

**Exploring the interchangeability of β -catenin binding sites of APC2 in the
 β -catenin destruction complex and in colon cancer.**

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I. Abstract

The Wnt signaling pathway is a conserved pathway that contributes to normal development and adult homeostasis, but its misregulation often results in human diseases such as colon cancer. Studies have found that 80% of colon cancer cases have truncated mutations in APC, a key component of the destruction complex. 30 years of research have shown that APC is a negative regulator of β -catenin by targeting β -catenin destruction in the destruction complex, but its mechanistic roles remain unclear. Several models for APC's roles have been proposed, many of which focus on the importance of β -catenin binding sites. Past research in Roberts's lab showed that APC2 that lacks all β -catenin binding sites fails to destroy β -catenin in the cells that receive the Wnt signal, suggesting that direct interaction between APC2 and β -catenin is required in those cells. We hypothesized these β -catenin binding sites' functions are simple to capture and recruit the β -catenin to the destruction complex; therefore, any β -catenin binding sites should be sufficient for this role. Hence, we tested this model by generating APC2 that deletes all β -catenin binding sites and adds back other proteins' β -catenin binding sites and performing functional studies in Drosophila embryos. In the APC2 single mutant background, our results pointed out that α -catenin's β -catenin binding sites fail to rescue the Wnt phenotypes, but Axin's β -catenin binding sites and TCF's β -catenin binding sites are able to partially rescue the Wnt phenotypes. Those β -catenin binding sites that partially rescue bind to Armadillo Region Repeat just like APC. In the APC2 APC1 double mutant background, none of these APC2 transgenes appear to rescue the β -catenin destruction, but some of them are able to rescue the Wnt signaling phenotype through cytoplasmic retention. We suggest that specific contacts to β -catenin might be required for cytoplasmic retention and complete overlapping with APC2's β -catenin binding sites might be required to rescue the β -catenin destruction.

II. INTRODUCTION

Colon cancer and its correlation to the Wnt signaling pathway

The Wnt signaling pathway is one of the six conserved pathways that regulate animal development but is frequently misregulated in human diseases (Clevers & Nusse, 2012). In fact, the Wnt signaling pathway plays a critical role in cell fate decisions and embryonic development as it controls cell proliferation, cell apoptosis, cell migration, and stem cell maintenance in adults. However, misregulation of the Wnt signaling pathway often results in uncontrolled cell proliferation and cellular immortality, dramatically contributing to cancer progression. Studies have found that truncating mutations in adenomatous polyposis coli (APC) - a tumor suppressor gene - inappropriately activate the pathway and account for more than 80% of colon cancer cases; therefore, it is essential to establish and study APC's function. Nearly 30 years of research have confirmed the role of APC as a negative regulator of Wnt signaling that participates in a multi-protein destruction complex that targets β -catenin for ubiquitination and destruction. β -catenin is a transcription factor responsible for the signal transduction in the nucleus to trigger the transcription of the Wnt-specific genes, such as cyclin D1 or c-Myc, which control cell fate decisions in many cells and tissues (Valenta et al, 2012). Therefore, the accumulation of β -catenin contributes to the hyperactivity of the cell cycle and uncontrolled cell proliferation, eventually leading to cancer development.

General outline for the Wnt signaling pathway

To understand how Wnt signaling's misregulations contribute to cancer progression, it is imperative to understand the fundamental mechanisms of the Wnt signaling pathway and a general outline is established with a focus on what occurs in the presence and absence of the Wnt signals. In the absence of Wnt signaling, the destruction complex assembles, which consists of APC, the scaffold protein Axin, the kinases Casein Kinase 1 (CK1), and Glycogen

Synthase Kinases 3 (GSK3) (Logan & Roel, 2004). Axin is considered to be the scaffold because it contains the binding site for APC, GSK3, CK1, and β -catenin. Once the destruction complex is formed, β -catenin is recruited to the destruction complex, allowing interactions between CK1, GSK3, and β -catenin. Specifically, β -catenin is phosphorylated first by CK1 at S45, which serves as a primed site for the phosphorylation by GSK3 at S33/S37/T41. These serine phosphorylations are then recognized by an SCF family E3 ubiquitin ligase containing the F-box protein β TrCP, which polyubiquitinates β -catenin and targets it for destruction in the 26S proteasome. Therefore, the level of β -catenin remains low, allowing the TCF/LEF family member to bind to the transcriptional repressor, Groucho, preventing the transcriptional activation of Wnt target genes.

On the other hand, in the presence of Wnt signal, the Wnt ligand binds to the receptor complex consisting of the seven-pass transmembrane protein Frizzled (Fz) and the single-pass LRP5/6 receptor (Li et al, 2012), leading to the inactivation the destruction complex. Specifically, the binding of Wnt to the Fz and LRP5/6 receptor complex results in the phosphorylation of the cytoplasmic tail of LRP5/6 by CK1 and GSK3, which is thought to recruit Axin away from the destruction complex, although other research suggests that the destruction complex remains intact. Moreover, the binding of Wnt ligands to Fz and LRP5/6 also results in the activation of the phosphoprotein Dsh, even though the exact mechanism of Dsh activation is unknown. Once Dsh is activated, it binds and inhibits the GSK3 enzyme, preventing GSK3 from assembling in the destruction complex. Since Axin serves as the scaffold of the destruction complex and GSK3 is needed to phosphorylate β -catenin, when they are recruited away, the destruction complex is inhibited, resulting in hyper-accumulation of β -catenin and translocation of β -catenin into the nucleus (Figure 1). However, the β -catenin translocation mechanism into the nucleus is poorly understood since β -catenin contains no consensus nuclear localization sequence (NLS) (Fagotto et al, 1998). Recently, it has been discovered that the Armadillo Repeats 10-12 can

mediate nuclear import and export through the interaction with nuclear pore complex (NPC) proteins such as cytoplasmic protein Nup 358 and central channel protein Nup62 (Jamieson et al, 2014). Once β -catenin enters the nucleus, it displaces the Groucho repressor and interacts with the TCF/LEF proteins, converting them from transcriptional repressors to activators. To be more specific, β -catenin and TCF/LEF function as bipartite transcription factors to induce the Wnt target gene expressions such as c-Myc, Cyclin D1, and many other target genes.

Even though APC is a well-established negative regulator of β -catenin, its mechanistic role in the destruction complex remains unclear. Several models for APC's role have been proposed and these have focused mainly on the β -catenin binding sites. Human APC is a 312-kDa multidomain protein that contains many domains, some of which are Armadillo repeats, which bind to Cytoskeletal Regulators such as KAP3 and ASEF, 15Rs and 20Rs, which bind to β -catenin, and SAMPs motifs, which bind to Axin (Figure 2). Each 15R binds to the structural groove formed by the Armadillo 5-9 of β -catenin in a phosphorylation-independent manner (Minde et al, 2011). The core 20Rs can also interact with the Armadillo 5-9 through the salt bridge interaction between two negatively charged residues in the N-terminal flanking region and K435 and K312 of β -catenin, in which this interaction is normally referred to as the charged button. In addition, the 20Rs require phosphorylations on the SxxxSLSSL motif to interact with β -catenin, and Axin also contains this motif to interact with Armadillo repeat 3-4 (Ha et al, 2004). In fact, biochemical studies have shown that CK1/GSK3 can phosphorylate the APC 20Rs and that phosphorylated 20Rs demonstrate a 1500-fold higher binding affinity for β -catenin than that of unphosphorylated 20Rs or the 15Rs (Liu et al, 2006). The researchers also demonstrated that 20R3 has the strongest binding to β -catenin, which increases 140-fold after phosphorylation. Once 20R3 gets phosphorylated, the phosphorylated motif plus N-terminal flanking sequences can occupy the entire groove that spans from Armadillo Repeat 1 to Repeat 12, which overlaps with other binding partners such as TCF and E-cadherin (Minde et al, 2011).

This result suggests that these proteins cannot bind β -catenin simultaneously, further suggesting competition in binding.

Proposed destruction complex models

Several models of β -catenin destruction complexes have been proposed, and all of them propose that 20Rs phosphorylation is crucial for the destruction complex. I will briefly present four destruction complex models, each suggesting different roles for APC.

Catalytic Cycle Model: This model was based on biochemical work that studied the molecular affinity of Axin and APC for β -catenin. Once APC, Axin, CK1, and GSK3 assemble, β -catenin is recruited and bound to Axin's single β -catenin binding site, and one of the three APC 15Rs repeats. The binding of β -catenin to Axin and 15Rs of APC positions the N-terminus of β -catenin in the proximity of CK1 and GSK3, leading to the β -catenin phosphorylation. Their data suggest that Axin's single β -catenin binding site has a higher binding affinity than unphosphorylated 20Rs of APC, so when CK1/GSK3 phosphorylates the 20Rs, phosphorylated β -catenin is then transferred from Axin to APC 20 Rs repeats, especially 20R3 due to its highest binding affinity to β -catenin based on Xu's group research (Liu et al, 2006). The β TrCP then binds to the N-terminus of β -catenin, resulting in β -catenin's ubiquitination. They further highlight that PP2A can dephosphorylate the 20Rs, causing conformational change to APC that releases the β -catenin to be degraded in the 26S proteasome (although Ha et al. demonstrate that the 20Rs bound to β -catenin cannot be dephosphorylated by PP2A over hours). Overall, by displacing phosphorylated β -catenin from Axin, APC allows adding new β -catenin to Axin. In summary, this proposed catalytic cycle suggests the main role of APC is to allow new cycles of recruiting β -catenin to Axin to be destroyed in the destruction complex.

Protection from Dephosphorylation Model: Su and colleagues proposed a different view of APC2's function in the destruction complex but agreed with Kimmelman and Xu's group

that 20Rs phosphorylation was crucial to APC's function. To test APC's function in regulating β -catenin phosphorylation and destruction, they decided to perform IP using DLD1 cell types, in which mutant APC cannot promote β -catenin degradation, either with or without myc-tagged APC. The IP result showed that β TrCP E3 ligase was not co-immunoprecipitated with β -catenin in cells without APC. Still, their interaction was restored with APC, indicating APC's role in mediating their interaction. By incubating free Flag-phosphorylated- β -catenin in DLD1 extract, they failed to detect phosphorylated β -catenin, suggesting β -catenin gets dephosphorylated during its incubation with DLD1 extracts and prompting them to believe APC protected the β -catenin from PP2A-mediated destruction. To evaluate their hypothesis, free and myc-APC phosphorylated β -catenin was incubated with PP2A core enzyme, and the result showed that free phosphorylated β -catenin was rapidly dephosphorylated, while the APC associated β -catenin was unaffected, indicating APC's role in preventing PP2A dephosphorylation of beta-catenin. APC 20 Rs phosphorylation has been suggested to increase APC binding and function, so they generated phosphorylated and unphosphorylated APC fragments containing both 20Rs and SAMP repeats and incubated them with phosphorylated β -catenin. As predicted, only phosphorylated APC could protect the phosphorylated β -catenin, while the other one failed to do (Su et al, 2008). Therefore, the APC's main role is to protect β -catenin from PP2A dephosphorylation and ensure that phosphorylated β -catenin is recognized by the SCF β TrCP E3 ligase and destroyed in the 26S proteasome.

Controlling β -catenin release rate model: Unlike the before-mentioned groups, Weis's group suggests that APC phosphorylation controls the rate at which β -catenin is released from the destruction complex (Ha et al, 2004). They suggest that the binding of Axin and CK1, and GSK3 to APC results in APC phosphorylation. In the absence of Wnt signals, cytosolic β -catenin binds to phosphorylated APC, resulting in the β -catenin-pAPC dimer getting recruited to the destruction complex. Because β -catenin binds tightly to pAPC, it slowly releases and sequesters

β -catenin. On the other hand, In the presence of Wnt signals, APC phosphorylation is decreased because of Axin recruitment away from the destruction complex, and the binding of β -catenin to unphosphorylated APC becomes significant. In the destruction complex, because of weak and non-specific interactions between APC and β -catenin, β -catenin gets phosphorylated, released quickly, and recognized by SCF β TrCP E3 ligase (Ha et al, 2004). In other words, the APC's main role in this model is not only to control the rate at which β -catenin is released from the destruction complex but also to control the rate at which β -catenin gets destroyed.

Regulating β -catenin's recruitment, phosphorylation, and ubiquitination: Rather than focusing on APC phosphorylation, Guettler's group focuses more on how Axin polymerization and APC modulate β -catenin capture, phosphorylation, and ubiquitylation (Ranes et al, 2020). They are one of the first groups to reconstruct the complex biochemically: adding all components of the destruction complex together in a virus and injecting the virus into insect cells. By conducting IP with WT Axin and non-polymerizable Axin, Axin was mutated in the Dix domain to avoid self-polymerization with itself, they discovered that the non-polymerizable Axin reduced β -catenin's recruitment, indicating that Axin polymerization is important to recruit β -catenin. To test the importance of APC, they conducted IP experiments with a reconstructed destruction complex containing WT APC, destruction complex containing mutant APC, and destruction complex without APC to observe different effects on β -catenin recruitment and β -catenin phosphorylation. The results showed that the destruction complex-WT APC had the highest level of β -catenin's recruitment and phosphorylation, whereas the destruction complex-lacking APC had the lowest β -catenin's recruitment and phosphorylation, further suggesting APC's role in mediating β -catenin's level in the destruction complex. Besides, APC can also mediate β -catenin's ubiquitination through a direct interaction with SCF $^{\beta-TrCP}$. Overall, Guettler's group suggests that APC plays an important role in regulating β -catenin's recruitment, β -catenin's phosphorylation, and β -catenin's ubiquitination.

A structure/ functional approach to investigate APC's function

Roberts's lab tested the importance of these different models of APC function by generating a series of Drosophila APC2 mutants that remove either some or all β -catenin binding sites. When testing an APC2 mutant that lacks all beta-catenin binding sites, they found that β -catenin was still destroyed in cells that don't receive the Wnt signals but accumulated in stripes in cells that receive the Wnt signals (Yamulla et al, 2014). These two results prompted us to believe that a direct binding between APC2 and β -catenin is crucial in the cells that do receive the Wnt, further highlighting the importance of β -catenin binding sites. We hypothesize that the β -catenin binding site's role. Prior work suggested that Axin that lacks β -catenin can fully rescue activity of the destruction complex, indicating that Axin's β -catenin binding site is also dispensable for β -catenin destruction in some cells (Peterson-Nedry et al, 2008). According to these two results, in the cells that don't receive the Wnt signals, it does not matter whether APC2 has β -catenin binding sites or not, but in the cells that do receive the Wnt signals, direct interactions between APC2 and β -catenin are required. In other words, why do APC2's β -catenin binding sites are required in the cells that receive the Wnt signals? We hypothesize that these APC2's β -catenin binding sites functions are to recruit and dock the β -catenin to the destruction complex, then it is possible to substitute APC2's β -catenin binding sites to other proteins' β -catenin binding sites. Therefore, we hypothesize that β -catenin binding sites are partially interchangeable between different proteins, so other proteins' β -catenin binding sites can substitute for the APC's β -catenin binding sites. The bulk of my project will focus on the interchangeability of β -catenin binding sites in the cell that do receive the Wnt signals.

Drosophila as a model to explore APC function in the destruction complex

To test our hypothesis, we decided to use *Drosophila Melanogaster* as our model organism because their Wnt signaling pathway is conserved throughout metazoans. *Drosophila*, similar to humans and other vertebrates, have two conserved APC proteins (dAPC1 and dAPC2) that both play an important role in regulating Wnt signaling in the embryonic epidermis and imaginal discs (Akong et al, 2002). Both dAPC2 and dAPC1 share a highly conserved region comprising Armadillo repeats, CID, a combination of 15 Rs and 20 Rs, and a series of SAMP motifs (Figure 2). Analysis of null alleles revealed that dAPC2 plays a more important role in regulating the Wnt signaling pathway than dAPC1. Specifically, Akong's et al. demonstrated that dAPC2 single mutants are embryonic lethal with Wnt activation phenotypes. In contrast, the dAPC1 single mutants are adult viable with Wnt phenotypes restricted to photoreceptors in their eyes (Akong et al, 2002) (Ahmed et al, 1998). Furthermore, in the dAPC2 dAPC1 double mutant background, β -catenin levels accumulate dramatically, whereas, in the dAPC2 single mutant, β -catenin levels only accumulate slightly, partly due to the residual dAPC1 activity, further suggesting that dAPC2 and dAPC1 are partially redundant (McCartney et al, 1999). In addition to sharing important domains and motifs of dAPC and human APC, *Drosophila* is a great genetics tool because there are specific experiments that can only be conducted using *Drosophila*. Besides, they can produce a huge amount of embryos in a short period of time.

These reasons further highlight *Drosophila* is a great tool for our proposed research and allow us to select both human colon cancer cell line SW480 and *Drosophila* as our model to study APC function in regulating β -catenin in the destruction complex with a focus on dAPC2 in the APC2 single mutant and APC2 APC1 double mutant background. In our lab, dAPC2 that removes all β -catenin binding sites are able to destroy β -catenin in SW480 cells, which suggests that dAPC2 may dimerize with the truncated APC as human APC are thought to self-dimerize (Joslyn et al, 1993) and dAPC2 has been shown to self-dimerize in immunoprecipitation experiment (Roberts et al, 2011). Therefore, to test our hypothesis that exogenous dAPC2 can

dimerize with truncated human APC, we conducted immunoprecipitation and found out that both truncated APC and dAPC2 can co-immunoprecipitate. Therefore, we decided to focus on focus on Drosophila rather than using the SW480 cell lines because, in the Drosophila system, we can generate flies that are completely devoid of endogenous dAPC2 and dAPC1, preventing endogenous dAPC2 from dimerizing and interfering with transgenes' functions. When designing dAPC2 mutants that delete all β -catenin binding sites, it is important to note that previous biochemical experiments showed that both phosphorylated and unphosphorylated 20R2 lacks β -catenin binding affinity (Liu et al, 2006) because the 20R2 is missing an upstream critical amino acid that can interact with the Armadillo groove of beta-catenin. Roberts's et al. demonstrated that the 20R2 is crucial for the β -catenin destruction complex, as embryos expressing dAPC2 Δ 20R2 have β -catenin accumulation across all cells, implying that the whole destruction complex is deactivated (Roberts et al, 2011). Moreover, 20R2 is a highly conserved sequence across various species, such as sea snails, fruit flies, and humans (Roberts et al, 2011). Therefore, these results indicate that 20R2 is crucial for the destruction complex functions and cannot be removed. In terms of designing dAPC2, these dAPC2 transgenes were generated by knocking out β -catenin binding sites but keeping the 20R2 intact.

Hypothesis

Previous research showed that APC2 lacking all β -catenin binding sites fail to destroy β -catenin in cells that do receive the Wnt signals, suggesting that direct interaction between APC2 and β -catenin is required for cells that receive the Wnt signals (Yamulla et al, 2014). We hypothesize that β -catenin binding sites' functions are simply to recruit and dock β -catenin in the destruction complex, then in theory, any β -catenin binding sites might be functional in cells that receive the Wnt signals, further indicating that APC2's β -catenin binding sites can be substituted with other protein's β -catenin binding sites. To test the interchangeability between different

proteins' β -catenin binding sites, we decide to conduct experiments with three candidate genes: Axin, which is a member of the destruction complex, TCF, which binds to β -catenin in the nucleus to activate the transcription, and α -catenin, which plays an important role in cell adherent junction with β -catenin. Therefore, we decided to generate APC2 constructs that remove all APC2's β -catenin binding sites and add back other proteins β -catenin binding sites. Specifically, these APC2 constructs are generated: APC2 $\Delta\beta$ -catenin, APC2 $\Delta\beta$ -catenin + Axin's β -catenin binding site, APC2 $\Delta\beta$ -catenin + TCF's β -catenin binding sites, APC2 $\Delta\beta$ -catenin + α -catenin's β -catenin binding sites (Figure 3). These transgenes are crossed with crossed into either $APC2^{g10}$ single mutant or $APC2^{g10}, APC1^{Q8}$ double mutant background. This background completely eliminates all endogenous APC functions in the embryo and provides the most stringent test of transgene functions. The research will allow us to determine if other proteins' β -catenin binding sites that can be substituted for APC's and Axin's binding sites, which would suggest that the major role of the β -catenin binding sites is to recruit β -catenin into the destruction complex. Understanding more about APC's β -catenin binding sites can help scientists better understand the mechanistic role of APC and how its mutation contributes to colon cancer development.

III. Methodology

Different methodologies to evaluate the functionality of these transgenes.

We use three functional tests to evaluate transgenes' functionality: assessing the embryonic viability, cuticle score, and immunofluorescent confocal microscopy of β -catenin and Engrailed levels.

Embryonic viability: embryonic viability is the percentage of embryos that can hatch into larvae. An embryonic viability number close to WT indicates that the transgene is functional

and allows the embryos to hatch into larvae. In contrast, a number close to the null mutation implies that the transgene is non-functional and unable to allow embryos to hatch into larvae.

Cuticle Score: The cuticle score is a functional test that allows us to determine the Wnt-mediated cell fate decisions in *Drosophila* embryos. Specifically, in stages 9 and 10 of *Drosophila* embryos, the embryonic epidermis provides a readout of Wnt activity as anterior and posterior cells of each segment are fated differently. In posterior cells, which do not receive the Wnt signal, β -catenin is destroyed to a basal level, so only a small amount β -catenin can enter the nucleus, leading to the production of denticles. Denticles are actin-based protrusions extending from the apical side of the cell, providing traction for the crawling larva after the embryo hatches (Rizzo & Bejsovec, 2017). In addition, they are arranged as trapezoid bands comprising five to six rows of cells with the same orientation, shape, and polarity (Peterson-Nedry et al, 2008). On the other hand, in anterior cells, which receive the Wnt signal, the destruction complex is inactivated, prompting a high level of β -catenin to enter the nucleus, which eventually produces naked cuticle cell fate. The cuticle patterning can be decided and assessed using a previously established method by the Peifer Lab (Figure 8). A cuticle score close to 0 indicates that the transgene is completely or nearly functional, indicated by 7-8 denticle bands and naked cuticle bands in the middle. On the other hand, a cuticle score close to 6 indicates that the transgene is non-functional or resembles the complete loss of APC activity in the $APC2^{g10}$, $APC1^{Q8}$ double mutant background, resulting in phenotypes devoid of denticles, failure of head evolution, and reduction in overall embryo size (Figure 5). Whereas in the $APC2^{g10}$ single mutant background, a cuticle score near 4 indicates that the transgene is not functional, leading to the phenotypes only containing merging 2-3 denticle bands, huge head holes, and size reduction (Figure 5).

β -catenin and Engrailed staining: Besides looking at the global signals like embryonic viability or cuticle score, we can also utilize the immunofluorescence confocal microscopy of

both β -catenin and Engrailed, which are molecular markers of Wnt signaling in Drosophila embryos (Peterson-Nedry et al, 2008). Engrailed is a Wnt target gene and an indicator of the level of β -catenin transcription instead of β -catenin protein levels. It is expressed by the presence of narrow stripes of two to three cells across the embryonic epidermis (Figure 11). In WT, in cells that don't receive the Wnt signals, the β -catenin levels are kept low, resulting in Engrailed not being expressed or no Engrailed bands. On the other hand, in the cells that receive the Wnt signals, the β -catenin levels accumulate and enter the nucleus to activate Wnt target gene transcriptions, leading to the presence of 2-3 stripes of Engrailed bands. On the other hand, in the $APC2^{g10}$, $APC1^{Q8}$ double mutant background, the destruction complex is completely inactivated, β -catenin protein levels hyper-accumulate uniformly throughout the whole embryos, leading to Engrailed expression bands strongly expanding (Figure 11). In the $APC2^{g10}$ single mutant background, β -catenin levels are somewhat elevated in all cells, resulting in Engrailed expression bands being somewhat expanded, compared to the WT (Figure 11).

IV. RESULTS

Investigating the interchangeability of APC2's and different proteins' β catenin binding sites

To test if different proteins' β -catenin binding sites are interchangeable with APC2's β -catenin binding sites in the cells that receive the Wnt signal, these transgenes were generated: APC2 $\Delta\beta$ -catenin + α -catenin β -catenin binding sites, APC2 $\Delta\beta$ -catenin + axin's β -catenin binding site, and APC2 $\Delta\beta$ -catenin + TCF's β -catenin binding sites and crossed into either single mutant: $APC2^{g10}$ or double mutant: $APC2^{g10}$, $APC1^{Q8}$ to assess transgenes' functions in a background completely devoid of endogenous APC protein. Transgene characterizations such as DNA PCR, RT PCR, and Western Blot were performed to confirm their expressions, localization experiments are conducted to ensure the transgenes are folded properly, and functional studies

are performed to assess transgenes' rescue activities.

Characterizing APC2 transgenes' localizations.

Before testing these functional studies, it is imperative to establish that adding back other proteins' β -catenin binding sites don't affect the protein folding by measuring APC2 transgenes' localizations because if APC2 transgenes do not fold properly, they tend to localize to different locations compared to the WT. Therefore, we decided to conduct immunofluorescence confocal microscopy to visualize the APC2 transgenes' localization. In the $APC2^{g10}$ single mutant background, the APC2 $\Delta\beta$ -catenin + α -catenin's β -catenin binding sites is localized to the cell cortex, which is similar to the WT APC2's localization, suggesting that adding back other protein's β -catenin binding sites do not affect protein's folding (Figure 6).

APC and Axin transgenes are expressed at the mRNA and protein levels.

In addition, to characterize APC2 transgenes' localizations, I also characterize APC and Axin from her lines of flies: GFP-APC2 in the $APC2$ single mutant background, Flag-Axin in the Axin single mutant background, and GFP-APC2 + Flag-Axin in the APC2 APC1 Axin triple mutant background. Therefore, genomic DNA PCRs are conducted to confirm the transgenes' presence, whereas the RT-PCRs and Western blot are performed to establish the transgenes' expression levels. Both APC and Axin transgenes were strongly detected at the DNA level and expressed at the mRNA level, respectively (Figure 7). We were able to confirm the Flag-Axin protein production through the Western Blot but failed to consistently detect the production of GFP-APC2 protein (Figure 7). This result contradicted the genomic DNA PCRs and RT-PCR analysis, suggesting that maybe GFP-APC2 was not possibly translated into protein. Therefore, we decide to conduct Immunofluorescence to determine the GFP-APC2 expression in the $APC2^{g10}$ single mutant background. As we predicted, the GFP-APC2 signal was clear and

expressed and localized to the cell cortex. (Figure 8). Therefore, we believe the problems have to reside in the Western Blot Method, so I have spent considerable time troubleshooting the Western Blot Protocol to find the optimal conditions to detect APC2 protein. We first decided to conduct Western Blot on flies with twist-GFP transgene: GFP expression driven by the twist promoter, which is expressed in the mesoderm starting at the early embryo (Pan et al, 1991). These twist GFP transgene flies serve as positive controls to ensure the Western Blot works properly since the twist promoter is highly expressed, so GFP should be easily detected in the Western Blot. The GFP band was clearly expressed and detected, but we still failed to detect the GFP-APC2 band in the Mixed Molecular Weight Setting (Figure 9). Being able to detect the twist GFP band using the Mixed Molecular Weight Setting in the Trans-Blot Turbo prompted us to think maybe the Mixed Molecular Weight Setting failed to transfer APC2 protein from the gel to the blot since APC2 is a huge protein, so we decided to try the High Molecular Setting to observe if the APC2 protein is successfully transferred or not. We detected the GFP band, but failed to detect the APC2 protein in the Western Blot in the High Molecular Setting (Figure 10). Another possible idea we tested was to try a different Ripa Lysis Recipe and order new detergents. Still, we were also unable to detect the APC2 protein in the Western Blot using different Ripa Lysis Buffers. Another potential idea we plan to try is to order new anti-GFP antibodies because anti-GFP antibodies are quite commercial, and there are a variety of them in the market. Therefore, future experiments on new anti-GFP antibodies must be conducted to validate our idea.

Some proteins' β -catenin binding sites are interchangeable with the APC2's β -catenin binding sites

α -catenin's β -catenin binding sites fail to rescue the Wnt phenotypes: Only 9% of embryos could develop into larvae in the $APC2^{g10}$ single mutant background, which is quite

close to 13.4% of APC2Δβ-catenin (Table 1). In addition, the mean cuticle score is 3.83, with a representative number of 5, indicated by the absence of denticles and head failure development (Figure 10). In stage 9-10 embryos expressing APC2Δβ-catenin + α-catenin's β-catenin binding sites, β-catenin protein levels were expanded in all cells (Figure 11), resembling phenotypes of embryos without transgenes observed in the APC2 single mutant background (Figure 12). Similar to β-catenin staining, the expanded Engrail stripes in cells that receive the Wnt signals also phenocopied phenotypes of no-transgene embryos in the APC2 single mutant background (Figure 11, Figure 12). These phenotypes further suggest that the α-catenin's β-catenin binding sites fail to rescue the embryonic viability, cuticle score, β-catenin, and Engrailed staining. Therefore, α-catenin's β-catenin binding sites cannot be substituted for APC2's β-catenin binding sites.

Axin's β-catenin binding site partially rescues the Wnt phenotypes: Approximately 79% of embryos could hatch into larvae, which is indistinguishable from the embryonic viability number of Full-length APC2 in the single mutant background (Table 1). The mean cuticle score was 0.31, with a representative number of 2, indicated by the presence of 6 denticle bands (Figure 10). In stage 9-10 embryos expressing APC2Δβ-catenin + Axin's β-catenin binding site, the β-catenin protein levels were slightly elevated in all cells, making it more difficult to observe the stripe patterns that are normally seen in the WT (Figure 11, Figure 12). Unlike β-catenin protein levels, the Engrailed stripes were clear and regulated close to the WT Engrailed bands. (Figure 11, Figure 12). These observed phenotypes further support our hypothesis that Axin's β-catenin binding site can partially rescue the Wnt phenotypes and substitute for APC2's β-catenin binding sites.

TCF's β-catenin binding site partially rescues the Wnt phenotypes: The APC2Δβ-catenin + TCF's β-catenin binding sites are also capable of rescuing the embryonic viability, cuticle patterning, β-catenin staining, and Engrailed staining in the single mutant, though Axin

seems to rescue the Wnt phenotypes better. 58% of TCF's β -catenin binding sites embryos were able to hatch into larvae with a mean cuticle score of 1.09 (Table 1). To be more specific, the cuticle patterning in Figure 9 was determined as a 2, indicated by the presence of 5 denticle bands, which only lacked two bands, compared to the WT (Figure 9). In stage 9-10 embryos expressing APC2 $\Delta\beta$ -catenin + TCF's β -catenin binding sites, the β -catenin protein levels were slightly elevated in all cells, making it more difficult to observe the stripe patterns that are normally seen in the WT (Figure 11, Figure 12). Unlike β -catenin protein levels, the Engrailed stripes were clear and regulated close to the WT Engrailed bands. (Figure 11, Figure 12). These observed phenotypes further support our hypothesis that TCF's β -catenin binding sites can partially rescue the Wnt phenotypes.

V. DISCUSSIONS

Adding other proteins' β -catenin binding sites do not affect APC2's folding.

Before conducting any functional studies, it is important to establish that APC2 folds properly because adding different domains or motifs to proteins tends to cause conformation changes, resulting in activity alterations. We decided to conduct immunofluorescent confocal microscopy to examine APC2 localization of APC2 transgenes in the single mutant background because localization alteration is a good indication that the protein does not fold properly. According to Figure 6, APC2 $\Delta\beta$ -catenin + α -catenin's β -catenin binding sites is localized to the cell cortex, which is similar to the localization of the WT APC2, further indicating that APC2 $\Delta\beta$ -catenin + α -catenin's β -catenin binding sites fold properly and are functional. One plausible explanation is that the APC 15Rs, APC 20Rs, and α -catenin's β -catenin binding sites are unstructured, suggesting the flexibility of these β -catenin binding sites (Minde et al, 2011). When we add unstructured α -catenin's β -catenin binding sites back to APC2, APC2's folding does not seem to change since APC2 transgene is localized in the cell cortex. Therefore, adding other

proteins' β -catenin binding sites should not be able to change APC2's folding and functions, further suggesting that these transgenes can be used for the interchangeability hypothesis.

The β -catenin binding sites on APC2 are partially interchangeable with some other β -catenin binding sites.

Given that other proteins such as α -catenin and TCF also possess β -catenin binding sites, we generated a series of Drosophila APC2 mutants that remove all β -catenin binding sites, keep 20R2 intact, and add other proteins: α -catenin, Axin, and TCF β -catenin binding sites and crossed these transgenes into the single mutant: $APC2^{g10}$ or double mutant: $APC2^{g10}$, $APC1^{Q8}$ background. We then conducted functional studies: embryonic viability, cuticle score, β -catenin staining, and Engrailed staining to observe if these transgenes can rescue Wnt phenotypes. According to Figure 10, Figure 11, Figure 12, and Table 1, $APC2\Delta\beta$ -catenin + α -catenin's β -catenin binding sites in the $APC2^{g10}$ single mutant failed to rescue the Wnt phenotypes. In fact, α -catenin's β -catenin binding sites do not seem to rescue any APC mutant phenotypes and largely resemble an APC2 transgene with no β -catenin binding sites. One plausible explanation for why this APC2 transgene fails to rescue is based on where α -catenin interacts with β -catenin. To be more specific, α -catenin binds to β -catenin through the N-terminal of the first Armadillo Repeats, and since there is no overlapping between where α -catenin and APC2 interact with β -catenin, it is reasonable to observe why α -catenin fails to rescue the Wnt phenotypes.

On the other hand, $APC2\Delta\beta$ -catenin + Axin's β -catenin binding sites and $APC2\Delta\beta$ -catenin + TCFs β -catenin binding sites are able to rescue the Wnt phenotypes. These observed Wnt phenotypes can be explained by where Axin and TCF interact with β -catenin. To be more specific, Axin interacts with the Armadillo repeats 3-4, which partially overlaps with APC2's β -catenin binding sites (Xing, 2003). Similar to APC2, TCF interacts with the Armadillo Repeats 3-10, indicating complete overlapping β -catenin binding sites. Specifically, TCF also contains charged

button interaction: two crucial negatively charged residues that interact with two positive lysine residues of Armadillo 5 to 9 of β -catenin (Graham et al, 2000). In addition, TCF also possesses the SxxxSLSSL motif to interact with Armadillo Repeat 3-4 (Graham et al, 2000). Therefore, both Axin's β -catenin binding sites and TCF's β -catenin binding sites are able to partially rescue the Wnt phenotypes, further indicating that only transgenes that bind to Armadillo Region Repeats like APC2 can rescue the Wnt phenotypes.

APC2 Transgenes are able to rescue the Engrailed phenotypes through cytoplasmic retention but not the β -catenin destruction.

The result from the APC2 single mutant background is a part of our group's bigger projects; other lab students have previously characterized some of these APC2 transgenes in the $APC2^{g10} APC1^{Q8}$ double mutant background. In the $APC2^{g10} APC1^{Q8}$ double mutant background, β -catenin protein levels hyper accumulated in the cells that receive the Wnt signals, observed in both α -catenin's β -catenin binding sites and Axin's β -catenin binding site (Figure 13). However, only Engrailed bands of α -catenin binding sites transgenes expanded, whereas the Engrailed bands of Axin binding site transgenes were regulated to the WT levels (Figure 13), further reinforcing the idea that cytoplasmic retention, which is the ability of APC to sequester some β -catenin in the cytosol, could account for the observed phenotypes. In our proposed model: APC regulates the activation of Wg/Wnt target genes through both β -catenin destruction and cytoplasmic retention. In cells that don't receive the Wnt signal, β -catenin is destroyed to a lower level, and APC sequesters additional β -catenin in the cytoplasm. In cells that receive the Wnt signal, the destruction complex is deactivated, β -catenin accumulates modestly. APC can still sequester some β -catenin in the cytoplasm, allowing a certain level of β -catenin to translocate to the nucleus and activate the Wnt target genes (Roberts et al, 2011) (Figure 14). In contrast, APC null embryos are defective in regulating the destruction complex and cytoplasmic retention,

resulting in hyper accumulated β -catenin in the nucleus and secreting naked cuticles (Yamulla et al, 2014).

In embryos that remove all β -catenin binding sites, keep 20R2 intact and add other proteins β -catenin binding sites, Axin's β -catenin binding site is able to retain cytoplasmic retention, whereas the α -catenin's β -catenin binding sites are defective in retention. Even though β -catenin protein bands were expanded, the Axin's β -catenin binding site was able to retain some β -catenin in the cytoplasm, allowing only a certain amount of β -catenin to enter the nucleus, resulting in Engrailed bands phenocopied the WT phenotype (Figure 13). This phenotype can be explained by the partial overlapping β -catenin binding sites between APC and Axin. To be more specific, Axin binds to β -catenin through Armadillo Repeats 3-4, whereas APC2 interacts with β -catenin through Armadillo Repeats 3-10. This suggests that specific interactions such as salt-bridge or polar bonds on Armadillo Region Repeat 3-4 are required to capture and retain some β -catenin in the cytosol. On the other hand, α -catenin's β -catenin binding sites failed to keep β -catenin in the cytoplasm, letting hyper accumulated β -catenin to enter the nucleus, causing expanded Engrailed bands, which are indistinguishable from the no transgene's Engrailed bands (Figure 12, Figure 13). Since α -catenin forms such a weak interaction with β -catenin through the Armadillo Repeat 1, it fails to restore APC2's ability to retain β -catenin in the cytosol. Overall, Axin's β -catenin binding sites might rescue the Wnt signaling phenotypes in the double mutant background through cytoplasmic retention, which requires specific interactions between Armadillo Region Repeat 3-4 of β -catenin. Therefore, since TCF also interacts through the Armadillo Region Repeats 3-4, then it is possible that the TCF's β -catenin binding sites can also rescue the Wnt phenotypes through cytoplasmic retention.

However, both of these transgenes' β -catenin binding sites failed to rescue β -catenin destruction, leading to β -catenin accumulation in cells that receive the Wnt signals. In WT, when the Wnt signals bind to the Fz and LRP5/6, these interactions recruit Axin away from the

destruction complex. It is commonly believed that even when Axin is compelled away, it can still bind to APC and a small amount of β -catenin. Therefore, we believe that Axin's β -catenin binding site and α -catenin's β -catenin binding sites cannot retain the full APC's function, allowing some β -catenin to evade the complex and accumulate in cells that receive the Wnt signal, resulting in hyper-accumulated β -catenin. Overall, in the double mutant background, these APC2 transgenes fail to rescue the β -catenin destruction, which prompts us to think maybe that the complete overlapping between APC2's β -catenin binding sites and other proteins' β -catenin binding sites is vital and required.

Future direction

In the APC2 single mutant background, we were able to confirm adding back α -catenin's β -catenin binding sites do not change APC2's folding through their localization in the cell cortex. In the future, we would love to conduct immunofluorescence confocal microscopy to confirm whether adding back *TCF*-catenin's β -catenin binding sites and Axin's β -catenin binding sites do not change APC2's folding. Similar to the single mutation, in APC2 APC1 double mutant background, we want to perform immunofluorescence experiments to verify if these APC2 transgenes fold properly. In addition, we hypothesize that complete overlapping between APC2's β -catenin binding sites and other proteins' β -catenin binding sites is required to rescue the β -catenin destruction; therefore, to test our hypothesis, immunofluorescence confocal microscopy experiments need to be conducted on the APC2 $\Delta\beta$ -catenin + TCF's β -catenin binding sites to verify our prediction. That is because TCF interacts with β -catenin through Armadillo Region Repeat 3-10, which is similar to APC. In fact, TCF contains 2 important negative residues that interact with the charged button in Armadillo Region Repeat 5-9 and SxxxSLSSL motifs to interact with Armadillo Region Repeat 3-4 (Graham et al, 2000). We expect that the TCF's β -catenin binding sites are able to rescue both β -catenin destruction and Wnt phenotypes. To test our

hypothesis about cytoplasmic retention, we will transfect SW480 cells with our panel of APC2 transgenes, inhibit proteasomal activity using MG132, and assess β -catenin's nuclear enrichment via immunofluorescence confocal microscopy since we can only visualize cell nuclei using human cell lines. From our prediction, it is expected that Axin's β -catenin binding sites and TCF's β -catenin binding sites are able to reduce the amount of detectable nuclear β -catenin, whereas α -catenin's β -catenin binding sites are defective in retention.

VI. LITERATURE CITED:

Ahmed, Yashi, Shigemi Hayashi, Arnold Levine, and Eric Wieschaus. "Regulation of armadillo by a Drosophila APC inhibits neuronal apoptosis during retinal development." *Cell* 93, no. 7 (1998): 1171-1182.

Akong, Kathryn, Elizabeth E. Grevengoed, Meredith H. Price, Brooke M. McCartney, Melissa A. Hayden, Jan C. DeNofrio, and Mark Peifer. "Drosophila APC2 and APC1 play overlapping roles in wingless signaling in the embryo and imaginal discs." *Developmental biology* 250, no. 1 (2002): 91-100.

Fagotto, François, Ursula Glück, and Barry M. Gumbiner. "Nuclear localization signal-independent and importin/karyopherin-independent nuclear import of β-catenin." *Current Biology* 8, no. 4 (1998): 181-190.

Graham, Thomas A., Carole Weaver, Feng Mao, David Kimelman, and Wenqing Xu. "Crystal structure of a β-catenin/Tcf complex." *Cell* 103, no. 6 (2000): 885-896.

Hankey, William, Wendy L. Frankel, and Joanna Groden. "Functions of the APC tumor suppressor protein-dependent and independent of canonical WNT signaling: implications for therapeutic targeting." *Cancer and Metastasis Reviews* 37, no. 1 (2018): 159-172.

Jamieson, Cara, Manisha Sharma, and Beric R. Henderson. "Targeting the β-catenin nuclear transport pathway in cancer." In *Seminars in cancer biology*, vol. 27, pp. 20-29. Academic Press, 2014.

Li VS, Ng SS, Boersema PJ, Low TY, Karthaus WR, Gerlach JP, Mohammed S, Heck AJ, Maurice MM, Mahmoudi T, et al. 2012. Wnt signaling through inhibition of b-catenin degradation in an intact Axin1 complex. *Cell* 149: 1245–1256.

Logan, Catriona Y., and Roel Nusse. "The Wnt signaling pathway in development and disease." *Annu. Rev. Cell Dev. Biol.* 20 (2004): 781-810.

Kimelman, D., and W. Xu. "β-Catenin destruction complex: insights and questions from a structural perspective." *Oncogene* 25, no. 57 (2006): 7482-7491.

Ha, Nam-Chul, Takashi Tonozuka, Jennifer L. Stamos, Hee-Jung Choi, and William I. Weis. "Mechanism of phosphorylation-dependent binding of APC to β -catenin and its role in β -catenin degradation." *Molecular cell* 15, no. 4 (2004): 511-521.

McCartney, Brooke M., Herman A. Dierick, Catherine Kirkpatrick, Melissa M. Moline, Annette Baas, Mark Peifer, and Amy Bejsovec. "Drosophila APC2 is a cytoskeleton-associated protein that regulates wingless signaling in the embryonic epidermis." *The Journal of cell biology* 146, no. 6 (1999): 1303-1318.

Pan, D. J., Jian-Dong Huang, and Albert J. Courey. "Functional analysis of the Drosophila twist promoter reveals a dorsal-binding ventral activator region." *Genes & development* 5, no. 10 (1991): 1892-1901.

Peterson-Nedry, Wynne, Naz Erdeniz, Susan Kremer, Jessica Yu, Shahana Baig-Lewis, and Marcel Wehrli. "Unexpectedly robust assembly of the Axin destruction complex regulates Wnt/Wg signaling in Drosophila as revealed by analysis in vivo." *Developmental biology* 320, no. 1 (2008): 226-241.

Ranes, Michael, Mariola Zaleska, Saira Sakalas, Ruth Knight, and Sebastian Guettler. "Reconstitution of the destruction complex defines roles of AXIN polymers and APC in β -catenin capture, phosphorylation, and ubiquitylation." *Molecular Cell* 81, no. 16 (2021): 3246-3261.

Roberts, David M., Mira I. Pronobis, John S. Poulton, Jon D. Waldmann, Elise M. Stephenson, Shahnaz Hanna, and Mark Peifer. "Deconstructing the catenin destruction complex: mechanistic roles for the tumor suppressor APC in regulating Wnt signaling." *Molecular biology of the cell* 22, no. 11 (2011): 1845-1863.

Rizzo, Nicholas P., and Amy Bejsovec. "SoxNeuro and Shavenbaby act cooperatively to shape denticles in the embryonic epidermis of Drosophila." *Development* 144, no. 12 (2017): 2248-2258.

Su, YunYun, Chunjiang Fu, Shinji Ishikawa, Alessandra Stella, Masayuki Kojima, Kazuhisa Shitoh, Emanuel M. Schreiber, Billy W. Day, and Bo Liu. "APC is essential for

targeting phosphorylated β -catenin to the SCF β -TrCP ubiquitin ligase." *Molecular cell* 32, no. 5 (2008): 652-661.

Stamos, Jennifer L., and William I. Weis. "The β -catenin destruction complex." *Cold Spring Harbor perspectives in biology* 5, no. 1 (2013): a007898.

Valenta, Tomas, George Hausmann, and Konrad Basler. "The many faces and functions of β -catenin." *The EMBO journal* 31, no. 12 (2012): 2714-2736.

Yamulla, Robert J., Eric G. Kane, Alexandra E. Moody, Kristin A. Polit, Nicole E. Lock, Andrew VA Foley, and David M. Roberts. "Testing models of the APC tumor.

Xu, Wenqing, and David Kimelman. "Mechanistic insights from structural studies of β -catenin and its binding partners." *Journal of cell science* 120, no. 19 (2007): 3337-3344.

Xing, Yi, Wilson K. Clements, David Kimelman, and Wenqing Xu. "Crystal structure of a β -catenin/axin complex suggests a mechanism for the β -catenin destruction complex." *Genes & development* 17, no. 22 (2003): 2753-2764.

VII. APPENDIX

FIGURES & TABLES

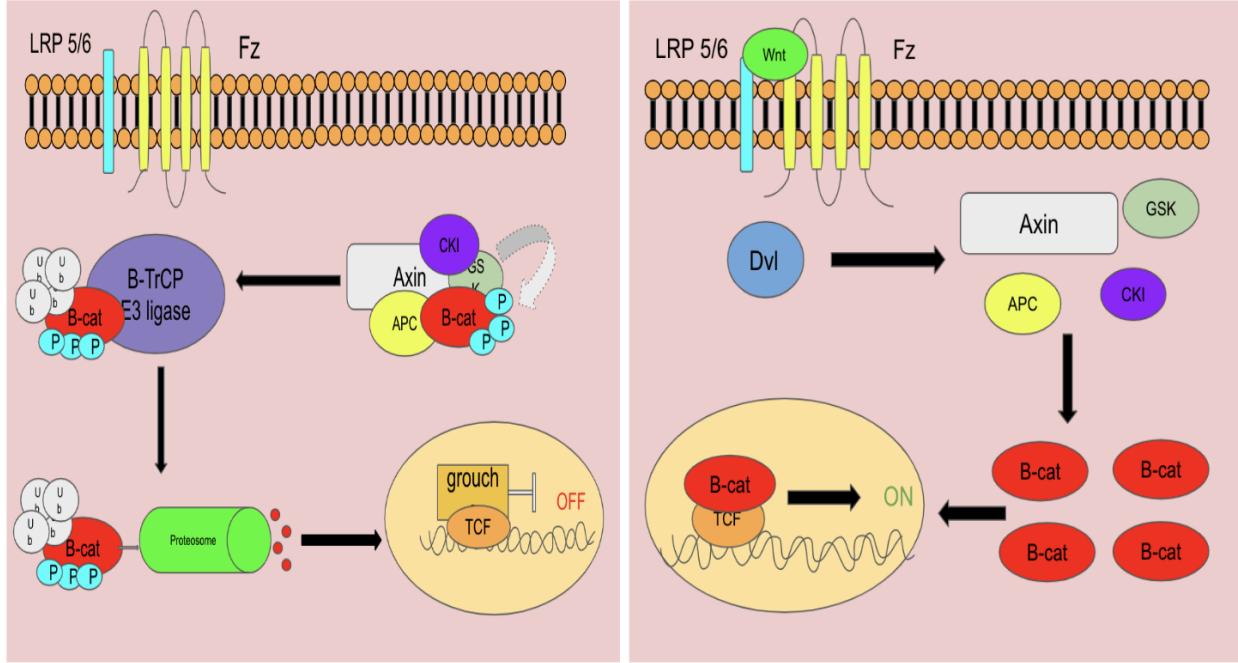


Figure 1: Diagram of Wnt pathways. With the absence of a Wnt signal, β -catenin is targeted for destruction. In the presence of Wnt, the β -catenin is not destroyed and translocates to the nucleus to activate the transcription. Modified from McCartney et al, 1999 and Najdi et al, 2011 papers.

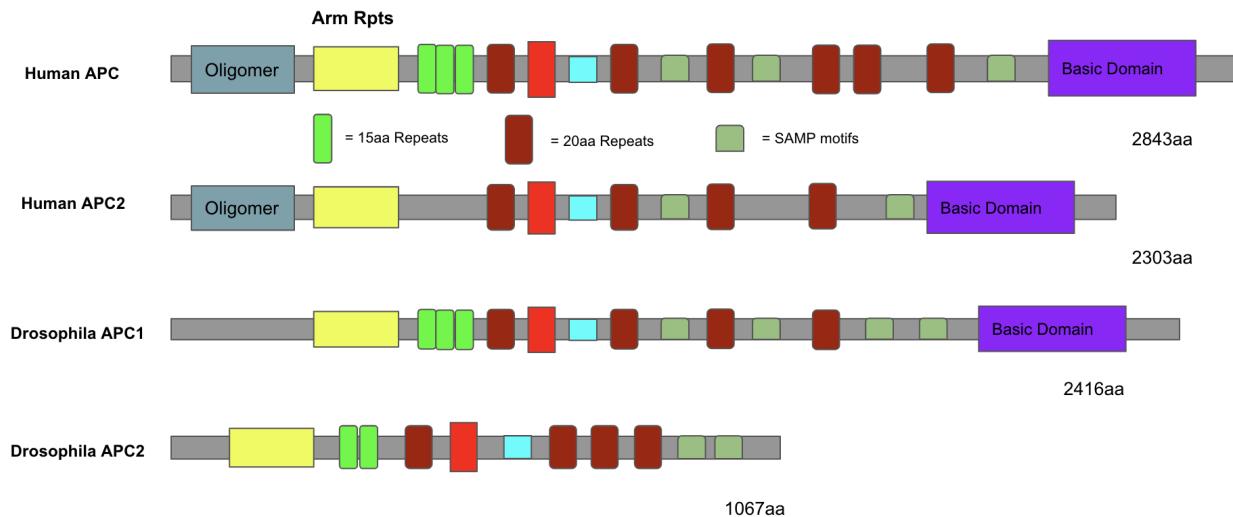


Figure 2: The schematics of human and Drosophila APCs. Both vertebrates and flies have two APC proteins that share some conserved region comprising Armadillo repeats, 15 amino acid repeats, 20 amino acid repeats, the catenin inhibitory domain(CID), and the SAMP motifs.

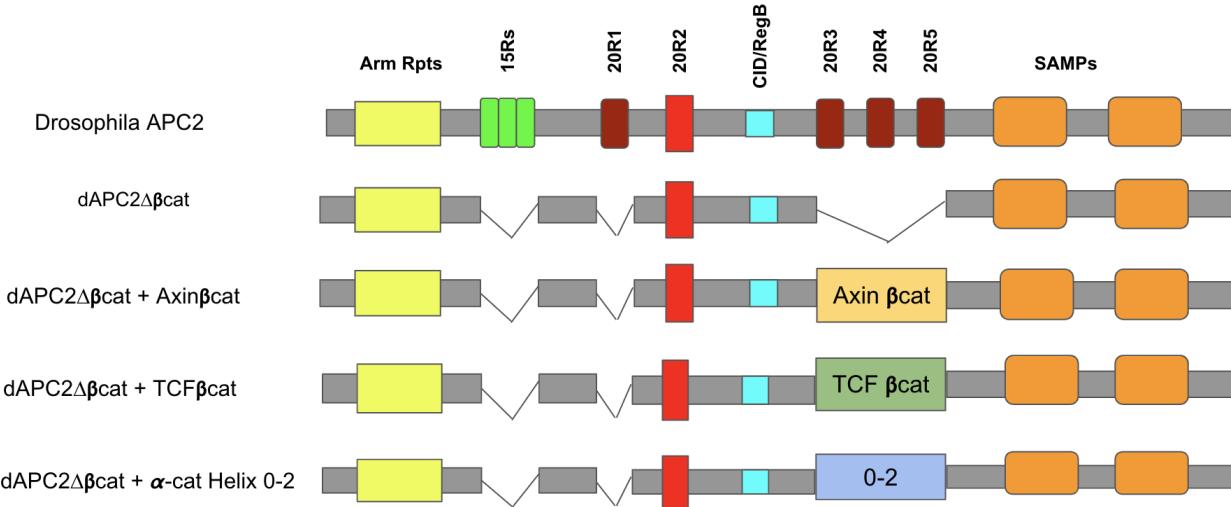


Figure 3: Drosophila APC2 deletion constructs were generated by removing all APC2's β -catenin binding sites and adding back other proteins' β -catenin binding sites. These transgenes are then crossed with either single mutant: $APC2^{g10}$ or double mutants: $APC2^{g10}$, APC^{Q8} in chromosome III.

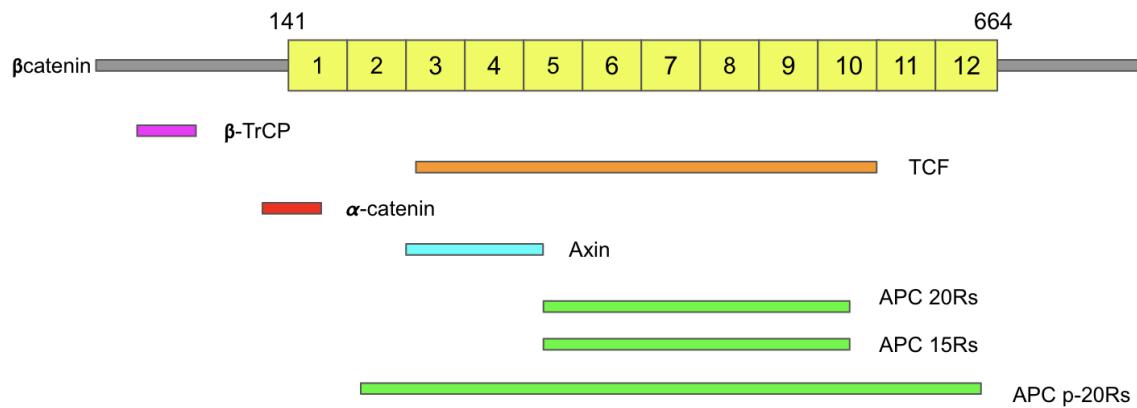


Figure 4: A summary of where different proteins interact with β -catenin. β -catenin contains the N-terminal region, 12 Armadillo Repeats, and the C-terminal region. APC 20Rs and APC 15Rs bind to the Armadillo

Repeats 5-9, while the phosphorylated APC2 extend their binding from Armadillo Repeats 3-10. TCF binds to Armadillo Repeats 3-10, Axin binds to Armadillo Repeats 3-4, and α -catenin binds to the groove of Armadillo Repeat 1.

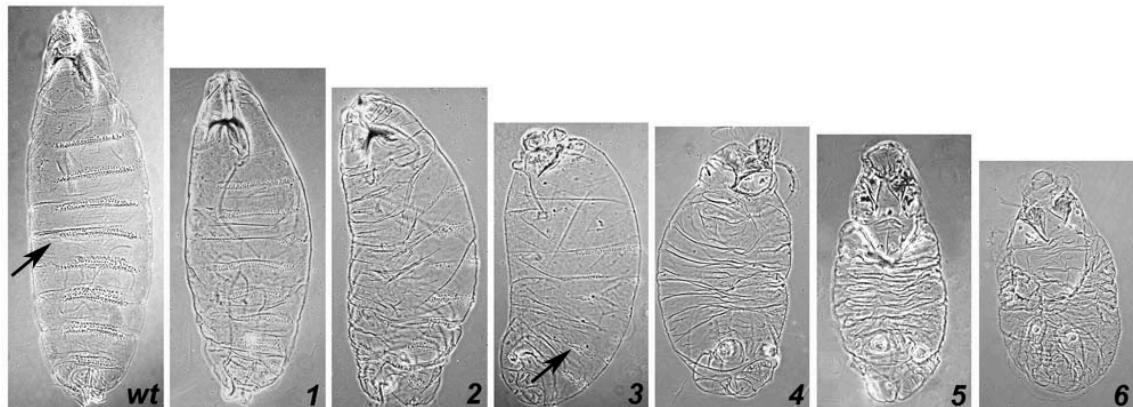


Figure 5: Established cuticle score grading method by the Peifer Lab at the University of North Carolina at Chapel Hill. Used for comparisons.

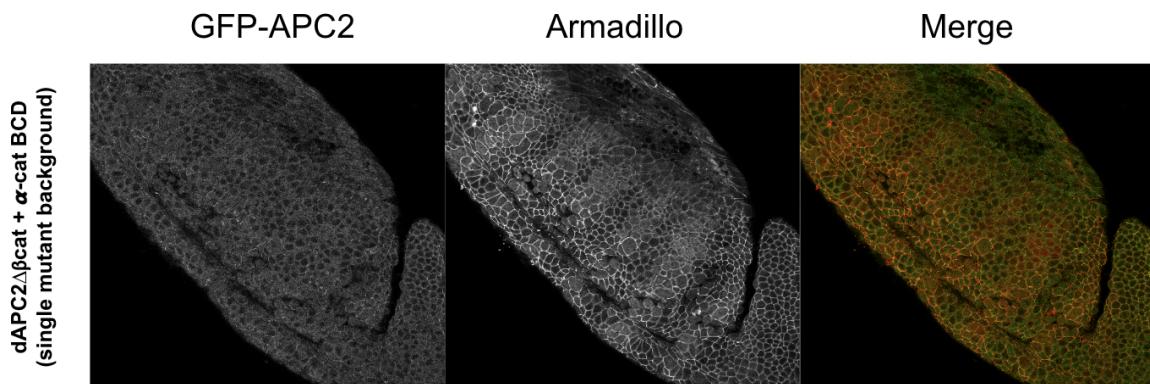


Figure 6: Immunofluorescence data include the GFP-APC2, β -catenin, and merged image of the APC2 transgenes in the $APC2^{g10}$ single mutant background 9-10 Drosophila embryos. GFP-APC2 is localized at the cell cortex.

K

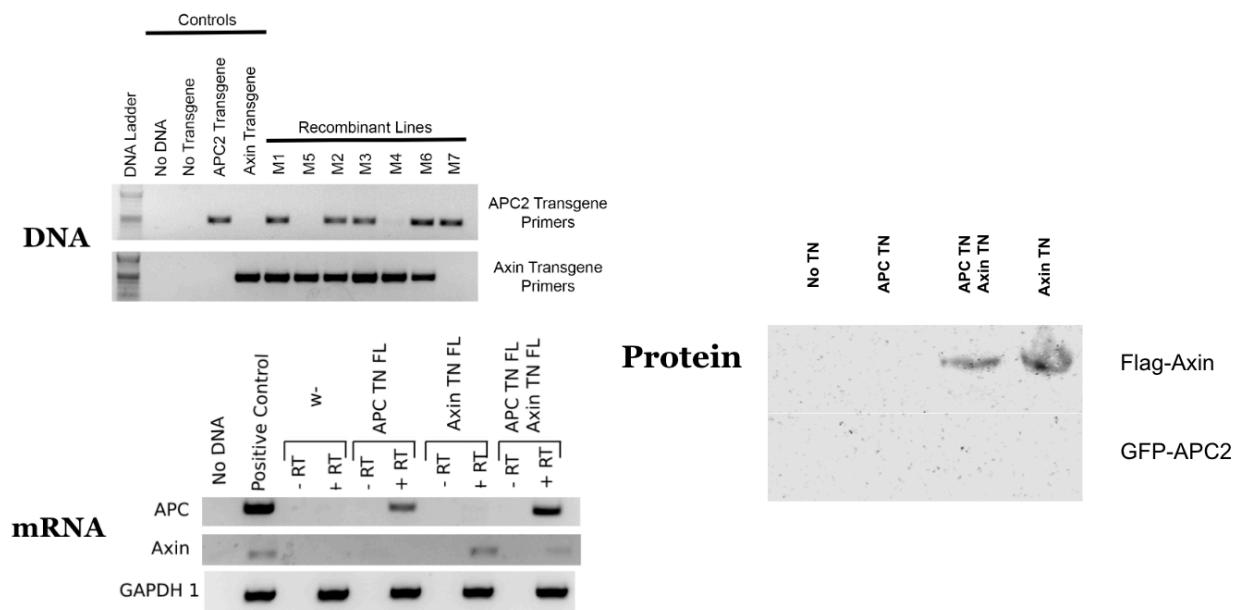


Figure 7: Transgenes' characterizations through the genomic PCR, RT-PCR, and Western Blot of GFP-Full length APC2 in the $APC2^{g10}$ mutant background, Flag-Full length Axin in the $Axin^{S044230}$ mutant background, and the GFP-Full length APC2 + Flag-Full length Axin in the $APC2^{g10}$, $Axin^{S044230}$, and $APC2^{g10}$ triple mutant background. All these transgenes were detected at the RNA level. Flag-Axin was detected in the Western Blot, but we failed to detect the GFP-APC2. This work is a joint collaboration with Julia Kiefer.

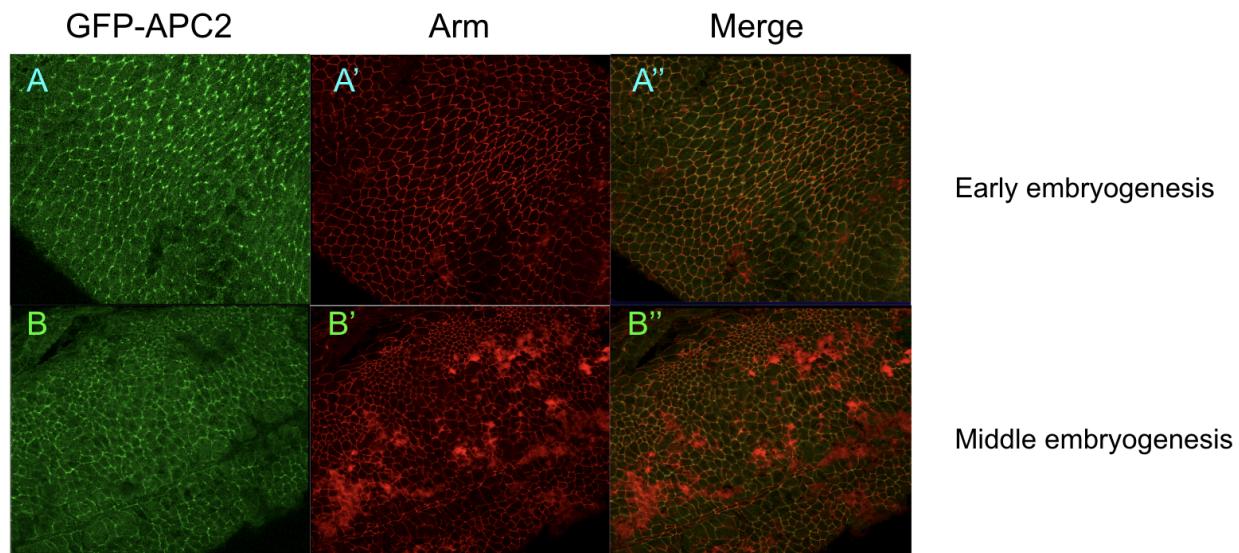


Figure 8: Immunofluorescence data include the GFP-APC2, β -catenin, and merged image (early and middle embryogenesis) in the $APC2^{g10}$ mutant background. GFP-APC2 is localized to the cell cortex.

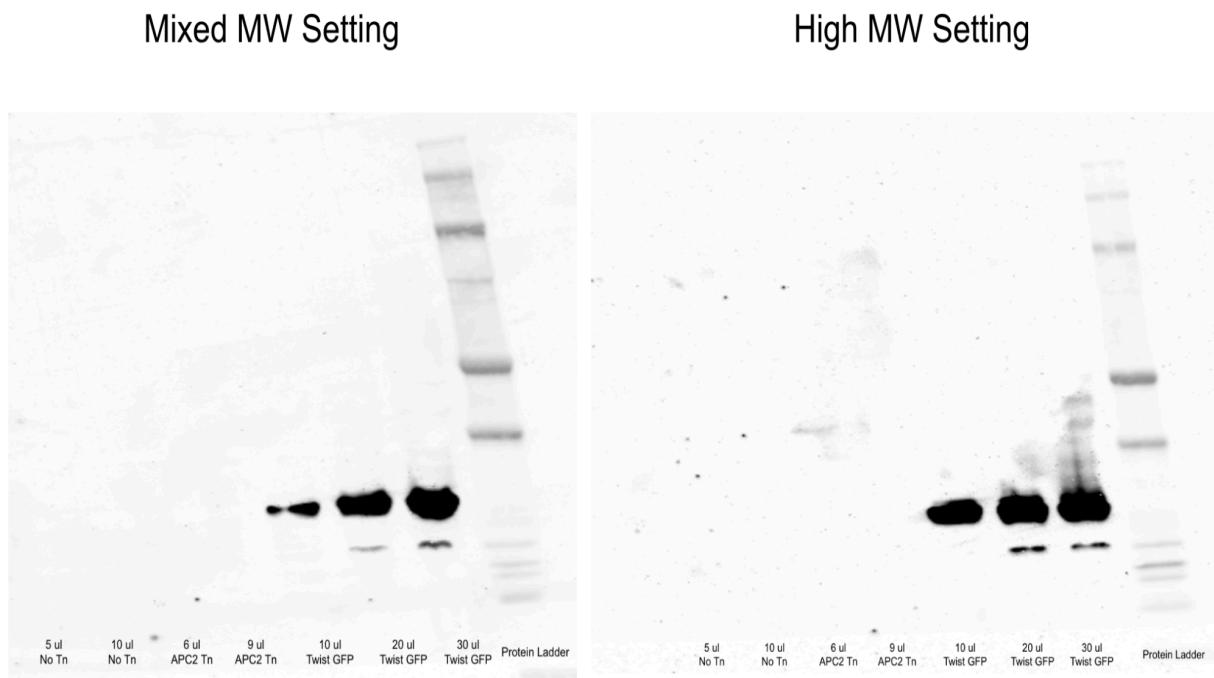


Figure 9: The Western Blot of the Wild-type, APC2 Transgene, and Twist GFP using the Mixed Molecular Weight and High Molecular Weight transfer system. The left panel was in the mixed molecular weight (MW) setting, and the right panel was in the high molecular weight setting.

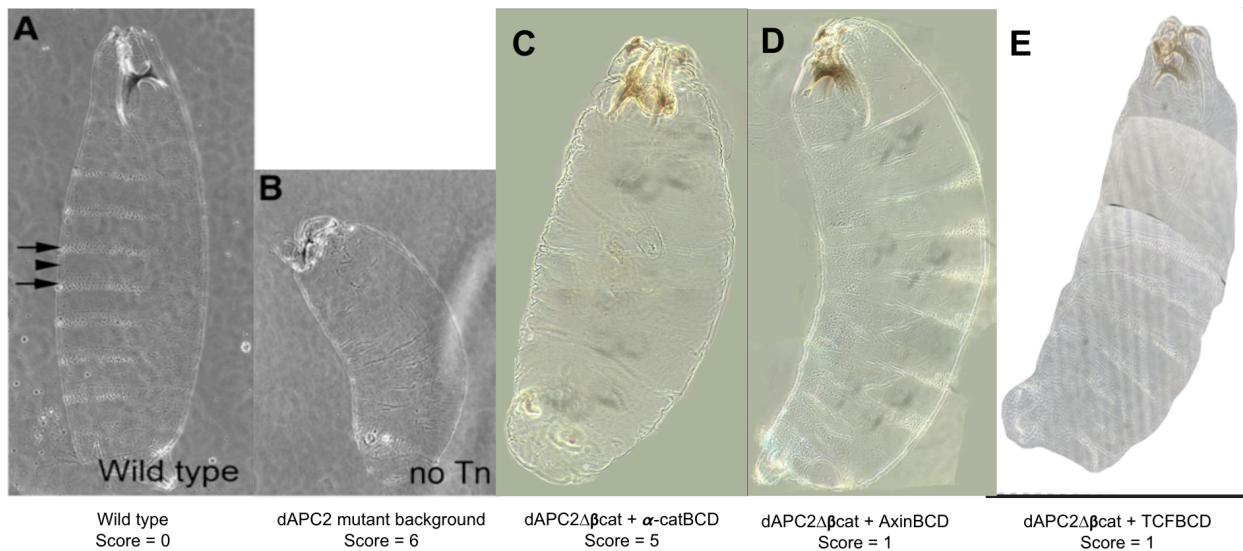


Figure 10: Cuticle analysis of Drosophila APC2 transgenes expressed in the $APC2^{g10}$ single mutant background. Panel A and E are extracted from Yamulla's et al, for comparison. The cuticle score was determined by using established guidelines in Figure 8. Arrow represents the cuticle bands, and the arrowhead represents the naked cuticle. Panel D is a joint collaboration with Julia Kiefer.

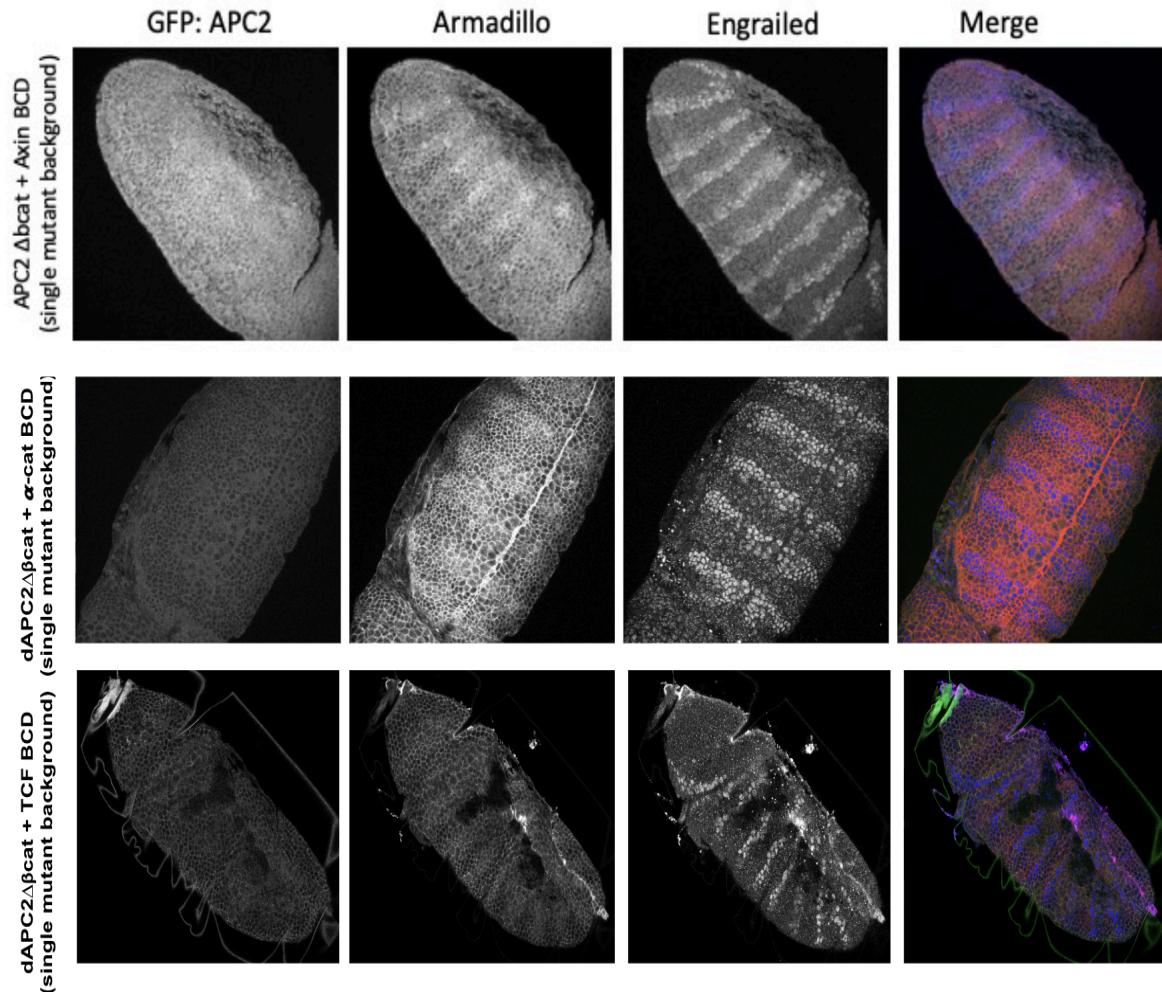


Figure 11: Immunofluorescence data include the GFP-APC2, β -catenin, Engrailed, and merged image of APC2 transgenes in the $APC2^{g10}$ single mutant background of stage 9-10 Drosophila embryos. The AxinBCD panel is done in collaboration with Julia Kiefer. In the first row and last row: β -catenin was slightly elevated and hard to see stripes pattern, and Engrailed stripes were regulated to the WT level. The α -catenin and TCF data were generated by me.

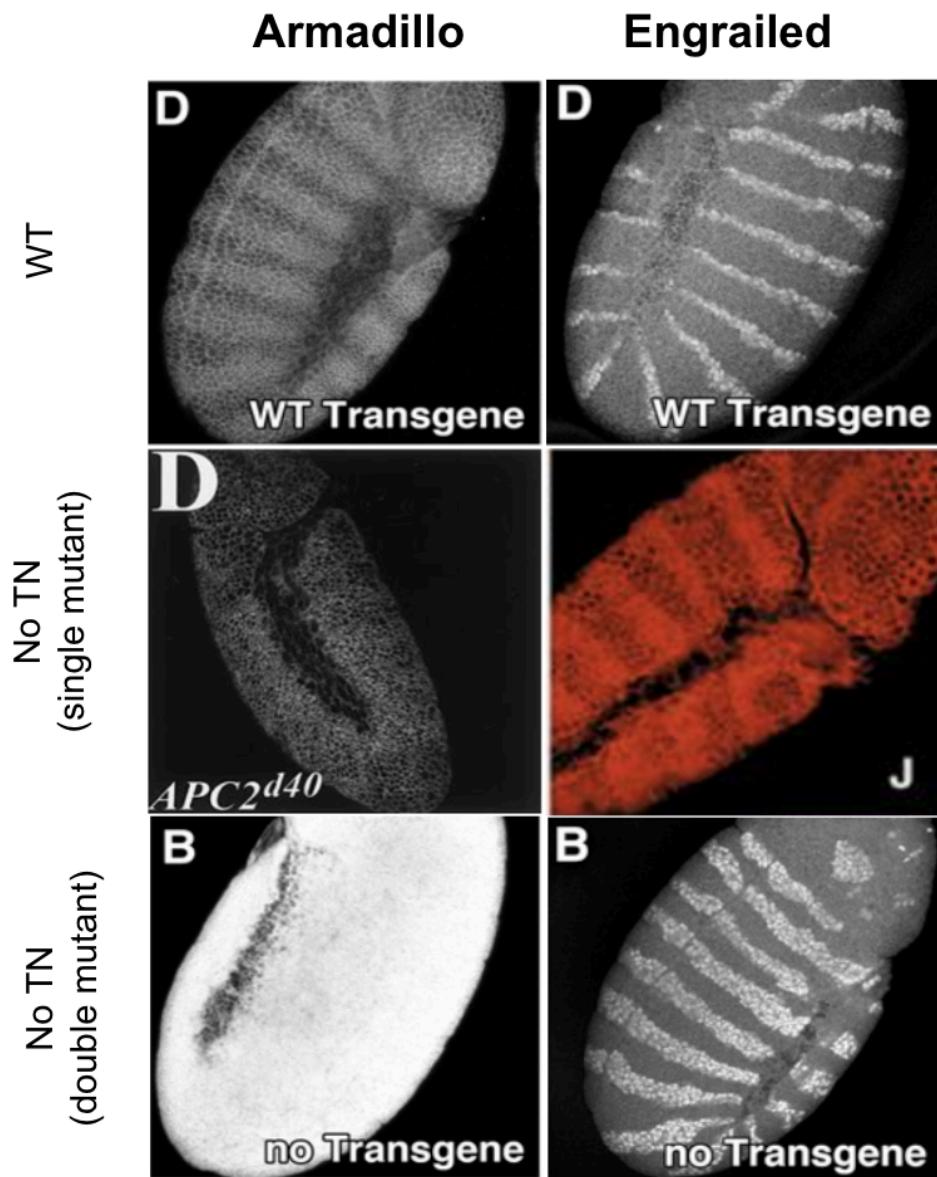


Figure 12: Immunofluorescence data of β -catenin and Engrailed bands of the Wild-Type, No Transgenes

in the *APC2* single mutant background, and No Transgenes in the *APC2 APC1* double mutant background of stage 9-10 Drosophila embryos. These panels are extracted from Akong's et al., Ahmed's et al., and Yamulla's et al., for comparison. In the single mutant background, the β -catenin bands were expanded in all cells and the Engrailed bands got slightly expanded. However, in the double mutant background, β -catenin levels were hyper-accumulated in all cells, with Engrailed expanded in the cells that receive the Wnt signals.

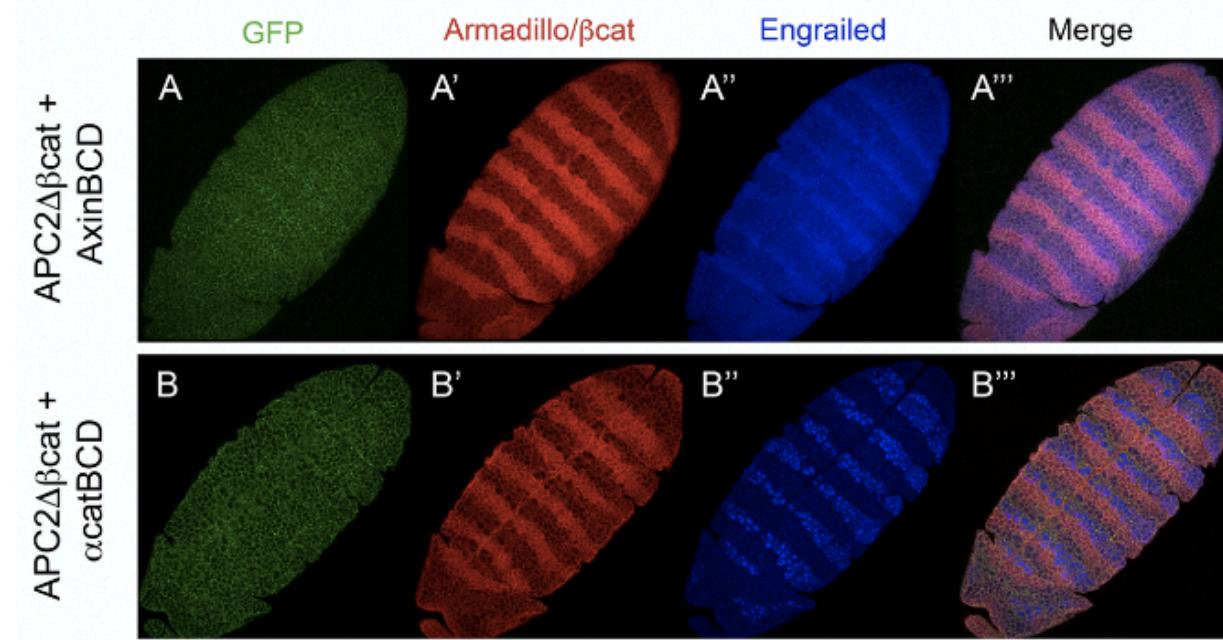


Figure 13: Immunofluorescence data include the GFP-APC2, β -catenin, Engrailed, and merged image of APC2 transgenes in the $APC2^{g10}$, $APC1^{Q8}$ double mutant background of stage 9-10 Drosophila embryos.

This figure is generated by Roberts's Lab at Franklin & Marshall College.

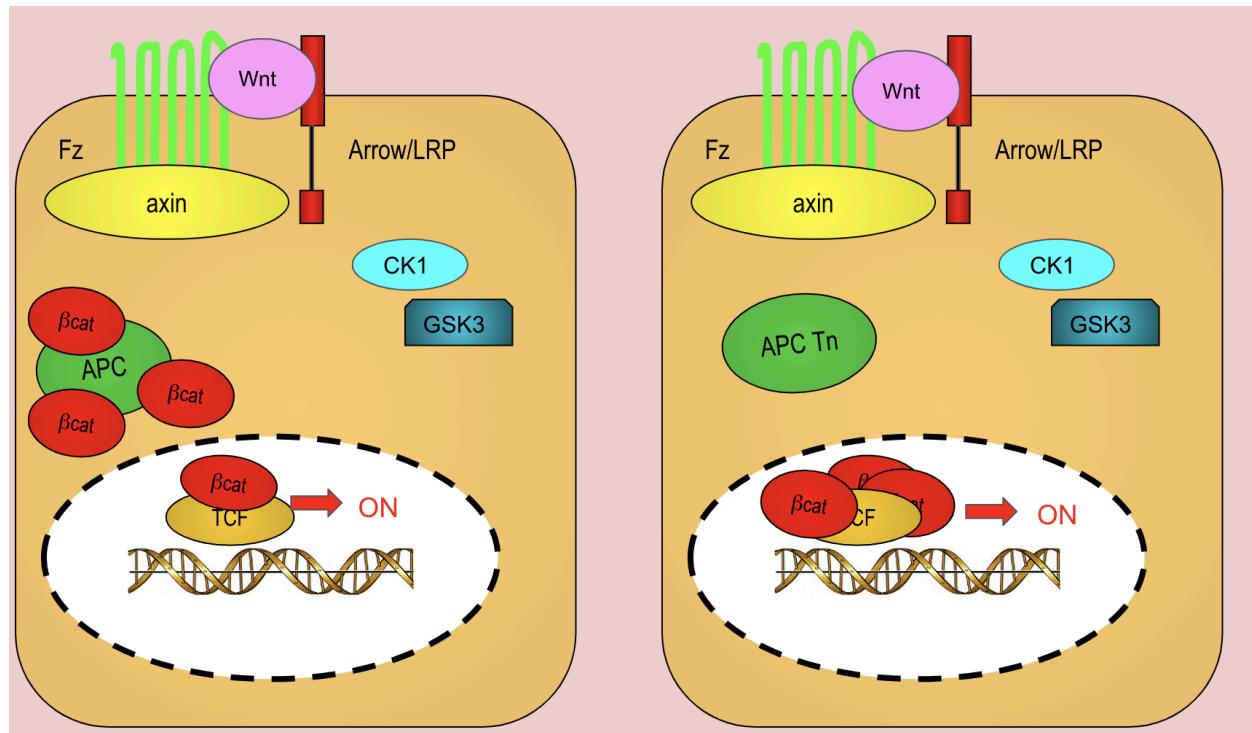


Figure 14: Model for the cytoplasmic retention in the cells that receive the Wnt signals. The left panel is a model for WT, and the right panel is a model for APC2 Tn.

	APC2 mutant background		
APC2 transgene	Embryonic Viability	Cuticle Score	Viable Stock
dAPC2 $\Delta\beta$ cat + α -catBCD	9%	3.83	No
dAPC2 $\Delta\beta$ cat + AxinBCD	79%	0.31	Yes, very weak
dAPC2 $\Delta\beta$ cat + TCFBCD	58%	1.09	Yes, very weak
dAPC2 $\Delta\beta$ cat	13.4%	3.7	No
Full length APC2	88.8%	0.01	Yes

Table 1: Summary table of embryonic viability, cuticle score, and viable stocks of APC2 transgenes in the *APC2^{g10}* single mutant background. The AxinBCD is a joint collaboration with Julia Kiefer, whereas the *dAPC2 $\Delta\beta$ cat* and Full-length APC2 were generated by other members in Roberts's lab. The α -catenin and TCF were generated by me.

Primer	Forward	Reverse
APC2	5'ACTTCAAGATCCGCCACAAC 3'	5' ATCTCACCTCCCGCTGG 3'
Axin	5' TTGTCAACAGTTGTCCGTGG 3'	5' GGTGCTTGTTCATCGTCATCC 3'
GAPDH	5' GAGCCGAGTATGTGGTGGAG 3'	5' ACGGAGACATTGGGCGTG 3'

Table 2: Primer sequences for PCR reactions. GAPDH is included as a positive control.

METHODS

Transgenic Drosophila flies

The transgenic Drosophila flies were generated using a previously published method (Roberts et al, 2011).

Immunoblotting

Transgenic flies eggs were collected and lysed in RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Igepal, 0.1% SDS, 0.5% DOC, and 5mM EDTA) supplemented with protease inhibitors to collect protein samples. Then proteins were added with 2 x Laemmli solution (Bio-Rad, Hercules, CA) and heated at 95[°]C, and then resolved by SDS-PAGE and transferred to the nitrocellulose membrane. Membranes were probed first with primary antibody: mouse anti-GFP (Clontech, clone JL8, 1:1000), mouse anti-Flag (clone M2, 1:1000), and mouse anti-tubulin (Sigma Aldrich, DM1A, 1:5000), and then with secondary antibody: goat anti-mouse antibody(1:10000). Signal was detected using SuperSignal West Dura Chemiluminescent and imaged on a Fluor Chem Q imager. Band intensity quantification was performed using Protein Simple Software.

Immunofluorescence.

The fly embryos were dechorionated in 50% bleach for 5 min and fixed in a mixture of an equal volume of heptane and 10% formaldehyde with EGTA for 20 min. The formaldehyde was removed and a 1:1 mixture of methanol and heptane was added to pop the vitelline membrane. Then, embryos were washed with methanol and hydrated in 1 x PBTN. Embryos are stained with anti-β-catenin (N27A1-DHSB) and anti Engrailed (4D9, DHSB). Images were collected by using the Leica SP8 scanning confocal microscope (Leica Microsystems, Buffalo Grove, IL).

Assessing embryonic lethality and cuticle score patterning.

Transgene function in the single mutant background was evaluated using embryos expressing the transgenes and maternally/zygotically null for APC2. The embryos were generated by creating a cross: APC2TN; $APC2^{g10}$ males and females. The transgene function of flies in the double mutant background, which was generated using a method called germ line clones, was evaluated using the FLP/FRT ovoD dominant female steroid method. *ywhsFlp/w; APC2TN/+*, FRT82B $APC2^{g10}$ $APC1^{Q8}$ /FRT82B ovoD female were crossed with *ywhsFlp/;; FRT82B APC2^{g10} APC1^{Q8}*/FRT82B *twiGFP* male. Heat shocks were performed 6 days after egg laying for 45 minutes in $37^{\circ}C$. The cuticle score was assessed using a previously established scoring guide developed by Peifer's lab.

Genomic DNA extraction

Genomic DNA was isolated from Drosophila fruit flies using the DNeasy Blood & Tissue Kit Protocol (Qiagen). The flies were first mixed and vortexed with detergent to lyse the cell membrane and proteinase K to digest the contaminating protein, and then with ethanol to precipitate the DNA to the spin column. Subsequently, the wash buffers were added to the spin column to remove any contaminants from the DNA, and an elution buffer was added to elute the DNA from the spin column to the microcentrifuge tube. PCR reactions were then conducted to quantify the DNA level. Primer sequences were included in Table 2.

Reverse Transcriptase-Polymerase Chain Reaction

The RNA was isolated from Drosophila flies using the Invitrogen Link RNA Mini Kit Protocol (Thermo Fisher). The RNA was extracted with similar steps from Genomic DNA extraction, but DNAase was also added to denature all released DNA. All produced RNA will be used to make cDNA by adding reverse-transcriptase and reaction mixture (primers, dNTPs, buffer, etc). PCR

reactions were then conducted to quantify the RNA level. Primer sequences were included in Table 2.