGF-β type II receptor localization

Because TGF-β is prevalent in many different tissues to promote many different processes, it must be tightly regulated. Establishing and maintaining differential localization of receptors and secreted ligand is crucial to prevent ectopic autocrine signaling. In polarized cell culture, the mammalian TGF-β type II receptor localizes specifically to the basolateral membrane, while TGF-β ligand is secreted apically (Murphy et al. 2004). Basolateral localization of the mammalian TGF-β type II receptor is dependent on a non-canonical, C-terminal basolateral targeting motif containing the residues LTAxxVAxxF (Murphy et al. 2007). Recent studies have shown that the C-terminus of the mammalian type II receptor interacts with the retromer complex and this interaction is necessary to maintain basolateral receptor localization (Yin et al. 2013).

Punt localization

<u>In cell culture</u>

After discovering that the mammalian TGF-β type II receptor is found basolaterally and contains a non-canonical basolateral targeting sequence, our lab decided to examine the *Drosophila* type II receptor. When Punt was expressed in mammalian cell culture, it was found specifically in the basolateral compartment (Steve Murphy and Ed Leof, unpublished). However, there is no discernible sequence within Punt that matches the mammalian type II receptor basolateral targeting motif, and truncation mutants that eliminate the general region in Punt that corresponds to the domain of the protein where the mammalian basolateral sequence is located failed to

prevent basolateral targeting. Additional truncations were made and one that removed most of the intracellular domain and left only the juxtamembrane 19 amino acids still remained basolateral. When this 19 amino acid sequence was deleted, however, Punt mislocalized apically. We called this 19 amino acid sequence the Punt targeting domain (PTD). The PTD contains the residues AHFNEIPTHEAEITNSSPL, which does not correspond with any known canonical or non-canonical basolateral targeting domains. This sequence is also quite different than the mammalian type II LTA targeting domain (Murphy et al 2007).

Although the PTD is a unique sequence not found in mammalian proteins, the mammalian cellular machinery still recognized it and properly sorts and traffics Punt to the basolateral surface. Understanding the PTD and discovering the machinery that acts on the PTD will help elucidate not only how basolateral sorting of TGF-β type II receptors is accomplished, but will also help us understand how conservation between mammalian and *Drosophila* sorting and trafficking systems is maintained despite different cis-acting targeting motifs.

In wing discs

After finding that Punt localizes specifically basolaterally in mammalian cell culture, our lab examined the localization of both Punt and Wit, the two type II TGF-β *Drosophila* receptors in wing imaginal discs. In agreement with the cell culture localization, Punt was found specifically within the basolateral domain of the wing disc epithelium. The other type II receptor, Wit, was found to be enriched apically (Figure 2). Since the type II receptors are not redundant during development and do not seem able to cross rescue defects caused by mutations in the other receptor (Marques et al. 2002),

we hypothesized that the differential localization of the two receptors was responsible for the inability to rescue. My experiments took advantage of mutations that altered wildtype receptor localization to test whether cross-typic rescue can be established by putting the homologous receptor in the correct subcellular location.

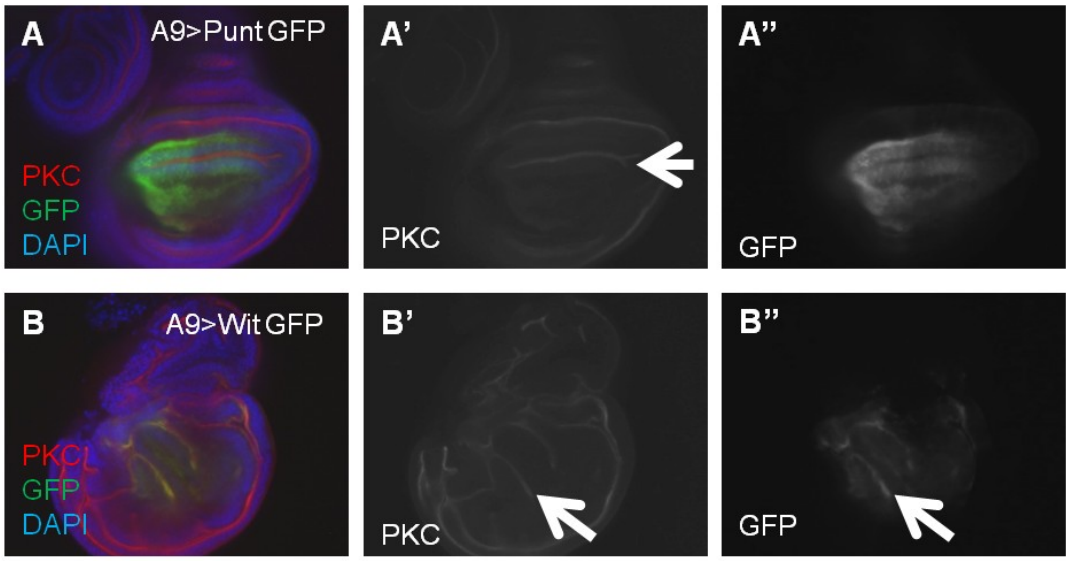

Figure 2. Punt is localized basolaterally, while Wit is localized apically. L3 wing discs were dissected and stained for apical marker PKC (red), GFP (green), and nuclear marker DAPI (blue). A.) Punt GFP staining is found only basolaterally, avoiding the apical stripe (red). A'.) Single channel image of PKC shows apical stripe (white arrow). A".) Single channel image of GFP shows GFP avoids the apical stripe. B.) Wit GFP staining colocalizes with the apical marker, resulting in a yellow stripe. B', B".) Wit GFP stripe overlaps with apical PKC stripe (white arrows).

Materials and Methods

Fly stocks

The following stocks were used: *wit*^{a12} (Marques et al. 2002), *wit*^{b11} (Marques et al. 2002), *punt*^{135} (Ruberte et al. 1995), *Δpunt* (Bloomington #9090), *da*>Gal4 (Bloomington), *arm*>Gal4 (Bloomington), *nub*>Gal4 (Calleja et al. 1996), *elav*>Gal4;*wit*^{b11} (Marques et al. 2002), UAS-*punt*-*GFP* (Guillermo Marques, unpublished), and UAS-*wit*-*GFP* (Guillermo Marques, unpublished).

Recombinants made for rescues

To test for rescue of a lethal allelic combination by altering the localization of Punt or Wit, recombinant stocks were made. The mutant alleles *wit*^{a12} or *punt*^{135} were recombined with the UAS-containing transgenic domain swap fly lines (Aidan Peterson, unpublished). These recombinant lines were then crossed to a Gal4 driver recombined with another mutant allele, either *wit*^{b11} or *Δpunt*. Homotypic rescues utilized versions of Punt with (Punt[punt] and Punt[apis]) or without the PTD (Punt[wit]) rescuing the lethal allelic combination of *punt*^{135} and *Δpunt* or versions of Wit with (Wit[punt]) or without the PTD (Wit[wit] and Wit[apis]) rescuing the lethal allelic combination of *wit*^{a12} and *wit*^{b11} (Marques et al. 2003). For heterotypic rescues, Punt with or without the PTD was recombined with *wit*^{a12}, while Wit with or without the PTD was crossed to and recombined with *punt*^{135}.

Constructs and transgenic fly lines

Domain swaps were made by Aidan Peterson and Melissa Ritter. Briefly, *PstI* and *HindIII* sites were introduced flanking the juxtamembrane sequence of Punt.

Digestion with *PstI* and *HindIII* removed the PTD, and primers containing *Apis* PTD or Wit juxtamembrane sequence with *PstI* and *HindIII* overhangs were ligated into pUAST attB.

For Wit, *NgoMIV* and *HindIII* sites were introduced and the construct digested with these restriction enzymes to remove Wit juxtamembrane sequence. Primers with *NgoMIV* and *HindIII* overhangs that contained either *Apis* juxtamembrane sequence or the PTD were ligated in. Once these were cloned into the *NotI* and *XbaI* sites of pUAST attb, transgenic fly lines were made (injection by GenetiVision).

HA-tagged Punt with a *PstI* site in pBlueScript (pBS HA-Punt-*PstI,* Aidan Peterson) was used as wildtype for all Punt mutants made. Point mutations were made to the Punt targeting sequence (PTD) using site directed mutagenesis (QuikChange kit, Agilent). The primers for site directed mutagenesis (QuikChange) to obtain point mutations (FNE, EIP, and PTE residues) of the PTD are:

FNE $_{forward}$ = 5’ CCTGCAGGCGCACGCCGCGGCGATACCCACGCAC 3’

FNE $_{reverse}$ = 5’ GTGCGTGGGTATCGCCGCGGCGTGCGCCTGCAGG 3’

EIP $_{forward}$ = 5’ GCGCACTTTAACGCCGCGGCCACGCACGAGGC 3’

EIP $_{reverse}$ = 5’ GCCTCGTGCGTGGCCGCGGCGTTAAAGTGCGC 3’

PTE $_{forward}$ = 5’ CTTTAACGAGATAGCTGCGCACGCGGCTGAGATAACA 3’

PTE $_{reverse}$ = 5’ TGTTATCTCAGCCGCGTGCGCAGCTATCTCGTTAAAG 3’

The primers for site directed mutagenesis (QuikChange) to change all charged residues from the PTD to uncharged residues are:

Charge $_{forward}$ = 5’

GCACTTTAACGCAATACCCACGCACGCCGCGGCGATAACAAACTCATC 3’

Charge $_{reverse}$ = 5’

CGATGAGTTTGTTATCGCCGCGGCGTGCGTGGGTATTGCGTTAAAGTGC 3’

After mutations were introduced by site-directed mutagenesis, Punt (containing the mutated PTD) was digested out of pBS using *NotI* and *XbaI* and cloned into the same sites in the pUAST attB vector and sent for microinjection to make transgenic fly lines.

For mutations that changed more than a few residues (conserved region and whole domain mutants) primers were ordered and sequences were changed using oligo dropin. Oligo dropin was performed by mixing single-stranded primers at 10 μM each in 1x NEB3 Buffer (New England Biosciences), heating to 75°C, and slowly cooling to allow annealing. This mix was then diluted 1:100 to be used in ligations. The vector used was pBS HA-Punt-*PstI*-*HindIII* (Aidan Peterson), which had *PstI* and *HindIII* flanking the PTD that allowed for removal of the PTD. The primers that were used to change the insect-conserved (10 residues) or whole PTD (19 residues) to all alanines contained 5’ *PstI* and 3’ *HindIII* overhangs, which allowed for direct cloning into the same sites in the pBS HA-Punt-*PstI*-*HindIII* vector.

Conserved $_{forward}$ = 5’

GGCTCACGCCGCGGCTGCTGCCGCGGCTGCTGCTGAGATAACAAACTCATCGCCA

TTGCTCAGCAACCGTCCCATTC 3’

Conserved $_{reverse}$ = 5’

AGCTGAATGGGACGGTTGCTGAGCAATGGCGATGAGTTTGTTATCTCAGCAGCAGC

CGCGGCAGCAGCCGCGGCGTGAGCCTGCA 3’

Whole $_{forward}$ = 5’

GGCAGCTGCCGCAGCGGCTGCAGCTGCAGCGGCGGCTGCAGCAGCCGCCGCTG

CGGCTCTCAGCAACCGTCCCATTC 3’

Whole $_{reverse}$ = 5’

AGCTGAATGGGACGGTTGCTGAGAGCCGCAGCGGCGGCTGCTGCAGCCGCCGCT

GCAGCTGCAGCCGCTGCGGCAGCTGCCTGCA 3’

Punt (containing the mutated PTD) was digested out of pBS using *NotI* and *XbaI* and cloned into the same sites in the pUAST attB vector.

To add C-terminal tags to Punt, primers were designed to add 5’ *NotI* overhangs and 3’ *XbaI* overhangs to clone into pUAST attB *NotI* and *XbaI* sites. Since the only change to the 5’ end of Punt was addition of the *NotI* overhang, the same forward primer was used and only the reverse primer was changed. Wit juxtamembrane sequence was added to the C-terminus of wildtype Punt using the Wit at Punt CTD reverse primer. PTD was added to the C-terminus of Δ10 or Δ19 Punt constructs using the 3’ PTD-Punt reverse primer.

5’ *NotI*-Punt $_{forward}$ = 5’

CCGCGGCCGCACAGGCGATAACAATGTCCAAATACGATCTGCTTTATCTAACG 3’

Wit at Punt CTD $_{reverse}$ = 5’

CGCTCTAGACTAGAGATTAGAACTGTAGCCAGGGCCCGATGGTGCCAGAGGCGAC

TCCTCCGGTTCCGGCTGTAAGCAATTCGTAGATTCCTTGGC 3’

3’ PTD-Punt $_{reverse}$ = 5’

CGCTCTAGACTAGAGCAATGGCGATGAGTTTGTTATCTCAGCCTCGTGCGTGGGTA

TCTCGTTAAAGTGCGCCTGTAAGCAATTCGTAGATTCCTTGGC 3’

After sequences were confirmed in the UAS vector, constructs were sent to GenetiVision for microinjection. To ensure consistent levels of expression, the same genomic insertion site was used for all Punt constructs. For Punt constructs, the VK20(3R)99F8 attB site was used for recombination using the ΦC31 integrase. Wit constructs utilized the VK31(3L)62E1 site.

UAS-Gal4 overexpression

To exogenously and tissue-specifically express different versions of Punt or Wit, I utilized the UAS-Gal4 system (Brand and Perrimon 1993). Different UAS-containing versions of Punt were crossed to either A9 or nub>Gal4 for wing disc-specific expression. Da or arm>Gal4 were used for constitutive expression. Elav>Gal4 was used for neuron-specific expression.

Dissection and immunohistochemistry

Wandering third instar larvae were picked into a glass dish containing 1x PBS. Larvae were inverted and fixed in 34% PFA for 25 minutes. After washes, primary antibody was added and incubated rotating overnight at 4°C. After primary antibody washes, wing discs were dissected from the larval carcass and secondary antibody was

added. After the secondary antibody washes, wing discs were mounted in 80%glycerol/20%PBT. Images were taken at 20x using a confocal microscope with a CARV attachment. Wing discs are shown as Z sections.

Antibodies used

Polyclonal rabbit anti-punt (Fabgennix Punt-112-AP) was used at 1:500. Goat anti-PKC (Santa Cruz SC-216) was used at 1:500. Since there is no good Wit antibody, a FLAG tag (DYKDDDK) was added to all Wit constructs. For Wit staining, monoclonal mouse anti-FLAG (Sigma F3165) was used at 1:500. Secondary AlexaFluor antibodies (Invitrogen) used include goat anti-mouse 555 and 568, goat anti-rabbit 488 and 555, goat anti-mouse 647, donkey anti-rabbit 488 and 647, donkey anti-goat 568, donkey anti-mouse 488 all diluted at 1:200.

Results

Our lab found that the *Drosophila* type II TGF-β receptor Punt is specifically basolaterally localized in wing discs (Figure 2). Subcellular localization to the basolateral surface is dependent on the 19 amino acid Punt targeting domain (PTD) found next to the transmembrane domain of Punt. My project characterized the PTD for crucial residues and determined how changes in localization affected fly viability.

The PTD is necessary for basolateral receptor localization

To test the requirement of the PTD for Punt localization at the basolateral surface, domain swap mutants were made by replacing the Punt juxtamembrane sequence with Wit juxtamembrane sequence or juxtamembrane sequence from *Apis* (honeybee) Punt (Aidan Peterson). The domain swaps were cloned into pUAST attB vectors and sent for microinjection (GenetiVision) to make transgenic fly lines. Once transgenic lines were established, they were crossed to various Gal4-mediated wing drivers to overexpress wildtype Punt, Punt with *Apis* sequence in the juxtamembrane location (Punt[apis]), or Punt with Wit sequence in the juxtamembrane location (Punt[wit]). Larval third instar wing discs were dissected out and used for immunostaining. Subcellular localization was determined by colocalization with apical or basolateral markers. Punt with wildtype or *Apis* juxtamembrane PTD localized to the basolateral junction, while Punt missing the PTD and replaced with juxtamembrane Wit sequence mislocalizes to the apical surface, where it colocalizes with the apical marker aPKC (Figure 3). This shows that the presence of the PTD is necessary for Punt

basolateral localization.

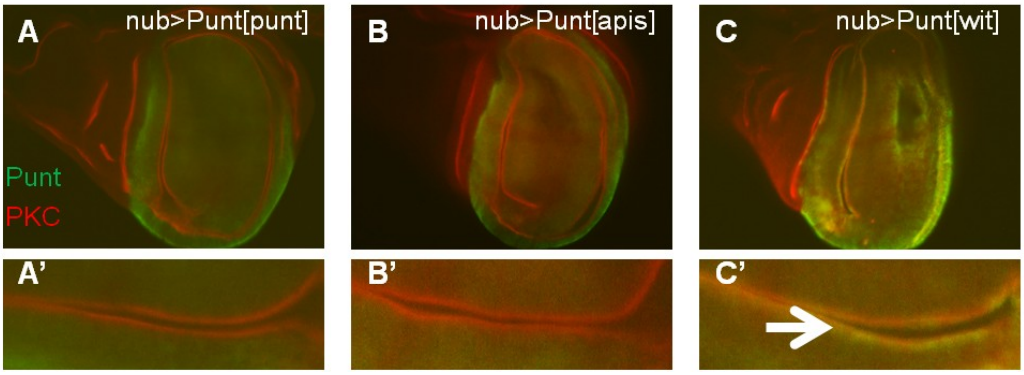

Fig 3. The PTD is necessary for basolateral localization. L3 wing discs were dissected and stained for colocalization apical marker PKC (red) and Punt localization (green). A.) nub Gal4 driving Punt with wildtype PTD shows Punt basolateral, with no overlap of receptor staining with the apical stripe. B.) nub Gal4 driving Punt with *Apis* (honeybee) PTD also shows basolateral localization only of Punt. C.) nub Gal4 driving Punt with PTD replaced by Wit juxtamembrane sequence shows mislocalization of Punt to the apical stripe (white arrow). A', B', and C' show enlarged views of the apical stripes. Images are Z sections taken at 20x on a confocal microscope with a CARV attachment.

To ensure that the loss of the PTD was responsible for apical mislocalization and that the addition of the juxtamembrane Wit sequence was not imposing an apical targeting sequence, I added the Wit juxtamembrane to the C-terminus of wildtype Punt and made a transgenic fly line. Immunostaining showed that Punt remained at the basolateral surface and that the Wit juxtamembrane sequence did not alter Punt localization (Figure 4).

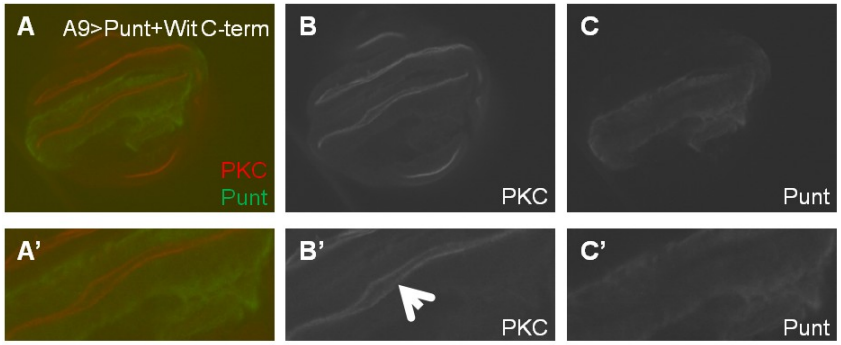

Figure 4. Addition of Wit juxtamembrane sequence to C-terminus of wildtype Punt does not alter Punt localization. Dissection and staining of L3 wing discs. A.) Punt staining (green) is basolateral and does not overlap with apical marker (red). B.) PKC staining shows apical stripe in wing disc. C.) Punt staining does not overlap with apical stripe. A', B', and C' are enlargements of the apical stripe. White arrow points to apical stripe. Images are Z sections taken at 20x on a confocal microscope with a CARV attachment.

The PTD is sufficient for basolateral receptor localization

Domain swaps were made to replace wildtype Wit juxtamembrane sequence with *Apis* juxtamembrane Wit or the PTD (Aidan Peterson). After transgenic lines were made, flies were crossed to wing-specific Gal4 drivers to overexpress Wit. Immunostaining was performed to look at Wit localization. Wildtype Wit (Wit[wit]) and Wit with *Apis* (Wit[apis]) sequence both colocalized at the apical surface with aPKC, while Wit with the PTD (Wit[punt]) mislocalized to the basolateral surface (Figure 5). This result showed that Punt is sufficient for basolateral localization and that the presence of the PTD can even override the wildtype localization of Wit to target the receptor to the basolateral surface.

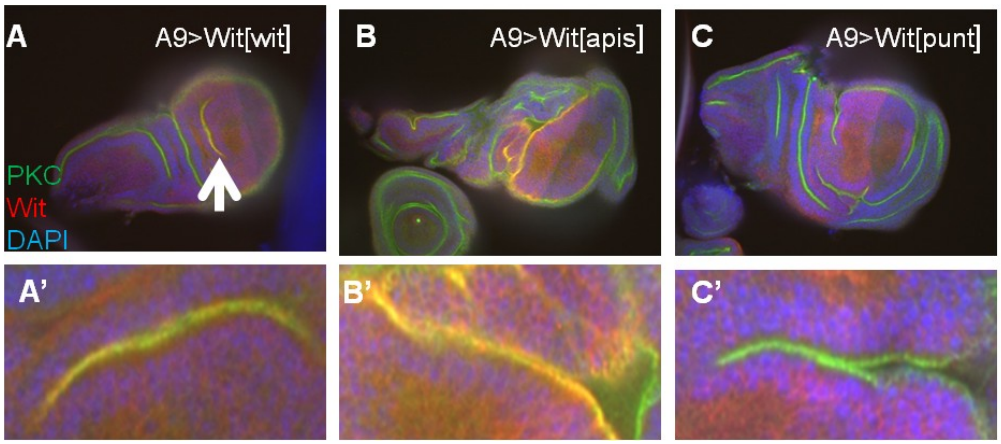

Figure 5. Addition of the PTD to Wit is sufficient for basolateral localization. L3 wing discs were dissected and stained for colocalization with the apical marker PKC (green), Wit (red), and nuclear stain DAPI (blue). A9 Gal4 was used to drive Wit overexpression specifically in the wing disc. A.) Wildtype Wit colocalizes with the apical marker, resulting in a yellow apical stripe (white arrow). B.) Wit with *Apis* (honeybee) juxtamembrane sequence also localizes apically. C.) Wit with juxtamembrane PTD is no longer found apically, as shown by the absence of colocalization at the apical stripe (no yellow stripe). Images are Z sections taken at 20x on a confocal microscope with a CARV attachment.

The position of the PTD within the protein affects function

Previous work from our lab has shown that deletion of the either the insect-conserved (Punt Δ10) or the whole PTD (Punt Δ19) from its juxtamembrane position within the Punt receptor results in apical mislocalization, both within *Drosophila* wing discs (Aidan Peterson, unpublished) and in mammalian cell culture (Steve Murphy and Ed Leof, unpublished). To determine whether the location of the PTD within the protein affects PTD function, a fly line was made in which the PTD was added to the C-terminus in the PTD deletion constructs (in both Punt Δ10 or Δ19). Wing discs were then dissected and stained to determine subcellular localization. Deletion of the PTD from its wildtype juxtamembrane position leads to apical mislocalization (Aidan Peterson, unpublished). When the full (19 amino acid) PTD was added to the C-terminus of the

PTD juxtamembrane deletion constructs (either Δ10 or Δ19), Punt was still mislocalized apically (Figure 6). In PTD deletion constructs with or without the PTD added to the C-terminus, it appears as though the Δ19 deletion results in more consistent apical mislocalization compared to the Δ10 deletion. In either case, however, the presence of the PTD was not sufficient to restore wildtype basolateral localization.

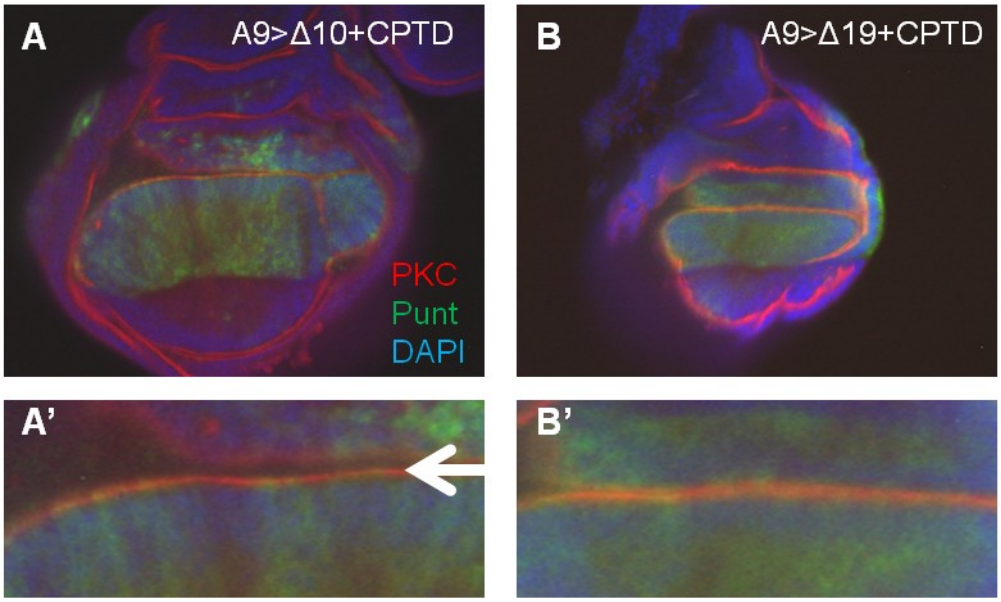

Figure 6. PTD needs to be in juxtamembrane portion of receptor to target basolaterally. L3 wing discs dissected and stained for apical PKC (red), Punt (green) and DAPI (blue). Constructs had either 10 or 19 residues from the PTD deleted and the PTD was added at the C-terminus. A.) When the insect-conserved residues of the PTD were deleted (Δ10) and the PTD was added C-terminally, apical mislocalization of Punt was observed. B.) Deletion of the entire PTD (Δ19) and addition of the PTD C-terminally resulted in apical mislocalization of Punt, as seen by the yellow stripe of colocalization. A' and B' are enlarged to better show the apical stripe (white arrow). Apical mislocalization is more prevalent in Δ19 construct, which matches what has been previously been observed by our lab. Images are Z sections taken at 20x on a confocal microscope with a CARV attachment.

Changes in receptor localization lead to differences in viability

Our lab found that within *Drosophila* wing discs, Punt and Wit are localized basolaterally and apically, respectively. Previous work with Punt and Wit has shown that these type II receptors cannot cross-rescue mutations of the other receptor (Marques et al. 2002). The domain swap constructs (Figures 3 & 5) were utilized to determine whether the different localization of Punt and Wit accounts for the inability of each receptor to rescue the other. The domain swaps were recombined with mutant Punt or Wit alleles and tested for the ability to rescue. Both homotypic and heterotypic rescues were performed.

In the first homotypic rescue, different versions of Punt (with wildtype PTD, *Apis* PTD, or Wit juxtamembrane sequence) were recombined with the $punt^{135}$ allele and crossed to *da* or *arm* Gal4 recombined with an allele that deletes the sequence on the right arm of the third chromosome that contains *punt* (*Δpunt* allele) (Table 1). Because Punt is crucial for both BMP and TGF-β signaling throughout the organism, constitutive expression was used for rescue (Simin et al. 1998). I found that attempting to rescue the lethal allelic combination of $punt^{135}$ and *Δpunt* by wildtype Punt resulted in lethality. Rescue by Punt with *Apis* PTD (Punt[apis]) resulted in an increase in viability compared to the wildtype rescue. Surprisingly, when Punt was mislocalized to the apical surface (Punt[wit]), full rescue was observed.

Table 1. Homotypic rescues of Punt by Punt. When Punt with or without the PTD is used to rescue the lethal allelic combination of *punt*^{135} and *Δpunt*, full rescue only occurs when Punt is mislocalized apically (Punt[wit]).

Phenotypes	**# Rescued**	**Total #**	**% Rescue**[1]
UAS-Punt[punt], *punt*^{135} *arm*>Gal4, *Δpunt*	3	64	15%
UAS-Punt [apis], *punt*^{135} *arm*>Gal4, *Δpunt*	27	242	33%
UAS-Punt [wit], *punt*^{135} *arm*>Gal4, *Δpunt*	27	79	100%

[1] Rescue is defined as the number of live adults divided by the Mendelian number of expected adults

The *wit*^{a12} allele was recombined with wildtype Wit (Wit[wit]), *Apis* Wit (Wit[apis]), or Wit with juxtamembrane PTD (Wit[punt]) and crossed to *elav*>Gal4; *wit*^{b11} to test the homotypic rescue of Wit (Table 2). Because Wit is mainly expressed and functional in the nervous system, *elav*>Gal4 was used for Wit rescue experiments (Marques et al. 2002). Full rescue was observed in all cases.

Table 2. Homotypic rescues of Wit by Wit. Full rescue occurs regardless of the juxtamembrane Wit sequence.

Phenotypes	**# Rescued**	**# Total**	**% Rescue**[1]
elav>Gal4; UAS-Wit[wit],*wit*^{a12} / *wit*^{b11}	31	108	>100% [2]
elav>Gal4; UAS-Wit[apis],*wit*^{a12} / *wit*^{b11}	56	267	>100%
elav>Gal4; UAS-Wit[punt],*wit*^{a12} / *wit*^{b11}	9	34	>100% [2]

[1] Rescue is defined as the number of live adults divided by the Mendelian number of expected adults

[2] Rescue above 100% is possible when working with balancers which are often inherited at less than expected Mendelian ratios.

To test for cross rescue of Wit by Punt, first recombinants were made. Wildtype Punt (Punt[punt]) and Punt with Wit juxtamembrane sequence (Punt[wit]) were recombined with wit^{a12} and then crossed to *elav*>Gal4;wit^{b11} to test for rescue (Table 3). Punt with wildtype PTD did not rescue the allelic lethality of the wit^{a12}/wit^{b11} combination. Punt with juxtamembrane PTD replaced by juxtamembrane Wit, which mislocalizes apically in wing discs, resulted in full rescue.

Table 3. Heterotypic rescue of Wit by Punt. Wildtype Punt is unable to rescue Wit, but apically mislocalized Punt can rescue the lethal allelic *wit* combination.

Phenotypes	**# Rescued**	**# Total**	**Rescue[1]**
elav>Gal4; UAS-Punt[wit],$\frac{wit^{a12}}{wit^{b11}}$	78	192	>100%[2]
elav>Gal4; UAS-Punt[punt],$\frac{wit^{a12}}{wit^{b11}}$	1	204	0%

[1] Rescue is defined as the number of live adults divided by the Mendelian number of expected adults
[2] As above, rescue above 100% is possible when working with balancers

To test for rescue of Punt by Wit, recombinants were made by combining the $punt^{135}$ allele with wildtype Wit (Wit[wit]), Wit with *Apis* juxtamembrane sequence (Wit[apis]), or Wit with juxtamembrane PTD (Wit[punt]). These recombinants were then crossed to *da*>gal4 recombined with *Δpunt* to test for rescue (Table 4). Wit with PTD did not rescue the Punt allelic lethality, while wildtype Wit did rescue. Wit with *Apis* juxtamembrane sequence did not give full rescue, but showed more viability than Wit with PTD.

Table 4. Heterotypic rescue of Punt by Wit. Wildtype Wit gives full rescue of Punt, while basolaterally mislocalized Wit is lethal.

Phenotypes	**# Rescued**	**# Total**	**Rescue**[1]
UAS-Wit[wit],*punt*^{135} da>Gal4, *Δpunt*	102	244	>100% [2]
UAS-Wit[apis],*punt*^{135} da>Gal4, *Δpunt*	14	138	30%
UAS-Wit[punt],*punt*^{135} da>Gal4, *Δpunt*	2	179	3%

[1] Rescue is defined as the number of live adults divided by the Mendelian number of expected adults
[2] As above, rescue above 100% is possible when working with balancers

Point mutations in the PTD do not alter basolateral localization

To determine whether a subset of residues within the 19 amino acid PTD were the critical residues for basolateral localization, overlapping sets of point mutations in the PTD were made and assayed for localization. When sets of three amino acids were mutated into three alanines, all of the mutants (FNE, EIP, and PTE) maintained wildtype basolateral localization and did not colocalize with the apical marker PKC (Figure 7). Non-canonical basolateral targeting sequences have been found that are charge dependent, but not sequence specific (Wolff et al. 2010). To determine if PTD function was dependent on charge, all charged residues within the PTD were simultaneously changed to alanines and transgenic fly lines were established. Staining with the apical marker PKC showed that Punt with no charged residues in the PTD still remained basolateral (Fig 7).

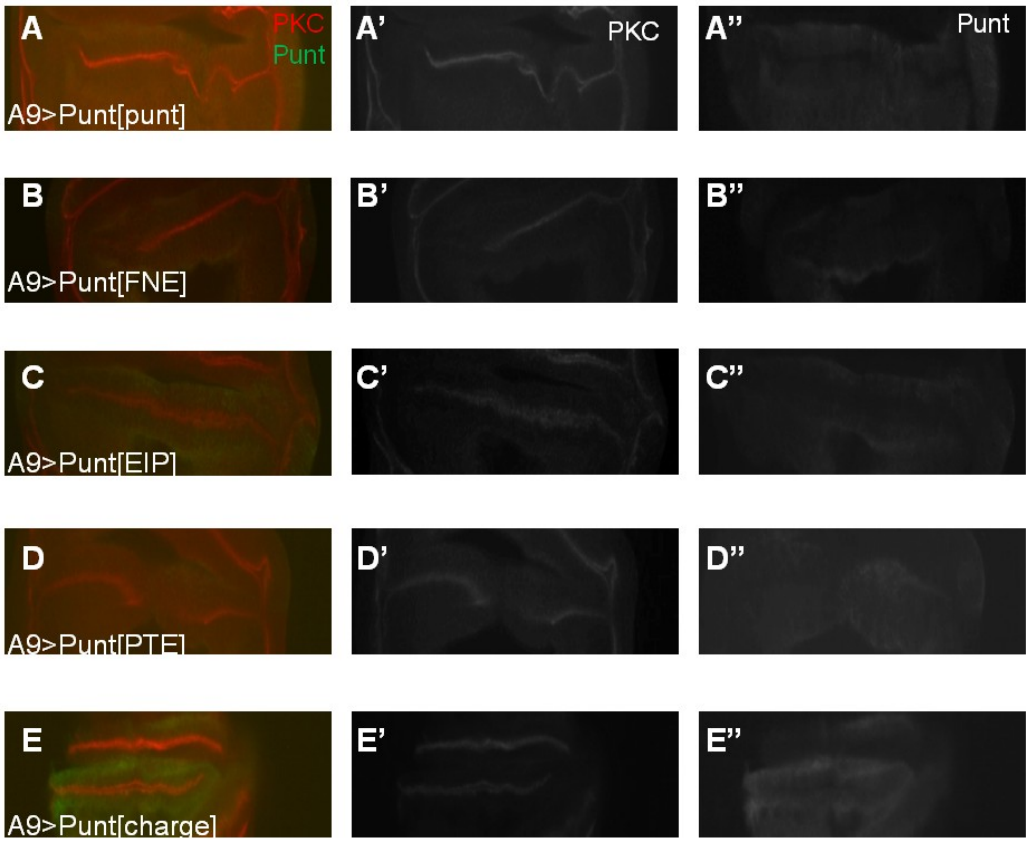

Figure 7. Point mutations to the PTD do no alter basolateral localization. L3 wing discs were dissected and stained for apical PKC (red) and Punt (green). A.) Wildtype Punt is basolateral A'.) Single channel shows apical PKC stripe. A".) Punt is basolateral, avoids the apical stripe. B.) Mutation of the FNE residues of the PTD did not alter basolateral localization. B', B".) Punt localization does not overlap apical stripe. C.) Mutation of the EIP residues of the PTD did not alter basolateral localization. C',C".) Again, Punt does not overlap with apical stripe. D.) Mutation of the PTE residues did not change wildtype basolateral localization. E.) Mutation of all the charged residues within the PTD (three Es) did not alter basolateral localization. E', E".) Punt is enriched throughout the disc but is absent from the apical stripe and does not overlap with the PKC marker. Images are Z sections taken at 20x on a confocal microscope with a CARV attachment. Images are cropped to only show pouch of wing disc containing apical folds (stripes).

Mutation of the insect-conserved portion or whole PTD leads to apical mislocalization

Because overlapping sets of residues within the PTD did not show sequence specificity, mutations were made to cover either the insect-conserved portion or whole PTD to determine whether any sequence specificity exists within the PTD. When the FNEIPTE sequence was changed to all alanines, mislocalization of Punt to the apical surface was observed, as indicated by colocalization with the PKC apical marker. When the entire PTD (AHFNEIPTHEAEITNSSPL) sequence was changed to alanines, apical mislocalization was also observed (Figure 8). As was observed with the Δ10 and Δ19 deletions mentioned above, the mutation of the whole PTD consistently showed more prevalent apical mislocalization compared to mutation of the insect-conserved portion.

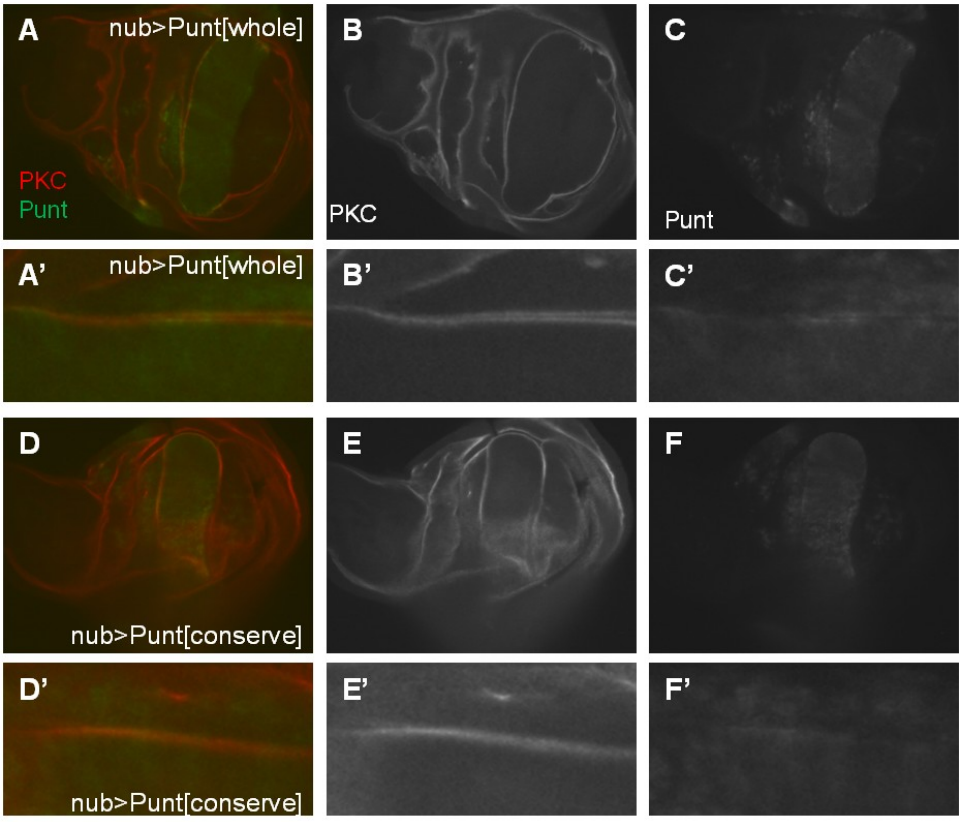

Figure 8. Mutation of the insect-conserved portion and whole domain of the PTD results in apical mislocalization. L3 wing discs were dissected and stained for PKC (red) and Punt (green). A, A') When the whole domain of the PTD was mutated to all alanines, speckles can be seen where the receptor colocalizes with apical PKC. B,B', E, E') Single channel images show PKC marking apical folds. C,C') Single channel image shows enrichment of Punt where apical stripe forms when the whole PTD is mutated. D,D'.) Mutation of the insect-conserved residues of the PTD results in mislocalized Punt at the apical fold. F,F') Single channel image again shows that Punt is enriched at the apical folds when the insect-conserved residues of the PTD were mutated.

Discussion

PTD and mammalian sequence comparison

The main goal of my work was to characterize a unique putative basolateral targeting sequence that our lab found within the *Drosophila* TGF-β type II receptor Punt. The Punt targeting domain (PTD) differs from the mammalian basolateral targeting domain of the type II TGF-β receptor in both location and sequence. The mammalian targeting domain was found at the C-terminus of the receptor (Murphy et al. 2007), while the PTD was found intracellulary, adjacent to the transmembrane domain of Punt. Additionally, the mammalian targeting sequence contained a consensus sequence of LTAxxVAxxR, while the PTD contains the residues AHFNEIPTHEAEITNSSPL, within which the FNEΦPTxE (Φ is a bulky hydrophobic acid) sequence shows conservation among insects. Both of these sequences differ from the known canonical basolateral targeting domains of YxxΦ or LL. This could mean that the non-canonical basolateral targeting sequences of the type II receptors are recognized by a different molecular trafficking complex than the canonical basolateral targeting sequences. Future studies will focus on the mechanism of function of the PTD in targeting and trafficking and may identify unique trafficking partners.

The PTD is necessary and sufficient for basolateral localization

To better understand how the PTD targets the receptor to the basolateral compartment, I relied on a series of domain swaps and mutations in the PTD. Deletion of the entire PTD resulted in apical mislocalization of Punt, both in mammalian cell culture and in wing discs (Steve Murphy & Ed Leof unpublished, Aidan Peterson unpublished). The PTD could have been a nonspecific spacer sequence that, upon deletion, changed the conformation of Punt and prevented proper Punt sorting and localization. To

eliminate the possibility that the missing juxtamembrane sequence was responsible for the mislocalization, a corresponding Wit juxtamembrane sequence was added in place of the PTD. When juxtamembrane Wit was added to Punt, Punt mislocalized to the apical surface, consistent with the behavior of the deletion constructs and illustrate that the PTD is necessary for basolateral Punt localization. To test whether the PTD is sufficient for basolateral transport, the PTD was substituted in the juxtamembrane location of Wit. The presence of the PTD was enough to override the wildtype apical localization of Wit and take it to the basolateral surface. Another test for the sufficiency of the PTD was conducted by adding the PTD at the C-terminus of Punt in which the endogenous juxtamembrane PTD was deleted. In this case, the C-terminal PTD did not direct Punt to the basolateral surface and Punt was found apically, as is seen with the juxtamembrane Punt deletions, perhaps indicating that in order to properly target to the basolateral surface, the PTD needs to be located adjacent to the transmembrane domain of the receptor. If the location of the PTD next to the membrane is important to its function, it might indicate that a membrane-associated factor acts with the PTD to target and traffic Punt to the basolateral surface. Future studies will aim to discover the cellular machinery that acts with the PTD by a biochemical or genetic screen.

Secondary structure of the PTD may contribute to function

It is curious that none of our three residue changes affected localization in *Drosophila* and that the *Drosophila* sequence was recognized by mammalian localization machinery. It is possible that the PTD forms a specific secondary structure that allows the PTD to function with cellular machinery. Mammalian cellular trafficking machinery might recognize the shape of the PTD and transport it as it would with mammalian cargo. Analysis of the secondary structure of the 19 amino acids of the PTD (by YASPIN secondary structure prediction, Centre for Integrative Bioinformatics VU) reveals that this

domain most likely forms a coil, with a beta strand possible at the AEI residues. It is interesting that the secondary structure changes from a coil to a beta sheet at the AEI residues, as this is the area just after the insect-conserved residues and is not conserved itself. If this beta sheet is important to function in targeting the receptor, we would expect conservation to preserve function. Further work on the structure of the PTD will be helpful to give insight into how it functions.

Homotypic Punt rescue

The attempt at homotypic rescue of Punt by replacing it with constitutively overexpressed Punt resulted in lethality, likely due to the generation of an ectopic, ligand-independent signal. Discovering that Punt without the PTD, which mislocalized apically, was viable was surprising, as the apical lumen has been reported to contain uniformly Dpp (Gibson et al 2002). We expected that the ligand would be able to access the mislocalized Punt and signal ectopically. I propose three possible explanations to explain the viability, 1) There is little or no type I receptor Tkv on the apical surface, 2) the level of Dpp in the lumenal compartment is lower than we think, 3) there is an unknown protein at the apical surface that prevents Dpp binding to Punt (and possibly Wit) or blocks the signaling activity of the receptor complex.

Heterotypic rescue of *punt* mutants *by* Wit

In the heterotypic rescue experiments of *punt* mutants by different Wit constructs, I also observed that basolateral localization led to ectopic signaling and lethality, since Wit with the PTD killed the flies, while wildtype Wit was viable. Wit is enriched apically but does leak basolaterally, while wildtype Punt always obeys the basolateral boundary. In the rescues, overexpression of wildtype Wit allows enough leaky Wit to get to the basolateral surface to rescue, but is not enriched there which prevents too much signaling and lethality.

Homotypic Wit rescue

Homotypic rescue of *wit* mutants by different Wit constructs did not vary between the three, showing that Wit juxtamembrane sequence is not necessary for viability.

Heterotypic rescue of *wit* mutants by Punt

When heterotypic rescues of Wit by different Punt constructs were performed, wildtype Punt showed lethality, likely due to ectopic signaling; the inability of Punt to rescue Wit matches what has been reported (Marques et al. 2002). Unlike wildtype Punt, Punt without the PTD (which mislocalized apically in wing discs) showed rescue in neurons. This result is exciting because Punt without the PTD seems to be behaving similarly to Wit to allow rescue, where rescue has not previously been observed. This could mean that in neurons, the presence of wildtype PTD in Punt prevents Punt from rescuing Wit defects. This agrees with our hypothesis that differential localization of the two type II receptors may be preventing them from substituting for each other, but future colocalization studies carefully characterizing both Wit and Punt subcellular locations in neurons must be performed to confirm this.

Although the results of the heterotypic rescue of Wit by Punt are promising, the rescues to determine if altering localization of Wit or Punt could allow for cross rescue need to be repeated using endogenous expression, to fully answer the question of whether differential localization is responsible for differences in receptor function. When the presence of the wildtype Punt is no longer lethal, we should be able to tease apart what is really going on to fully understand the importance of the different localizations in determining lethality or viability.

Separation of ligand and receptor controls signaling

Trafficking signaling components to specific subcellular compartments represents a level of control in signaling pathways. In mammalian cell culture, TGF-β type II

receptors are found basolaterally and signaling occurs at the basolateral surface (Murphy et al. 2004, Murphy et al. 2007). Basolateral TGF-β receptor localization is important to prevent ectopic autocrine signaling that might lead to developmental defects including lethality. In *Drosophila*, the TGF-β ligand Decapentaplegic (Dpp) is expressed at the midline of the developing wing disc, while the type II TGF-β receptors Punt and Wit are found basolaterally and apically, respectively. Dpp signaling in the wing disc appears to primarily invoke Punt (Letsou et al. 1995, Ruberte et al. 1995). The opposing localizations of Punt and Wit may account for the functional differences between the two. If Wit is not in the right location to signal through Dpp, it cannot rescue Punt lethality. Future rescue experiments will seek to endogenously express mislocalized receptors to test for cross-typic rescue to answer this question.

Punt may be sorted after internalization

Another interesting aspect of the juxtamembrane location of the PTD is its similarity to internalization sequences. In the mammalian type II TGF-β receptor, mutations in the juxtamembrane sequence delay internalization and as a result enhance TGF-β signaling (Park et al. 2012). It is possible that Punt is sorted only after internalization, mediated by the PTD. There could be factors associated with the plasma membrane that interact with the PTD to sort and traffic specifically to the basolateral surface. If this were the case, transient apical localization of Punt may be observed. This seems to be an unlikely scenario, as in mammalian cell culture neither the mammalian type II receptor nor Punt show transient apical localization. In wing discs, wildtype Punt is clearly absent from the apical compartment, but with the limited resolution of our colocalization assay, we cannot definitively rule out at this point the possibility of transient apical localization in wing discs.

Because the juxtamembrane sequences of the mammalian type II TGF-β receptor and Punt are vastly different, the presence of an internalization sequence in the mammalian receptor does not mean Punt juxtamembrane sequence (the PTD) behaves the same. Interestingly, the proposed mechanism of internalization of the mammalian type II TGF-β receptor is lipid raft-dependent and not clathrin-dependent (Park et al. 2012). Canonical basolateral targeting sequences work in a clathrin-dependent manner, while some apical targeting sequences work via lipid raft-dependent mechanisms (Carmosino et al. 2010, Cao et al. 2012). It seems unlikely that the basolateral targeting PTD would behave like the mammalian juxtamembrane sequence, and utilize lipid rafts, to arrive at the basolateral surface.

Future work should look at the effects of endocytosis on Punt localization by utilizing endocytosis blocking factors to determine if Punt transport to the basolateral surface requires internalization. Differences in endocytosis between wildtype Punt and Punt with a mutated PTD should also be examined. Also, future experiments could look for Punt, with or without the PTD, colocalization with endosomal markers to determine if the function of the PTD depends on internalization. If Punt basolateral localization is found to be internalization dependent, known factors involved in internalization will be tested for interaction with the PTD.

The PTD function may be wing disc-specific

My studies have looked exclusively at localization in the wing discs, but there are several other polarized epithelial tissues in *Drosophila* that could be used to examine if, like the wing disc, these tissues also show the differential localization of Punt and Wit. Wit is the type II TGF-β receptor responsible for signaling in the neuromuscular junction, while Punt is the more prominent receptor elsewhere (Marques et al. 2002). It is possible that differential localization of Punt and Wit may be responsible for the inability of Punt to

rescue Wit mutations. While the rescue experiments I performed address this issue, final answers about rescue ability will only be answered when the receptors are expressed at endogenous levels. It is possible that the PTD works specifically in wing discs, although this would be a peculiar finding, because Punt localizes properly to the basolateral surface in mammalian cell culture, showing that although there is no sequence conservation between the PTD and mammals, there is functional conservation and the mammalian trafficking machinery that recognizes the PTD.

Summary

In summary, Punt is localized to the basolateral membrane by the PTD, thereby limiting Dpp signaling to the basolateral surface. This is important for growth and patterning of the wing disc during development because inappropriate Dpp signaling leads to wing defects and lethality. Additionally, my results have shown that the unique PTD is both necessary and sufficient (when in the proper membrane location) for Punt basolateral localization. The insect-conserved portion of the sequence (10 amino acids) is the minimal sequence for basolateral targeting but to get specific basolateral targeting the whole 19 amino acid domain may be required. There is no single, specific residue within the PTD that is crucial for basolateral transport. My findings also show that differential localization of the two type II *Drosophila* TGF-β receptors Punt and Wit may contribute to the inability of each receptor to substitute for the other. Future work on the PTD will be of interest to determine the trafficking mechanism utilized for basolateral transport. Uncovering the trafficking components will give insight into the functional conservation that is observed between Punt and the mammalian type II receptor as both localize basolaterally, although the targeting sequences each receptor utilizes are very different.

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