UNIVERSITY OF CALIFORNIA, SAN DIEGO

Daple Modulates Wnt/Frizzled Signaling *via* a Novel G Protein Regulatory Function

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by

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The Thesis of Jeremy Y. Lin is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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University of California, San Diego

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DEDICATION

I dedicate this thesis to my friends and family for their encouragement and support.

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ACKNOWLEDGEMENTS

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ABSTRACT OF THE THESIS

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Wnt signaling is a key pathway in developing organisms for cell growth, embryonic patterning, and organogenesis. However, a dysregulation of the Wnt signaling pattern is

associated with cancer progression in 90% of colon cancer and 50% of breast cancer patients. Since its initial discovery over 30 years ago, even today we are still uncovering the specifics on the mechanisms and processes that govern Wnt signaling. Based on our results currently undergoing publication, we know that that Daple, a non-receptor GEF protein, possesses a G-protein Binding and Activating (GBA) motif which can interact with and activate G-proteins during non-canonical Wnt stimulation to disassociate the heterotrimer into Gα and Gβγ subunits, linking the Wnt pathway to G-protein signaling. This initiates downstream non-canonical Wnt responses, such as PI3k/Akt enhancement through Gβγ subunit release. In this study, we examine Daple's GBA motif and its effect through the Wnt pathway on cell polarity and membrane stability using epithelial MDCK cells. We confirmed that Daple's GBA motif is a key regulator of PI3k/Akt signaling, β-catenin localization, and 3D cyst development to modulate adherens junction integrity and cell polarity. Future work in uncovering the mechanisms of Daple signaling would more clearly reveal its potential as a prime target in the Wnt pathway to thwart cancer progression and cell invasion.

Introduction

1. The Wnt Pathway

1.1 Background and Significance

The Wnt protein was first discovered as a proto-oncogene in 1982 when Roel Nusse and Harold Varmus mutated a random assortment of mouse genes to see which could potentially cause tumors when mutated. They identified a new murine proto-oncogene, naming it Int1, for Integration site 1. Overexpression of this gene was discovered to produce spontaneous mammary hyperplasia and neoplasia, causing an uncontrolled proliferation of cells leading to the development of cancer (Fresno Vara, Casado et al. 2004). Upon further characterization of this protein, it was discovered to be the same protein as Wingless (Wg), already discovered about a decade earlier in 1973 to be involved in body axis growth and positioning during embryonic development. As a compromise, the protein was then renamed to Wnt as a combination of Int1 and Wg. After more than three decades of research in various model organisms, the Wnt pathway has been found to play crucial roles in embryonic axis formation (Niehrs 2012), bone mass and strength (Baron and Kneissel 2013), type II diabetes (Garcia-Jimenez, Garcia-Martinez et al. 2013), and adult homeostasis, making Wnt signaling an essential pathway to study for its clinical implications. However, controversy still exists on the specifics of the mechanisms in the Wnt signaling pathway, which will be addressed below.

Most mammals have an assortment of 19 Wnt genes, categorized into 12 subfamilies. Some Wnts are identified as primarily activating the canonical pathway (Wnt 1, Wnt 3a, Wnt 7, and Wnt 8), while others initiate the non-canonical Wnt pathways (Wnt 5a and Wnt 11) (Rao and Kuhl 2010). As we go further back evolutionarily, there is less Wnt gene involvement, with 11 subfamilies in the Cnidaria, fewer in sponges, and completely absent in single-celled organisms, indicating that Wnt proteins play a vital role in the evolutionary process of multi-cellular development (Katanaev 2010; Clevers and Nusse 2012). Extensive research (primarily in *Drosophilia*) has been done to show that the Wnt pathway is consistently exploited in embryonic

patterning and organogenesis during early growth, and tapers off as the organism matures, becoming predominantly dormant in adults (Katanaev 2010).

In the cell, Wnt proteins are first produced in the endoplasmic reticulum (ER) to 40kDa in size, then undergo post-translational lipid modification by Porcupine (an O-acyltransferase) for proper signaling during secretion and receptor binding. The primary receptor for Wnt ligands is the Frizzled (Fzd) receptor, a GPCR-like protein with 7 trans-membrane domains (Malbon 2004) that can transduce its signal through G-proteins (Huang and Klein 2004; Kim, Kim et al. 2013). The N-terminus of Fzd is located on the extracellular side with a Cysteine Rich Domain (CRD), which acts as a receptor with high affinity to Wnt ligands (Dann, Hsieh et al. 2001). The C-terminal part instead is found in the cytoplasm and contains a PDZ-domain Binding Motif (PBM) sequence (Lys-Thr-X-X-X-Trp) (Umbhauer, Djiane et al. 2000; Wawrzak, Luyten et al. 2009). There are 10 different Fzd genes found in humans with varying degrees of similarities between the isoforms (Huang and Klein 2004), with an average of 30% sequence similarity between the Frizzled groupings (Fredriksson, Lagerstrom et al. 2003). The variation between Fzd receptors contribute to their functions, as some can initiate the canonical or non-canonical Wnt pathways (Huang and Klein 2004; Kohn and Moon 2005).

1.2 The Canonical Wnt Pathway

1.2 The Canonical Wnt Pathway

The most described Wnt pathway is the canonical pathway, which utilizes β-catenin proteins to act as a transcriptional activator in the nucleus for production of Wnt targeted genes. (He, Sparks et al. 1998; Zhang, Gaspard et al. 2001; Anastas and Moon 2013). At steady state in the absence of Wnt stimulation (**Fig. 1A**), cytoplasmic β-catenin undergoes degradation through a destruction complex composed of Axin, APC, GSK3, and CK1. β-catenin becomes phosphorylated by the two kinases (CK1 and GSK3) (MacDonald, Tamai et al. 2009), which is

the signal recognized by E3 ubiquitin ligase β-TrCP to target β-catenin for ubiquitination-mediated degradation *via* the proteosome. In the absence of nuclear β-catenin, transcription of Wnt targeted genes is inhibited by Groucho/TLE repressors and histone deacetylases.

In the presence of Wnt ligands (**Fig. 1B**), Wnt attaches to both the Fzd receptor and co-receptor LRP 5/6 (Low-density lipoprotein receptor-related protein 5/6), in order to recruit Disheveled (Dvl). Dvl has 3 primary domains (Penton, Wodarz et al. 2002): a PDZ domain, flanked by a DIX domain on the N-terminal region and a DEP domain located towards the C-terminal end (Boutros and Mlodzik 1999). After Wnt ligand stimulation, Dvl binds to Fzd via the PBM-PDZ domain interaction (Gao and Chen 2010), prompting Axin and GSK3 to adhere to the membrane (MacDonald, Tamai et al. 2009). These processes result in a dismantling of the destruction complex, allowing β-catenin to accumulate. β-catenin can then drift from the cytoplasm to the nucleus and act as a transcription co-activator for genes in the TCF/LEF family, such as c-Myc and cyclin-D1 to ultimately control cell proliferation and cell survival (Kohn and Moon 2005; Komiya and Habas 2008).

1.3 Non-Canonical Wnt Pathways

There are also two other Wnt signaling pathways that are independent of the β-catenin protein, known as the non-canonical Wnt pathways: the Wnt/Planar Cell Polarity (PCP) pathway and the Wnt/Calcium pathway (**Fig. 2**).

The Wnt/PCP pathway organizes the arrangement of cells on multi-cellular structures for a coordinated orientation and polarization along a specific axis (Seifert and Mlodzik 2007; Goodrich and Strutt 2011; Gao 2012). For example, epithelial cells are uniformly oriented with distinct apical and basal domains, either facing the lumen or adhering to the basal surface, respectively (Simons and Fuller 1985). Mesenchymal cells are also polarized, with a leading and trailing edge in order to facilitate cell movement (Ridley, Schwartz et al. 2003). In the Wnt/PCP

pathway, non-canonical Wnt ligands (like Wnt 5a) bind to Fzd receptors and activate Dvl. Dvl then initiates downstream signaling cascades, including RhoA and Rac1 GTPase activation, which ultimately results in actin/cytoskeleton rearrangement and cell migration (Komiya and Habas 2008; Sugimura and Li 2010).

The non-canonical Wnt/Calcium pathway utilizes Fzd and Dvl to induce G-protein activation (Kilander, Petersen et al. 2014). Heterotrimeric G-proteins are a family of proteins that function as potent molecular switches for signal transduction within the cell. They are primarily governed by Guanine nucleotide Exchange Factors (GEFs), which exchanges the G-protein's Guanosine Di-Phosphate (GDP) to Guanosine 5-Tri-Phosphate (GTP) (Kilander, Dijksterhuis et al. 2011). The result is a disassociation of the G-protein's trimeric complex into two smaller and distinct subunits, Gα and Gβγ, which can continue to amplify their responses in a variety of signaling pathways. The Gα monomer can trigger Phospholipase C (PLC) to operate through its signal transduction pathway and stimulate Protein Kinase C (PKC) activity, resulting in elevated intracellular calcium levels. (Luna-Ulloa, Hernandez-Maqueda et al. 2011). Simultaneously, Gβγ heterodimers can directly activate the Phosphoinositide 3 Kinase (PI3K)/Akt pathway, a prominent signaling cascade for cell growth and stability, nutrient metabolism, cell polarity, protein synthesis, and cell migration (Morgensztern and McLeod 2005; Song, Ouyang et al. 2005; von Maltzahn, Bentzinger et al. 2012). To downregulate G-protein signaling, GTPase Activating Proteins (GAPs) can hydrolyze the GTP back into a GDP, allowing for reformation of the heterotrimer (Gilman 1987; Bourne 1995).

Calcium is used as a second messenger in the non-canonical pathway following G-protein activation. An increase in intracellular calcium from the endoplasmic reticulum (ER) can activate various proteins, notably Calcineurin and Calcium/Calmodulin-dependent Kinase II (CaMKII). Calcineurin imports NFAT (Nuclear Factor of Activated T-cells) into the nucleus to regulate gene expression, while CaMKII signals TAK1 (TGFβ-Activated Kinase 1) and NLK (Nemo-like

Kinase) to antagonize canonical β-catenin in a negative feedback loop (Ishitani, Ninomiya-Tsuji et al. 1999; Komiya and Habas 2008; Inestrosa, Montecinos-Oliva et al. 2012).

2.0 Daple

2.1 Non-receptor GEFs and Daple

The GEFs needed to activate G-proteins are most commonly found in ligand activated G-Protein Coupled Receptors (GPCRs). Since our Frizzled receptor has a congruent topography to GPCRs, with an outer facing N-terminal receptor, a 7 trans-membrane domain, and a cytosolic C terminus (Kohn and Moon 2005; Koval and Katanaev 2011), some researchers have concluded the Fzd receptor is a *bona fide* GCPR, and found G-proteins to be involved with both the canonical and non-canonical pathways. However, in order to be classified as a GPCR, the Fzd receptor needs the ability to act as GEFs for heterotrimeric G-proteins, and research has yet to conclude a direct interaction between G-proteins and the Fzd receptor (Petersen and Reddien 2009; Clevers and Nusse 2012). In this research, we seek to bridge the gap of understanding by establishing the role of non-receptor GEFs, which operate independently from the classical view of G-protein activation with its ability to disassociate the G-protein heterotrimer without external ligand stimulation (Tall, Krumins et al. 2003). Now in our lab, GIV has been identified as the first non-receptor GEF that activates Gαi subunits *via* a defined, evolutionarily conserved motif, known as the Gα-Binding and Activating (GBA) motif (Garcia-Marcos, Ghosh et al. 2009).

Daple (also known as CCDC88C) is a large 250kDa protein, discovered as an orthologue of GIV, with a high frequency of similar amino acids. Analogous to GIV, Daple has a HOOK and a coiled-coil domain toward its N-terminus, which allow for microtubule binding and homo-oligomerization, respectively (Kobayashi, Michiue et al. 2005). In the C-terminal end lies a G-protein binding domain (GBD) (Le-Niculescu, Niesman et al. 2005), a proposed GBA motif we seek to identify, and an extreme terminal PDZ-binding motif (PBM) domain capable of

interaction with Dvl's PDZ domain. In 2003, Daple was identified to be a binding partner of Dvl as a negative regulator of the canonical Wnt signaling pathway. When murine cells were stimulated with Wnt 3a, Daple stalled β-catenin accumulation and subsequent TCF activation. Additionally, Daple is also known to play a role in the non-canonical Wnt/PCP pathway as a regulator of Dvl-induced activation of Rac, which promoted actin remodeling and cell migration (Ishida-Takagishi, Enomoto et al. 2012). As we stand, there is still much more to uncover about Daple, as a current search in PubMed yields only eight related results. Our lab decided to take on the challenge of elucidating more on Daple and the role it plays in the Wnt pathway. The results and conclusions presented are currently undergoing final publication.

2.2 Daple Possesses a Gα-Binding and Activating (GBA) Motif and Binds to Gαi Subunits

In our search for the putative GBA domain in Daple, a sequence analysis comparison was performed between GIV and Daple across various species (including zebrafish, mice, and chicken), and as expected, we saw high residue similarity between the two in the left sided N-terminus. However, toward the C-terminal end, there was much divergence in the sequences, which can account for the specificity of protein binding between the two, as GIV and Daple associate with different proteins. Upon further analysis, there is a stretch of highly conserved residues located at Daple's 1668-1683, which is congruent to the G-Binding and Activating (GBA) motif previously identified in GIV and other related synthetic peptides (**Supplementary 1A**) (Johnston, Ramer et al. 2005; Garcia-Marcos, Ghosh et al. 2009; Garcia-Marcos, Jung et al. 2011). To characterize this GBA domain in Daple, we wanted to first verify its binding capabilities. Cos7 cells were obtained and double transfected with HA-tagged Gαi3 (the preferred binding partner of the GBA domain in GIV), and Daple, as normal Cos7 cells do not endogenously express quantifiable levels of Daple. A whole cell lysate and co-

immunoprecipitation was done to pulldown the HA tag of Gαi3, and found Daple to be a binding partner, revealing their interaction (**Supplementary 1B**).

We next investigated if the interaction between Daple and G-proteins was consistent with previous GBA motifs, in that Daple binds G-proteins with high affinity when bound to the inactive GDP form, but not to the active GTP conformation. We saw in our pulldown that recombinant purified GST-tagged Gαi3 bound robustly to His-Daple-CT (composed of amino acids 1650-2028, containing the GBA motif) when preloaded with GDP, but not when preloaded with GDP/AlF_4^- or GTPγS, both of which mimic the active GTP-bound configuration (**Supplementary 1C**). These results show that Daple possesses a highly conserved and functional GBA motif capable of specific interaction with inactive GDP:Gα proteins *in vitro*.

2.3 Identification of Critical Determinants for Effective Interaction between Gαi and Daple

To further understand the specific interaction mechanism between Daple and Gαi proteins, a homology model was built to replicate the binding between the GBA and Gαi3 domains. We utilized the atomic structure of KB-752, a synthetic GEF peptide with a sequence matching the GBA motif (Johnston, Ramer et al. 2005) to generate Gαi3's binding conformation, and then substituted the synthetic peptide with Daple. A hydrophobic cleft was revealed, formed by aromatic residues W211 and F215 on the SwII (SwitchII) domain, and the α3 helix on the Ras-like region of Gαi3. In tandem, Daple contains a highly conserved hydrophobic phenylalanine on amino acid 1675, capable of forming a major molecular contact with the hydrophobic cleft on Gαi3. We hypothesized that mutation of the phenylalanine to a non-hydrophobic residue, like alanine, would disrupt interaction due to a loss of binding forces. A site-directed mutagenesis experiment was carried out on His-Daple CT to mutate the 1675 phenylalanine into an alanine (signified by F1675A, or FA). When these constructs were incubated with recombinant purified

GST-Gαi3, only the Wild-Type (WT, non-mutated sequence) was able to bind, while the FA mutation was unable to associate with Gαi (**Supplementary 2B**). This result demonstrated that the phenylalanine is critical for binding onto to the hydrophobic cleft of Gαi3, and afforded us a negative control to use in our future experiments.

2.4 Daple is a *bona fide* Guanine Nucleotide Factor (GEF) for Gαi *in vitro*

Now knowing that Daple possesses a GBA motif that binds Gαi, we decided to check its GEF function. The definition of a GEF is the ability to accelerate the rate of nucleotide exchange in G-proteins, from a GDP subunit to a GTP subunit. In the inactive state, the G-protein is bound to a GDP, which naturally dissociates very slowly. However, when a GEF binds to the G-protein, it catalyzes GDP nucleotide release, allowing for GTP to bind in its place (Bourne, Sanders et al. 1990; Mukhopadhyay and Ross 2002). Therefore, to verify if a GEF is functional or not, we can analyze the quantity of nucleotide exchange (GDP to GTP) against baseline activity. Our experiments performed analyzed this nucleotide exchange rate by incubating either WT or FA Daple-CT constructs with radioactive-labeled [γ-^{32}P] GTP:Gαi3, then quantifying the [γ^{32}Pi] released through scintillation counting. As a result, WT-Daple-CT with His-Gαi3 was able to accelerate the rate of exchange ~3 fold over the inactive FA mutation (**Supplementary 2c**).

2.5 Daple Activates Gαi in Cells Responding to Wnt 5a

In order to substantiate our hypothesis that Daple is the mediator between the Wnt pathway and G-proteins, we needed to link Daple activation and subsequent GEF activity to Wnt stimulation. In this regard, we followed closely along previously described experiments of non-canonical Wnt stimulation (Yamamoto, Yoo et al. 2007; Sato, Yamamoto et al. 2010), using HeLa cells as the model cell line, and Wnt 5a as the principle Wnt ligand for non-canonical signaling. Our HeLa cells were transfected with Daple-WT or Daple-FA, then analyzed for Gαi

activation using an anti-Gαi:GTP antibody that recognized specifically the active GTP-bound form of Gαi (Lane, Henderson et al. 2008; Lopez-Sanchez, Dunkel et al. 2014). The resulting immunoprecipitation showed that Daple's GBA motif is necessary for activation of Gαi after Wnt 5a stimulation (**Supplementary 2D**).

2.6 Daple Activates PI3K-Akt Signaling via Release of Free Gβγ Subunits.

To reinforce Daple's activity as a GEF, we looked into the structural mechanisms of its heterotrimeric separation between the Gα subunit and the free Gβγ subunits released. A crystal structure comparative analysis was performed and there we saw an overlap in the binding interaction site on Gαi, with either Daple or Gβγ (**Supplementary 3A**). We hypothesized that binding of Daple to Gαi would structurally and forcefully evict the Gβγ subunits in order to facilitate downstream signaling. With this in mind, a competition assay was performed on Gαi3 preloaded with Gβ, then incubated with rising concentrations of Daple-WT. The result showed increasing displacement of Gβ from Gαi as higher doses of Daple-WT were added; furthermore, it was noted that FA mutation which was not able to bind G-proteins, did not result in a separation of the Gαi3:Gβ complex, even at maximum concentration (**Supplementary 3B**).

To determine if the Gβγ released by Daple's GBA motif affected downstream pathways, we decided to analyze PI3k/Akt signaling, as previous studies consistently linked both non-canonical Wnt signaling and free Gβγ to PI3k/Akt enhancement (Ohigashi, Mizuno et al. 2005; von Maltzahn, Bentzinger et al. 2012). In our HeLa cells, we found Akt activation (based on Ser473 kinase phosphorylation) present in Wnt 5a stimulated Daple-WT, but not in the FA mutant (**Supplementary 3C**), indicating that Daple's GBA motif is essential for Wnt pathway mediated Akt signaling. To validate that the enhancement in Akt signaling was based solely on the release of free Gβγ subunits, we used a Gallein, a Gβγ inhibitor which specifically blocks the active site on Gβγ to disrupt its interaction with key downstream effectors (Smrcka 2008;

Seneviratne, Burroughs et al. 2011). We found that during incubation of Daple-WT with Gallein under Wnt 5a stimulation, the Akt stimulation had vanished, while for Fluorescein (the inactive analogue serving as a negative control), the Akt pathway had remained enhanced. These results conclude that the Daple-mediated release of free Gβγ subunits positively affects downstream PI3k/Akt signaling.

3. Hypothesis

All taken together, we know that Daple's GBA motif is a crucial player in the Wnt pathway, acting as a GEF to disassociate heterotrimeric G-proteins into the active form of GTP:Gα and releasing free Gβγ. This goes to activate a variety of downstream effects, one of which being enhancement of the PI3k/Akt pathway. In this study, utilizing the MDCK epithelial cell line and 3D matrix techniques, we move forward and hypothesize that Daple's GBA motif enhances the PI3k/Akt pathway to affect downstream cell junction stability and cell polarity to ultimately impact cancer formation.

Results

1. The PI3k/Akt Pathway is Enhanced by Daple's GBA Motif

Cell polarity is defined as the asymmetric differentiation of cellular components to determine a specific cell function and cell type, e.g., epithelial cells have an apical domain which lies above the tight junctions and faces the outer lumen surface, and a specific basolateral region which adheres to the basal lamina (Goodrich and Strutt 2011). Dysregulation of cell polarity has been known to cause developmental disorders, cell transformation, and cancer development (Bornens 2008; Bryant and Mostov 2008). Control of cell polarity is through many means, one of which is PI3k/Akt signaling, known to organize junction formation and epithelial cell differentiation (Laprise, Chailler et al. 2002; Rivard 2009). Now, in order to study Daple's effect on PI3k/Akt activation and cell polarity, we obtained MDCK II (Madin-Darby Canine Kidney type II) epithelial cells to use as a model cell line, as they grow with distinct apical-basal orientations, have well defined cell junctions, and polarize in both 2D and 3D cell cultures. (Dukes, Whitley et al. 2011). The MDCK cells were split into two cell lines, each of which transfected with different pcDNA3-MYC-Daple plasmids: MYC-Daple-WT, and MYC-Daple-F1675A mutation, which has its phenylalanine on residue 1675 mutated to an alanine to mimic a loss of GEF function (**Supplementary 2B**). The plasmid also contains the MYC tag, a commonly used 10 amino acid sequence which functions to specifically visualize the location and concentration of MYC-proteins within the cell, without disrupting function. After transfection of the MDCK cells with the plasmid, stable cell lines were generated by inducing a selective pressure with the addition of G418 media, which has Geneticin as the active ingredient to block peptide synthesis. The pcDNA3 plasmid contains the neomycin gene to provide resistance against this antibiotic (Rinaldi, Suttiprapa et al. 2012), selecting for only the MDCK cells that received and are expressing the plasmid to survive and reproduce.

Using our generated MDCK stable cell lines, a whole cell lysate immunoblot was performed and probed for phosphorylated Akt (on Ser473). The parental (untransfected) and WT

both displayed a consistent and even band for Akt phosphorylation, while only in the MDCK-Daple-FA stable cell line do we see a decrease in Akt enhancement (**Fig. 3**). This result shows that the GBA function in Daple is necessary for amplifying downstream Akt activity.

2. Daple's GBA Motif Regulates β-catenin Localization at Cell Junctions

Next, after confirming that Daple's GBA motif enhances Akt phosphorylation, we wanted to look at some downstream physiological consequences. We know that the PI3k/Akt pathway promotes adherens junction assembly (Laprise, Chailler et al. 2002) and has a controlling effect on cell growth, morphology, and migration (Chae, Yang et al. 2009). The adherens junction helps to form strong physical mechanical attachment in the apical portion of epithelial cells, using a tight network of cadherins, actin, and catenins (including β-catenin) (Nelson 2008). Functionally, the adherens junction serves to fortify epithelial barriers, define cell polarity with an apical and basal orientation, and also maintain contact inhibition (Smalley and Dale 1999; Howard, Varallo et al. 2003; Kam and Quaranta 2009; Baum and Georgiou 2011). Normally, cells divide until they physically touch one another, which then the growth factor signals terminatee. However, upon loss of contact inhibition, a transformation occurs, known as Epithelial-Mesenchymal Transition (EMT), in which cells grow in an uncontrolled manner and morph into migratory and invasive cells with a loss of polarity (Puliafito, Hufnagel et al. 2012). To study if Daple plays a potential role in reinforcing cell barriers at the adherens junctions, an immunofluorescence under 10% Fetal Bovine Serum (FBS) media was conducted on the previously generated MDCK stable cell lines, and probed for β-catenin as an integral adherens junction protein. The results suggest that under these conditions, there were no major phenotypic effects of Daple's F1675A mutation compared to the wild-type (**Supplementary 4**).

After procuring the baseline standard localization of β-catenin at 10% FBS, we wanted to verify if there could possibly be a phenotypic change under a different environment. We took

advantage of the serum starvation technique which is commonly utilized to detect signaling in cells, as it helps to synchronize the cell cycle of proliferation/quiescence, slow down cell growth and development, and also downplay the growth factor stimulants found in normal serum levels (Jackman and O'Connor 2001; Chen, Huang et al. 2012). Also, since we know that GEFs are signal amplifiers, their activity is best appreciated when ambient signals are low. For example, in GIV, an orthologue of Daple, we typically see the effect of signal amplification due to the GEF activity when the studies were carried out at steady-state with low serum levels (Ghosh, Garcia-Marcos et al. 2008). A new experiment was performed, with a 2% FBS serum deprivation overnight before the immunofluorescence, and β-catenin was probed to visualize the effects at the adherens junction (**Fig. 4**).

The results showed that compared to the WT at 2% FBS (**Fig. 4A**), which had proper β-catenin localization at the adherens junction, the FA stable cell line revealed a substantial loss of β-catenin at the cell junctions (**Fig. 4B**), signifying that Daple's GBA motif plays a role in the localization of β-catenin and overall junctional integrity.

3. The GBA Motif in Daple Positively Regulates Cyst Formation

The last question we asked was if the GBA motif in Daple affected cell junctions and polarity in a 3-dimensional (3D) culture, in order to more closely mimic the development of normal *in vivo* epithelial cells. In a 3D culture, which has been exploited by researchers for decades to study cell morphology, polarity and development (Thomas, Schultz et al. 1982; Rana, Mischoulon et al. 1994; Cukierman, Pankov et al. 2001; Hunter and Zegers 2010), the cells are submerged in an extra-cellular matrix in order to spontaneously form spherical cysts, which are characterized by a hollow lumen encircled by a layer of polarized cells with defined apical and basal domains (Elia and Lippincott-Schwartz 2009; Wells, Yarborough et al. 2013). This complex multicellular formation requires each cell to have its own standardized orientation and well-

established junctions for proper cross talk and coordination during 3-dimensional structure development. Our experiment was performed according to the procedure outlined by Elia and Lippincott (Elia and Lippincott-Schwartz 2009), but modified to include a 2% serum deprivation for 7 days before observation. The development for both of our MDCK-Daple stable cell line constructs were photographed with pictures representing the average cyst size, shape, and overall formation (**Fig. 8)**. The WT stable cell lines had proper cyst development with a distinct apical axis around the inner lumen and a basolateral outer edge. In contrast, the Daple-FA cells were stunted in growth and formed much smaller individual colonies lacking the synchronization to develop into a proper 3-dimensional cyst. These results demonstrate that Daple's GBA motif plays a vital role to upregulate epithelial cell development and cell polarity.

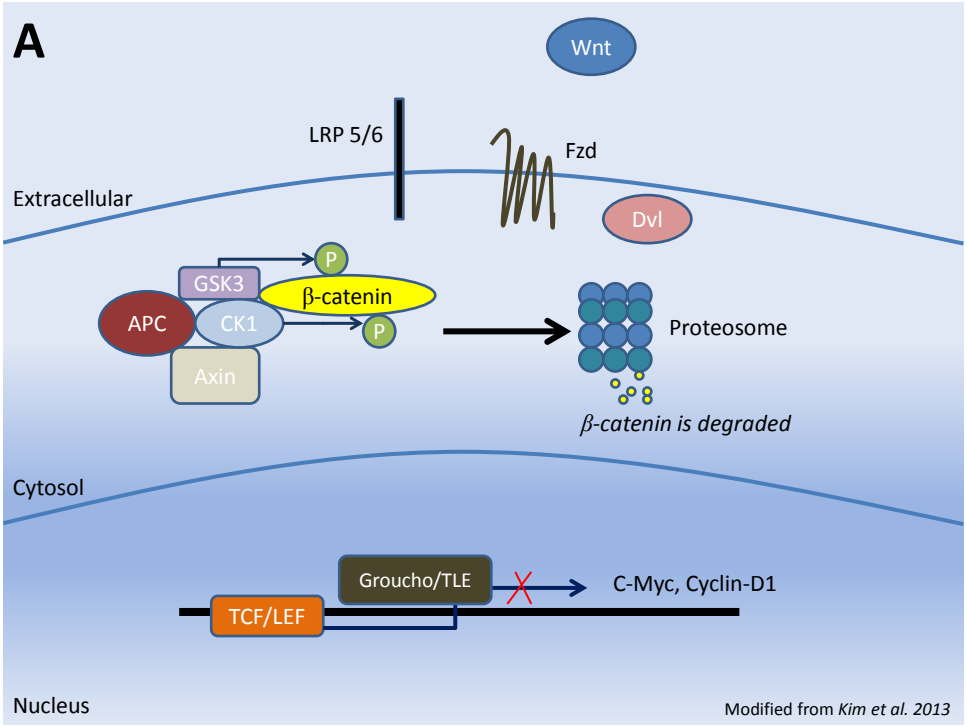


Figure 1a. Diagram of the Canonical Wnt Pathway involving β-catenin, non-stimulated. In the absence of Wnt ligands, β-catenin enters a destruction complex composed of APC, Axin, GSK3, and CK1. GSK3 and CK1 phosphorylate β-catenin, which gets ubiquitin tagged (not shown), and sent to the proteosome for degradation. There is no β-catenin entering the nucleus and its transcription target, TCF/LEF, is hindered by Groucho/TLE repressors.

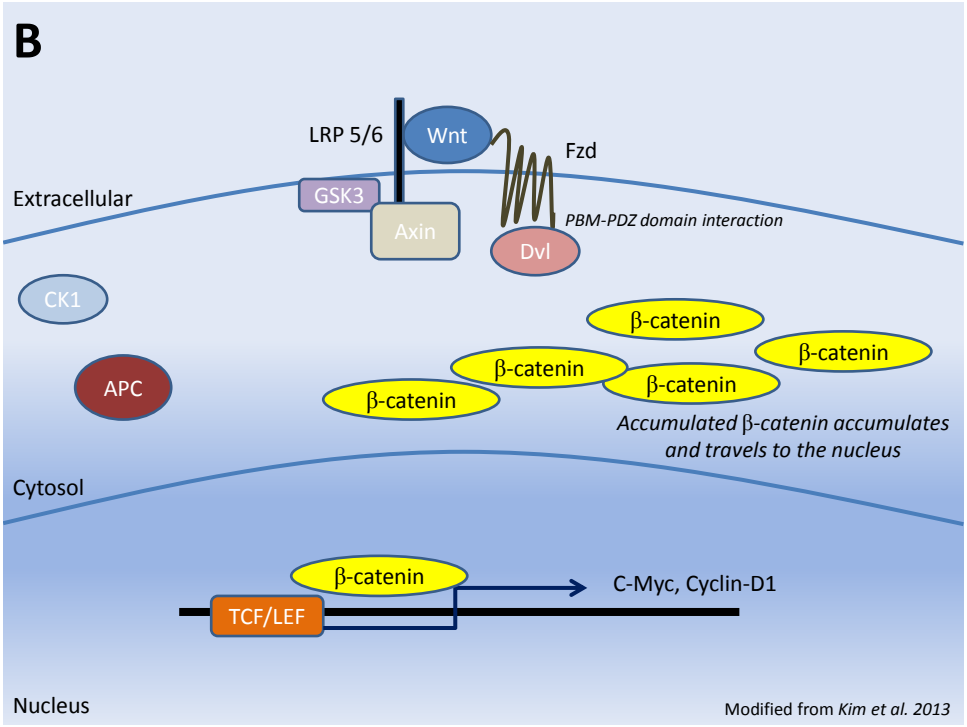


Figure 1b. Diagram of the Canonical Wnt Pathway involving β-catenin, stimulated. During Wnt stimulation, Wnt binds to Fzd and co-receptor LRP5/6 to initiate canonical signaling. Dvl binds to Fzd and recruits Axin and GSK3 to the cell membrane, dismantling the destruction complex. β-catenin accumulates and progresses into the nucleus, where it removes the Groucho/TLE repressors and acts as a TCF/LEF transcriptional activator to generate Wnt transcription products, including C-Myc and Cyclin-D1.

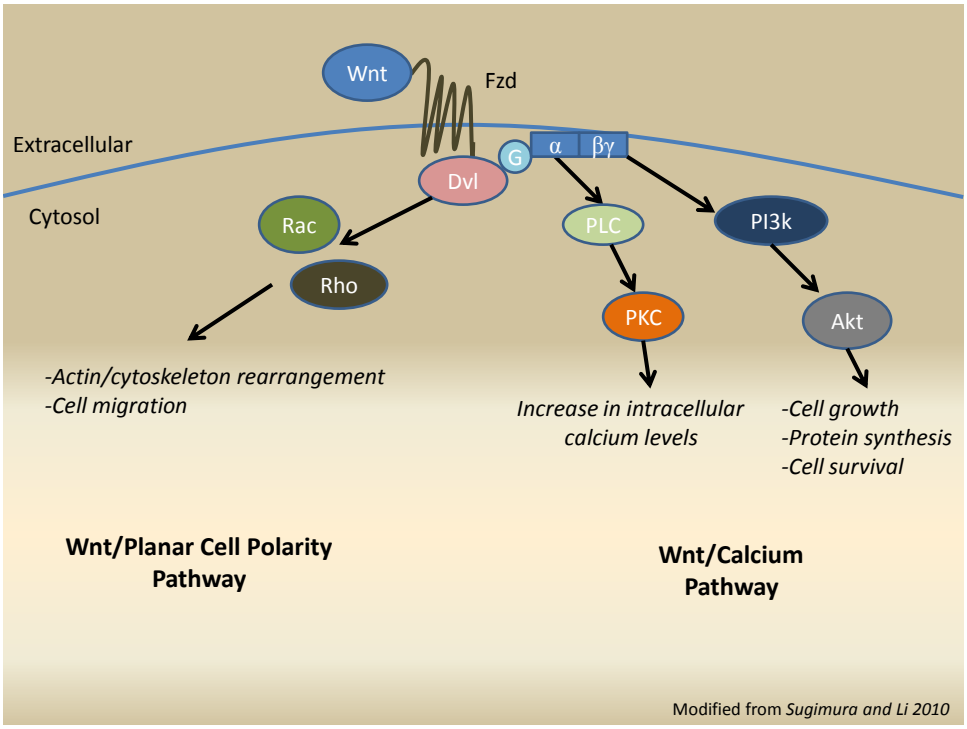


Figure 2. The Non-canonical Wnt pathways. The non-canonical Wnt pathways do not utilize β-catenin in their signal transduction. In the Wnt/PCP pathway, non-canonical Wnt ligands adhere to the Fzd receptor, which binds Dvl to stimulate Rho and Rac, ultimately affecting actin/cytoskeleton rearrangement and cell migration. In the Wnt/Calcium pathway, heterotrimeric G-proteins are recruited by Fzd and Dvl to be disassociated into its subunits, Gα and Gβγ. These go on to initiate a variety of signaling pathways, including elevating intracellular calcium levels and triggering the PI3k/Akt pathway.

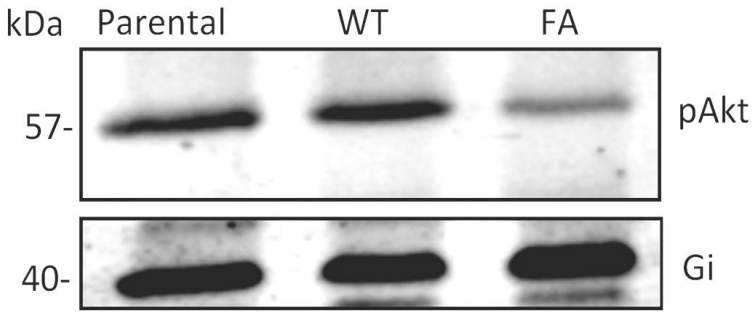


Figure 3. Daple Mutants without a Functional GBA Motif Results in Decreased Akt Phosphorylation. Whole cell lysis was performed on parental MDCK and MDCK cells stably expressing MYC-Daple-WT and FA plasmids in 10% FBS serum. The cell lysates were loaded and an SDS-PAGE was performed. The membrane was immunoblotted and probed for phosphorylated Akt, while Gi was used as a loading control.

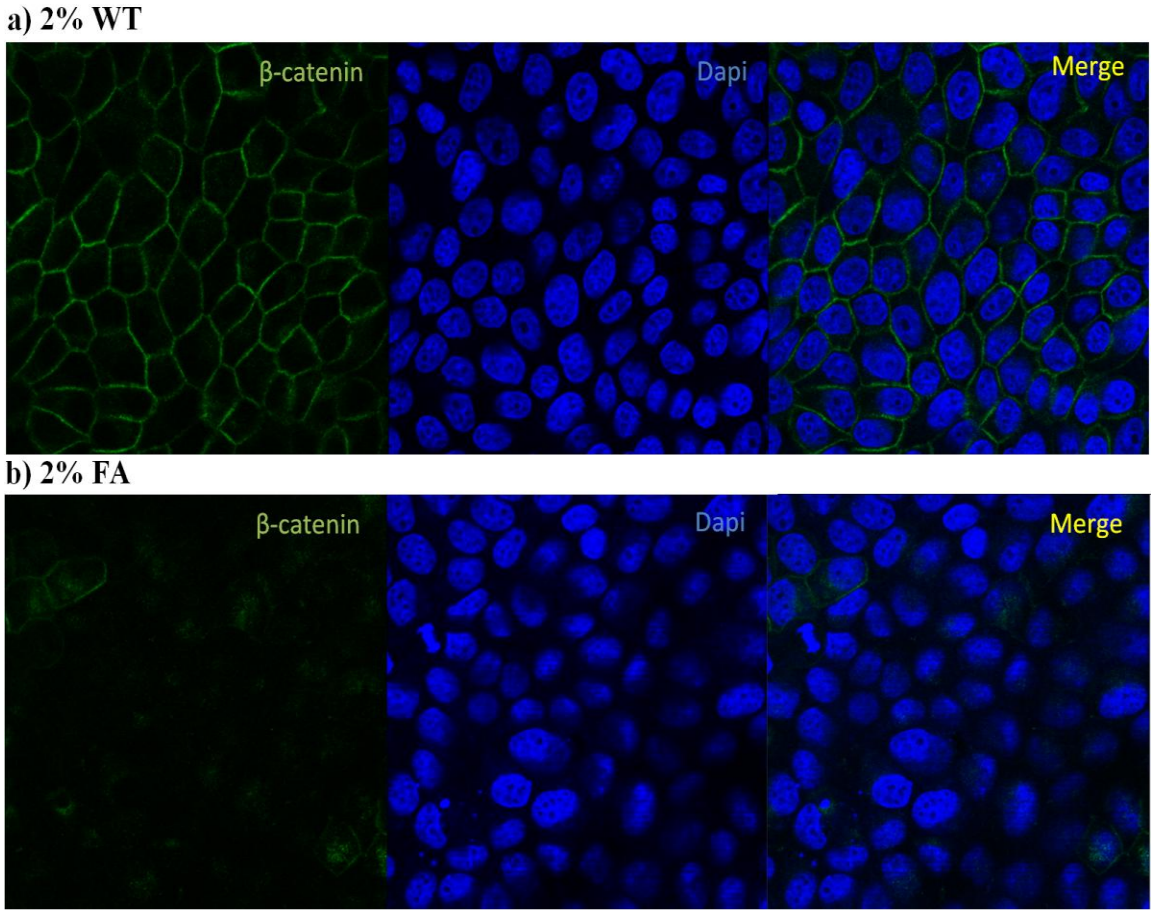


Figure 4. β-catenin Localization in MDCK Daple Stable Cell Lines Under 2% serum Starvation. MDCK stable cell lines with MYC-Daple-WT and FA plasmid constructs were grown in serum deprived 2% FBS (**4a** and **4b** respectively) overnight before fixation. Immunofluorescence was performed using β-catenin and Dapi for adherens junction and nuclear markers, respectively. The 2% MYC-Daple-FA mutant shows lacking β-catenin in its cell adherens junctions compared to the WT, signifying that Daple's GEF function plays a controlling role in the localization of β-catenin.

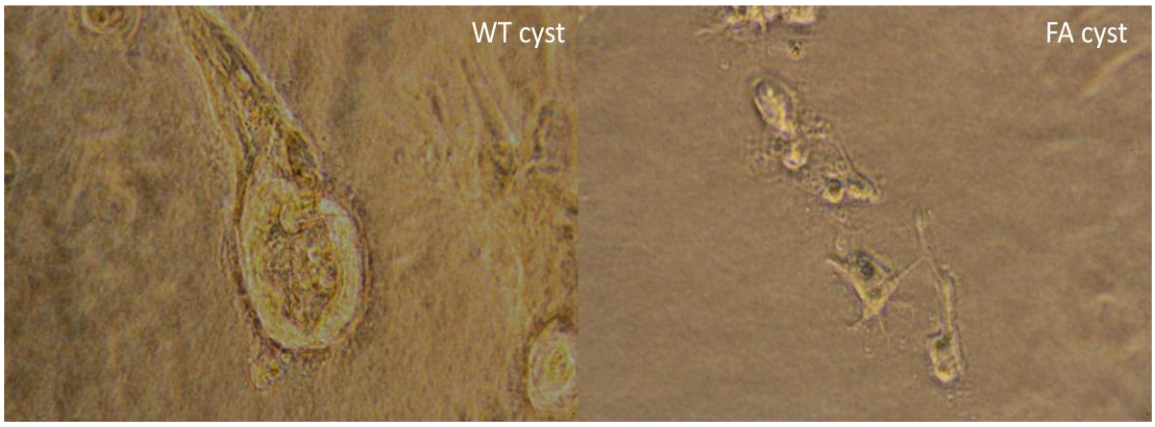


Figure 5. The GBA Motif in Daple is Essential for Proper Cyst Formation. Stable MDCK cell lines with MYC-Daple-WT and FA plasmids were grown in a 3-dimensional rat collagen matrix under 2% FBS serum deprivation for 7 days before fixation. Pictures were taken representing the average cyst size, shape, and formation for each of the stable cell lines. The WT cysts grew normally, forming a hollow lumen circumscribed by polarized cells with specific apical and basal domains, while the FA cysts lost their polarity and formed individual colonies without an appropriate cell orientation in the 3D matrix.

Discussion

Both the canonical and non-canonical Wnt pathways have yet to be fully defined, even though they have been studied extensively for the past three decades. In 2003, a yeast two-hybrid experiment using a mouse brain cDNA library was screened, and the Daple protein was identified as a binding partner of Dvl (Oshita, Kishida et al. 2003). Since then, however, there have been relatively few publications that describe the role of Daple in the Wnt pathways (Ishida-Takagishi, Enomoto et al. 2012). Our lab took on the task of unveiling Daple's contribution to the Wnt pathway: first, by identifying its putative GBA motif, then, by characterizing its role as a GEF in Wnt signaling. We concluded that Daple possesses a GBA motif that binds to and activates Gαi subunits in response to Wnt 5a to initiate downstream signaling patterns, one of which is PI3k/Akt enhancement. This study builds upon the aforementioned experiments and taking a deeper look to identify some consequences of Wnt-induced, Daple-mediated activation of the PI3k/Akt pathway. Using the MDCK epithelial cell line, it was confirmed that Daple's GBA motif is necessary for PI3k/Akt enhancement, which affects β-catenin localization, cell junction stability, and proper cell polarity, to ultimately play its distinct role in cancer progression.

1. Daple Possesses an Active GBA Motif that Upregulates PI3k/Akt Activity

Based on previous results in our lab, we know that Daple's C-terminus contains a highly conserved and specific GBA motif similar to the proteins in its related family (most notably GIV) (**Supplementary 1A**), in that they preferentially bind to Gαi3 G-proteins with the inactive GDP conformation over the active GTP conformation (**Supplementary 1C**). Consequently, when the phenylalanine on Daple's residue 1675 was mutated to an alanine, its G-protein binding ability (**Supplementary 2B**) and GEF activity (**Supplementary 2C**) was abolished, validating the functional GBA motif in Daple capable of G-protein interaction and activation. From here, we connected Daple's GBA motif to non-canonical Wnt signaling enhancement using the Wnt 5a ligand. HeLa cells were transfected with Daple-WT and FA, and we were only able to see G-

protein activation in Wnt 5a stimulated Daple WT cells (**Supplementary 2D**). Notably, on a side project, we attempted to use Wnt 3a ligands, a known canonical Wnt pathway activator, but no response was elicited, signifying an exclusive use of Daple's GBA domain in non-canonical Wnt signaling. A downstream consequence of Daple-mediated Wnt signaling was the structural displacement of Gβγ (**Supplementary 3A, 3B**), invoking the enhancement of the PI3k/Akt pathway in both HeLa and MDCK cell lines, as seen by phosphorylation at Serine 437 on Akt (**Supplementary 3C**, **Fig. 3,** respectively). In HeLa cells, we verified that the PI3k/Akt upregulation was brought into effect *via* the free Gβγ subunits using Gallein, a Gβγ active site inhibitor, and saw the Akt enhancement vanish. What we found was in accordance with previous publications, as GIV has also been shown to also promote PI3k/Akt signaling through similar mechanisms (Garcia-Marcos, Ghosh et al. 2009; Ghosh, Garcia-Marcos et al. 2011). With this, we can conclude that Daple utilizes its GBA motif to activate G-proteins for PI3k/Akt enhancement. One experiment to back up our findings in MDCK cells, is to again use a Gallein-induced inhibition of Gβγ activity. First, we would obtain a baseline level of phosphorylated Akt in the Daple-WT and Daple-FA MDCK stable cell lines, expecting to see a decreased concentration of activated Akt in the GEF-deficient cells. Next, treat the cells with Gallein, a proven disruptor of Gβγ subunits in order to inhibit their downstream signaling (Lehmann, Seneviratne et al. 2008; Seneviratne, Burroughs et al. 2011). We would now expect to see a reduction in phosphorylated Akt concentrations in Daple-WT, displaying a phenotype similar to the FA, and proving that Daple activates PI3k/Akt in MDCK epithelial cells through Gβγ release. The next step would be to link the increase in Akt phosphorylation directly to Wnt signaling. To do so, we could repeat the previous experiment, but starting with Wnt 5a stimulation for the non-canonical pathway. If the Akt phosphorylation is indeed Wnt linked, then we would see a greater increase in phospho-Akt in the WT-MDCK stable cell line, but little to no effect for the FA-MDCK stable cell line.

On a larger picture, we can pivot our focus in another direction and analyze the roles of Daple-mediated PI3k/Akt signaling in cell mobility, which could play a tremendous role in the later stages of cancer progression (Fresno Vara, Casado et al. 2004; Morgensztern and McLeod 2005). To identify how Daple could affect cell migration, a simple scratch-wound assay could be performed (Yarrow, Perlman et al. 2004; Liang, Park et al. 2007). First, obtain Daple-WT and F1675A MDCK stable cell lines and grow them until confluency on a 6-well dish, then scratch a thin wound across the middle using a pipette tip. Time-lapse pictures would be taken as the cells along the wound edge polarizes and reforms into the scratched space (Cory 2011). If Daple's GBA domain affects cell motility, then we should see a better restoration in the WT cell lines against the FA cell lines. If true, it is quite possible that overexpression of Wnt/Fzd signaling would utilize Daple to activate the PI3k/Akt pathway and upregulate cell metastasis, as a majority of colon cancer and breast cancer cases are associated with hyper-activation of the Wnt pathway (Katanaev 2010).

2. Daple's GBA Motif Affects β-catenin Localization

The adherens junction plays a vital mechanism to reinforce epithelial cell borders, maintain cell polarity, and establish contact inhibition to prevent Epithelial-Mesenchymal Transition (EMT), which is when cells lose their growth inhibition signals and gain oncogenic and invasive properties (Behrens, Mareel et al. 1989; Yang and Weinberg 2008). β-catenin is an essential protein at the adherens junction, linking the cadherin proteins to α-catenin associated actin (Yamada, Pokutta et al. 2005). As we looked into Daple's role in junction stability at 2% FBS in order to reduce background ambient noise (Jackman and O'Connor 2001; Ghosh, Garcia-Marcos et al. 2008; Chen, Huang et al. 2012), the FA-MDCK cell line revealed a striking absence of β-catenin from the adherens junction in the GEF deficient cells, while the WT had maintained its cell borders. At first, we thought the lack of β-catenin at the junction was due a decrease in

overall β-catenin expression. However, a whole cell lysate SDS-PAGE was performed on MDCK cells with the same treatment, and the concentration of β-catenin was consistent for both the cell lines (**Supplementary 5**). Instead, another explanation suggests a re-localization of β-catenin away from the cell adherens junction, and into the cytoplasm or nucleus. We know from canonical Wnt signaling that when β-catenin enters the nucleus, it displaces the Groucho/TLE repressors and acts as a transcription factor for Wnt gene products. Therefore we could investigate β-catenin relocalization by checking the concentrations of Wnt transcription gene products, such as C-Myc or Cyclin-D1. MDCK-WT and FA stable cell lines would be cultured at 2% FBS, then a Reverse-Transcription Quantitative Polymerase Chain Reaction (RT-qPCR) would be performed to test the expression levels of C-Myc. If the hypothesis is true, then the FA mutant should have higher concentrations of the transcription product, caused by increased nuclear β-catenin levels.

An interesting observation is that this phenotype is only seen at 2% FBS starvation (**Fig. 4**), and not at 10% FBS (**Supplementary 4**). One could go farther into studying this phenomenon and assess how the localization of β-catenin from the adherens junction to the nucleus is regulated. Some research has already been done in attempts to detail the specific events that cause β-catenin to either remain in the adherens junction or migrate into the nucleus (Nelson and Nusse 2004; Gavert and Ben-Ze'ev 2007; Heuberger and Birchmeier 2010; Valenta, Hausmann et al. 2012). This regulation is critical to our understanding of cancer, as the same pool of β-catenin is used for both epithelial cell junction stability and canonical Wnt signaling for cell growth (Meng and Takeichi 2009; Shapiro and Weis 2009; Royer and Lu 2011). One approach to gain a deeper understanding would be to perform a calcium switch assay, which is commonly used for testing junction integrity and cell junction recovery, as the cadherin clusters disassemble without a calcium supply (Tobey, Argote et al. 2004; Hong, Troyanovsky et al. 2011). MDCK-WT and F1675A stable cell lines would be grown under 2% serum. After confluency, the cells would be

switched to a EGTA-treated calcium-free 2% FBS media for 16 hours to deplete calcium stores and consequently destabilize the cadherin-mediated cell junctions (Zhang, Li et al. 2006). After 16 hours, add back the calcium-rich media to allow junction reformation. In order to visualize the cell junctions, adherens junction antibodies would be used (β-catenin or E-cadherin), and time-lapse pictures would be taken immediately after calcium depletion, and every 30 minute period until 24 hours after the calcium-rich media was added. Our hypothesis would suggest diminished junction stability and restoration in the FA cell line compared to the WT cell line, as the GEF-deficient cells would have reduced β-catenin at the adherens junction according to our results (**Fig. 4B**).

3. The GBA Domain in Daple Regulates 3-Dimensional Cyst Formation

3-dimensional culturing has been exploited by researchers for decades in order to study cyst formation and characterize cell development, polarity, and survival in conditions more closely mimicking *in vivo* environments (Saelman, Keely et al. 1995; Bryant, Datta et al. 2010; Buchholz, Teschemacher et al. 2011; Yuajit, Homvisasevongsa et al. 2013). Our MDCK epithelial cells were submerged in an extra-cellular rat collagen matrix, and allowed to spontaneously form cysts, which naturally require each cell to have its own established junctions and proper orientation, with an apical membrane facing the lumen circumscribed by an outer-facing basolateral perimeter (Saelman, Keely et al. 1995; Buchholz, Teschemacher et al. 2011; Yuajit, Homvisasevongsa et al. 2013). When the MDCK-WT and FA cell lines were grown in a 3D rat collagen matrix at 2% FBS for seven days, the WT had proper cyst development with a distinct apicobasal axis, representative of proper cell growth and orientation. In contrast, the Daple-FA developed into much smaller colonies and had obstructed cyst formation without a proper lumen (**Fig. 5**). Taken together, these results demonstrate that Daple's GBA motif plays a role to upregulate epithelial cell development and cell polarity. This result adheres to previous

research, as literature states that the PI3k/Akt pathway inhibits apoptosis and drives cell growth in epithelial cells (Testa and Tsichlis 2005; Chae, Yang et al. 2009). Another plausible explanation for the diminished cysts in the GBA-mutated cells is due to the absence of β-catenin at the cell junctions. Without β-catenin securing the adherens junction as shown in Figure 4, the F1675A MDCK cells could lose their cell polarity and organization (Meng and Takeichi 2009), thereby disallowing proper 3D cyst formation as they mature.

When we use MDCK 3D cells to study cyst formation, we have to take into consideration the limitations of using such a model. MDCK cells are not composed of developmental pluripotent cells, but rather adult canine kidney epithelial cells, which are already specialized to an extent and may lack the potentiation and plasticity of embryonic or oncogenic cells (Zegers, O'Brien et al. 2003). Our experiment is also, ultimately, *in vitro*, even as we come closer to imitating *in vivo* conditions. The next step to gain further insight into our results and extend our scope would be to implement animal models, such as *Drosophila* and *Xenopus* organisms. There have been extensive history using these animal models (Hedgepeth, Conrad et al. 1997; Sakanaka, Sun et al. 2000), and it would be prudent to build upon the decades of research in order to study the Wnt pathway and how it could utilize Daple's GBA domain to affect cell junction formation, polarity, and oncogenesis (Kikuchi 2013; Munnamalai and Fekete 2013).

4. The Relationship Between Daple and Cancer

Our results for Daple's role in cancer formation closely align with previously published research and conclusions. Currently, it is established that the non-canonical Wnt pathway plays a dualistic role in cancer progression. Initially, it act as a potent tumor suppressor to repress cell transformation and carcinogenesis. However, after transformation has already occurred to turn the cell towards malignancy, then non-canonical Wnt signaling can incite cell migration, leading to metastasis and invasion (Kobayashi, Michiue et al. 2005; Ishida-Takagishi, Enomoto et al. 2012).

The results we generated follow this binary role, as diagrammed (**Supplementary 6**). We discovered that Daple's GBA domain regulates β-catenin localization, as the MDCK F1675A cells yielded a reduced concentration of β-catenin in the adherens junction, affecting cell junction integrity. We also know in 3D development, the GBA motif is essential for proper cell polarity and coordinated cyst formation. All these aspects of cancer formation induced by Daple's GBA domain are vital to oppose Epithelial-Mesenchymal Transition (Yang and Weinberg 2008). Conversely, we know Daple enhances the PI3k/Akt pathway, which can promote cell migration to initiate cell invasion. Since the Wnt pathway modulates a cascade of oncogenic signals and cancer progression using Daple's GBA motif, we can see its potential as a therapeutic target for drug interventions to regulate cancer development and its associated pathologies.

Materials and Methods

Cell Transformation

50ng of our vector was added to 50µl of competent DH5α cells in an eppendorf tube and incubated on ice for 30 minutes. A heat shock transformation was induced at 42^0C for 45 seconds, then placed back on ice for 2 minutes before adding 1mL of fresh LB media (without antibiotics) and incubated on a shaker for 60 minutes at 37^0C. 100µl of this cell culture was pipetted onto LB plates with the ampicillin antibiotic to select for the transformed cells that uptook the plasmid.

Purification of DNA Plasmid

One colony from the selective LB plate with the transformed DH5α cells was picked and grown in a 20mL LB+ampicillin broth for 4 hours. Then all 20mL was transferred into a larger 500mL LB+ampicillin broth and incubated overnight at 37^0C. The next day, we used and followed the protocol from the HiPure Plasmid Filter Midiprep Kit by Invitrogen to purify our desired DNA plasmids.

Measuring Concentration of DNA plasmids

We used the Nanodrop equipment and software from Thermo Scientific to measure DNA concentration.

Generation of MDCK Stable Cell Lines

Type I MDCK cells obtained from ATCC were cultured according to ATCC guidelines. For normal culturing media, 1x DMEM (containing 4.5 g/L glucose and L-glutamine) from Gibco was used, supplemented with 10% FBS (Fetal Bovine Serum), 1% P/S/G (Penicillin-Streptomycin-Glutamine), and for stable cell lines, selected with G418 Sulfate solution at 800µg/mL. The MDCK cells were incubated in an open system incubator at 37^0C with 5% CO_2.

Stable cells lines were established by transfection of plasmids pcDNA3-MYC Daple WT and FA into our MDCK cells. Transfection was performed following Novagen Company directions, using Novagen's Genejuice transfection reagent (3µl/µg DNA) and 100µl/µg DNA of Opti-MEM from Gibco. The reagents were mixed with our 2µg of desired DNA, and incubated at RT for 20 minutes before transfecting the MDCK cells. After successful transfection, stable cells lines were cultured in culturing media containing 800µg/mL G418 sulfate solution to select for only the cells that received and are expressing the plasmid. To maintain the cells when they reached confluency, ATV at 1:10 was added and used to split the cells into a new 6-well flask with 2mL cell culture. From the Type I MDCK cells obtained, type II MDCK cells were generated by culturing the cells to passage 30, and then utilized for the experiments.

Whole Cell Lysis Procedure

When our MDCK epithelial cells were confluent, the 6-well plate was put on ice while the media was drained. A quick rinse with cold PBS was done to wash away the excess leftover media. The cells were scraped cleanly and transferred into an eppendorf tube, which was then centrifuged for 13 seconds at 13.4k in 4^0C to obtain a cell pellet. The supernatant was removed by suction, and 30-50µl of Lysis Buffer was added, depending on cell pellet size. After vortexing, an equal about of 5x Sample Buffer was added and the entire tube was vortexed. Lastly, the sample was boiled at 100^0C for 10 minutes, when it was ready to be put into a gel for an SDS-PAGE.

SDS-PAGE Procedure

The sample was loaded into a 10% SDS-PAGE (Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis) gel with 1X running buffer at 90v-120v for 1.5 hrs or until the proteins were properly separated. A protein marker (Precision Plus Protein All Blue Standard, Bio-Rad Laboratories, Inc.) was utilized as reference. The proteins from the gel were then

transferred onto a PVDF (PolyVinylidene Fluoride) membrane with 1X transfer buffer at 35V overnight. After the transfer completion, the PVDF membrane was first rehydrated with dH_2O for 2 minutes, then temporarily stained with Ponceau staining solution from Sigma Aldrich for 10 minutes at RT. This process reversibly binds the proteins of interest with a red-pink marker and allows us to cut the membrane at our desired specific protein size. After cutting, the PVDF membrane was soaked in 1X PBS to remove the Ponceau staining. Next, the membrane was blocked with 1X PBS, supplemented with either 5% BSA (Bovine Serum Albumin) for phosphorylated proteins, or 5% dry non-fat milk for all other proteins. Membranes were incubated overnight in desired primary antibodies, then three 10-minute washes were done in 1X PBST (1X PBS supplemented with 1:1000 Tween-20). The membrane was placed in desired secondary fluorescent antibodies for 60 minutes at RT and washed again in PBST, with only the last wash in PBS. To scan, the Li-Cor Odyssey imaging system was used to detect and quantify the membranes in two-color infrared imagine. Image J software from the National Institute of Health was used to process and assemble Odyssey images.

2D Immunofluorescence

From a fresh cell culture, 200µl was pipetted onto a 6-well plate with 5 sterile circular coverslips and incubated at 37^0C until the cells were confluent. The cells on the coverslip were fixed with 2mL of fresh 3% paraformaldehyde (PFA), then incubated 10 minutes on ice, followed by a 15 minute incubation at room temperature. To store, the 3% PFA was removed, and 2mL of 0.2% PFA was added to maintain coverslip integrity for up to two weeks. The desired coverslips were taken out and placed into a 12-well plate, and washed three times for 15 minutes each with 1X PBS to remove the PFA in the cells. The cells were permeabilized and blocked with 500mL of fresh IF permeabilization buffer for 30 minutes. 6µl of primary antibodies (with appropriate dilution mentioned in Table 1) were added to the cell coverslips and incubated for 60 minutes.

After primary incubation, the coverslip was washed again (three 15 minute washes in 1X PBS), and then 6µl of secondary antibodies (with appropriate dilution mentioned in Table 1) were incubated for 60 minutes. Afterwards, the coverslip was washed again, three times for 15 minutes each in 1X PBS, and then placed on 6µl ProLong Gold antifade reagent (Invitrogen) and secured with clear nail polish around the coverslip edges. Images were acquired using a Leica CTR4000 Confocal Microscope using a 63x objective, and the images were processed using the Image J-software provided.

3D Collagen Matrix Procedure

The 3D collagen matrix procedure was adapted from the instructions outline by Elia and Lippincott-Schwartz (Elia and Lippincott-Schwartz 2009). Briefly, confluent MDCK Type II epithelial cells were washed, then suspended in a buffered rat collagen matrix at a specific concentration of $3x10^4$ cells/ml for optimal growth in a 24-well plate. The cells were first incubated in a 37^oC no CO2 oven for 30 minutes for proper collagen polymerization, then 1ml of 2% FBS was added to each well and maintained in a 37^oC with CO2 incubator, and media changed every 3 days.

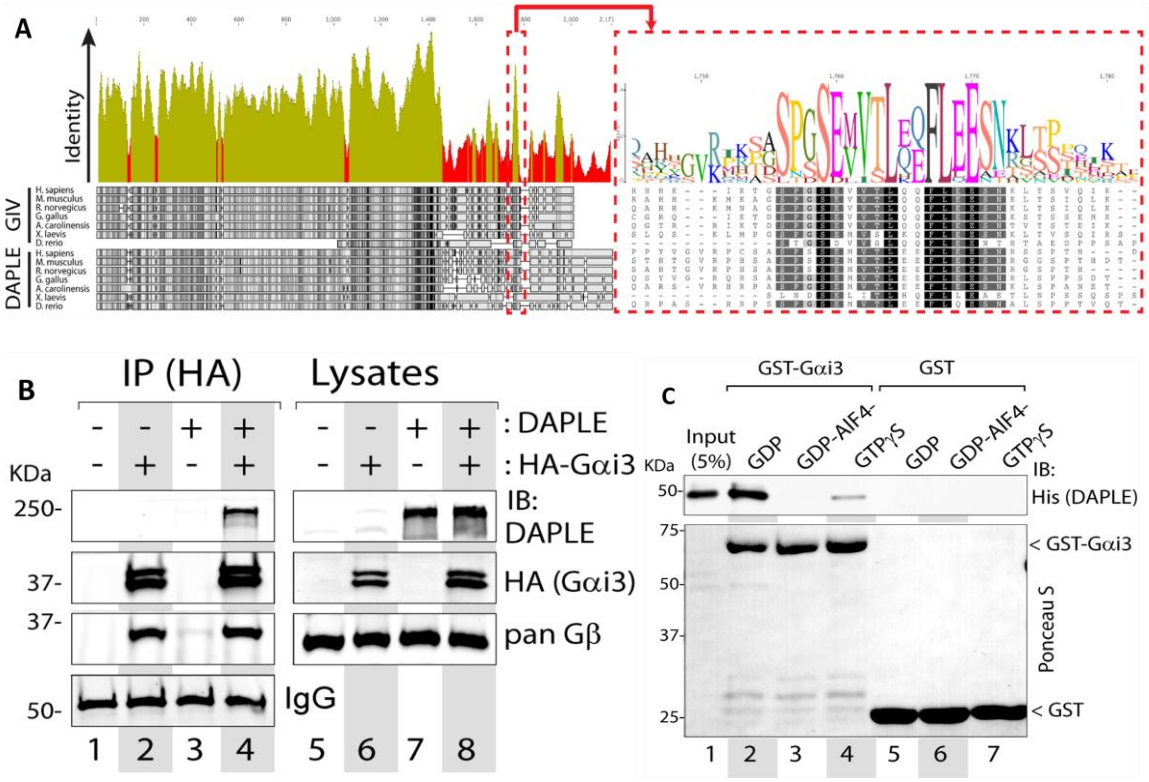
Table 1. Antibodies

Name	**Isotype**	**Dilution**	**Company**
β-catenin (Rabbit)	Rabbit polyclonal IgG	1/500	Inke Nathke Lab
β-catenin (Mouse)	Mouse polyclonal IgG	1/500	Invitrogen
Daple	Rabbit polyclonal bleed	1/500	Non-commercial Bleed
Phospho-Akt (Ser473)	Rabbit polyclonal IgG	1/250	Cell Signaling
Gi3	Polyclonal rabbit IgG	1/333	Santa Cruz
Gβ	Polyclonal rabbit IgG	1/250	Santa Cruz
Actin	Mouse monoclonal IgG	1:1000	Sigma Aldrich

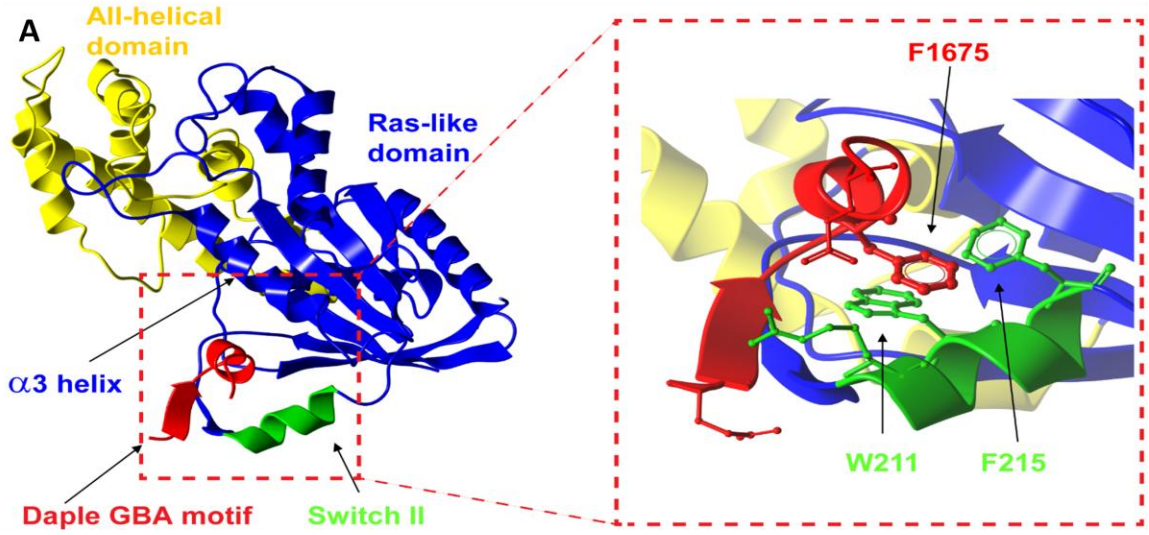
Table 2. Buffers

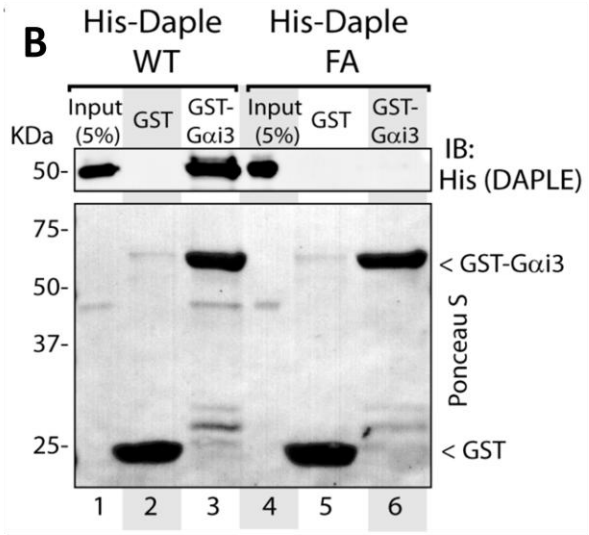
Name	Ingredients	Preparation
Lysis Buffer	20 mM Hepes pH 7.2, 125 mM K-acetate and 5 mM Mg-acetate	To 10 ml buffer, add 2 mM DTT, 1 tablet of Roche Protease inhibitor cocktail (Complete, EDTA-free), 0.4 % Triton X-100, 1X phosphatase inhibitor cocktail 2 and 3, 0.2 mM Sodium Orthovanadate
10X Running Buffer	0.25 M Tris, 1.92 M Glycine, 1% SDS, H_2O	Add 1 mL of 10% SDS to 1 L of 1X Running Buffer
10X Transfer Buffer	0.2M Tris, 1.29 M Glycine, H_2O	
5X Sample Buffer	5% SDS, 156 mM Tris, 25% glycerol, 0.025% Bromophenol Blue, 25% 2-Mercaptoethanol (BME), H_2O. pH 6.8	Add BME to aliquot prior to use
Immunofluorescence Permeabilization Buffer	2mg BSA/ml, 0.1% TritonX,	

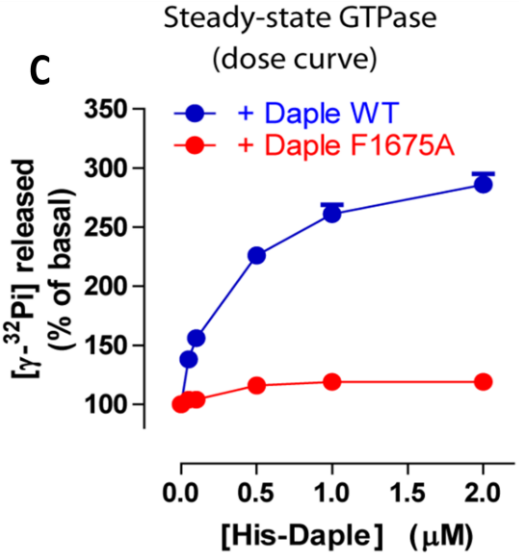
Supplementary

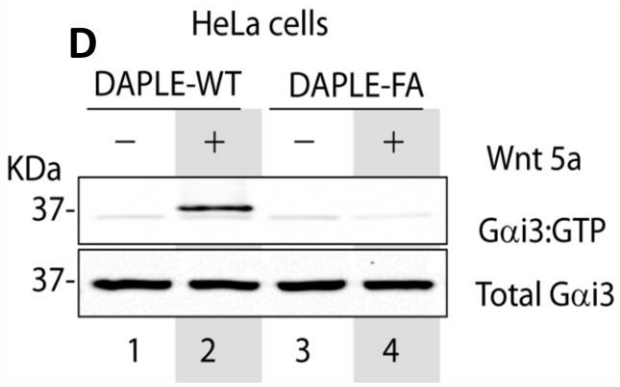


Supplementary 1. Daple Possesses a GBA motif and Binds to Gαi Subunits
(A) Sequence analysis comparison of GIV and Daple across various species. We see a high degree of conservation between the two proteins on Daple's residues 1668-1683. **(B)** Whole cell lysate immunoprecipitation performed on Cos7 cells expressing HA-Gαi3 revealed that Daple interacts with Gαi3. **(C)** Recombinant purified GST-Gαi3 incubated with purified His-Daple-CT (aa 1650-2028) shows Daple has a preferential binding to Gαi3 when preloaded with inactive GDP form, but not when preloaded with GDP-AlF_4^- or GTPγS, both of which mimic the active GTP-bound conformation.


A
All-helical domain
Ras-like domain
α3 helix
Daple GBA motif
Switch II
F1675
W211
F215

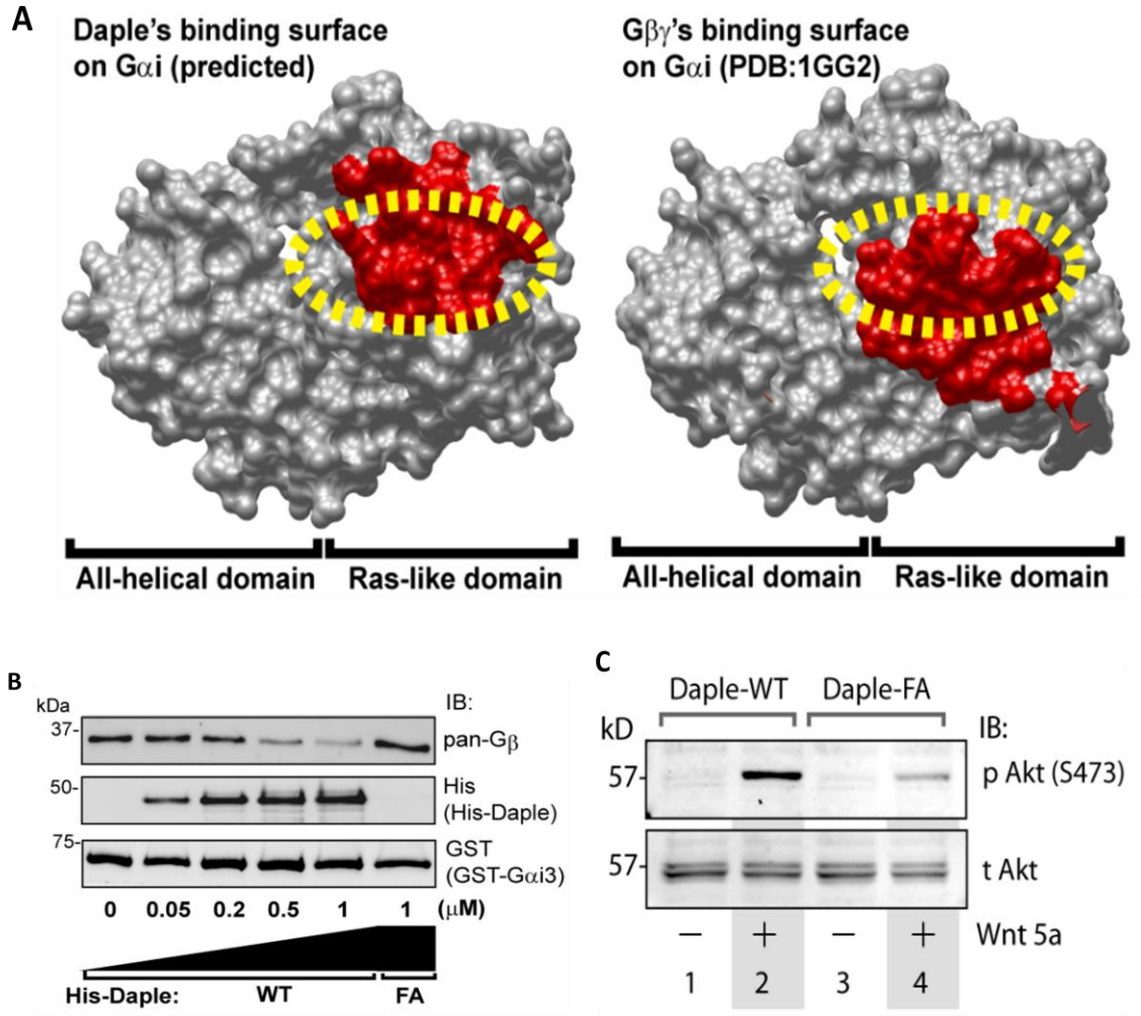

B
His-Daple WT
His-Daple FA
Input (5%)
GST
GST-Gαi3
KDa
50-
75-
50-
37-
25-
IB: His (DAPLE)
< GST-Gαi3
Ponceau S
< GST
1 2 3 4 5 6


C
Steady-state GTPase (dose curve)
+ Daple WT
+ Daple F1675A
[γ-32Pi] released (% of basal)
350
300
250
200
150
100
0.0 0.5 1.0 1.5 2.0
[His-Daple] (μM)

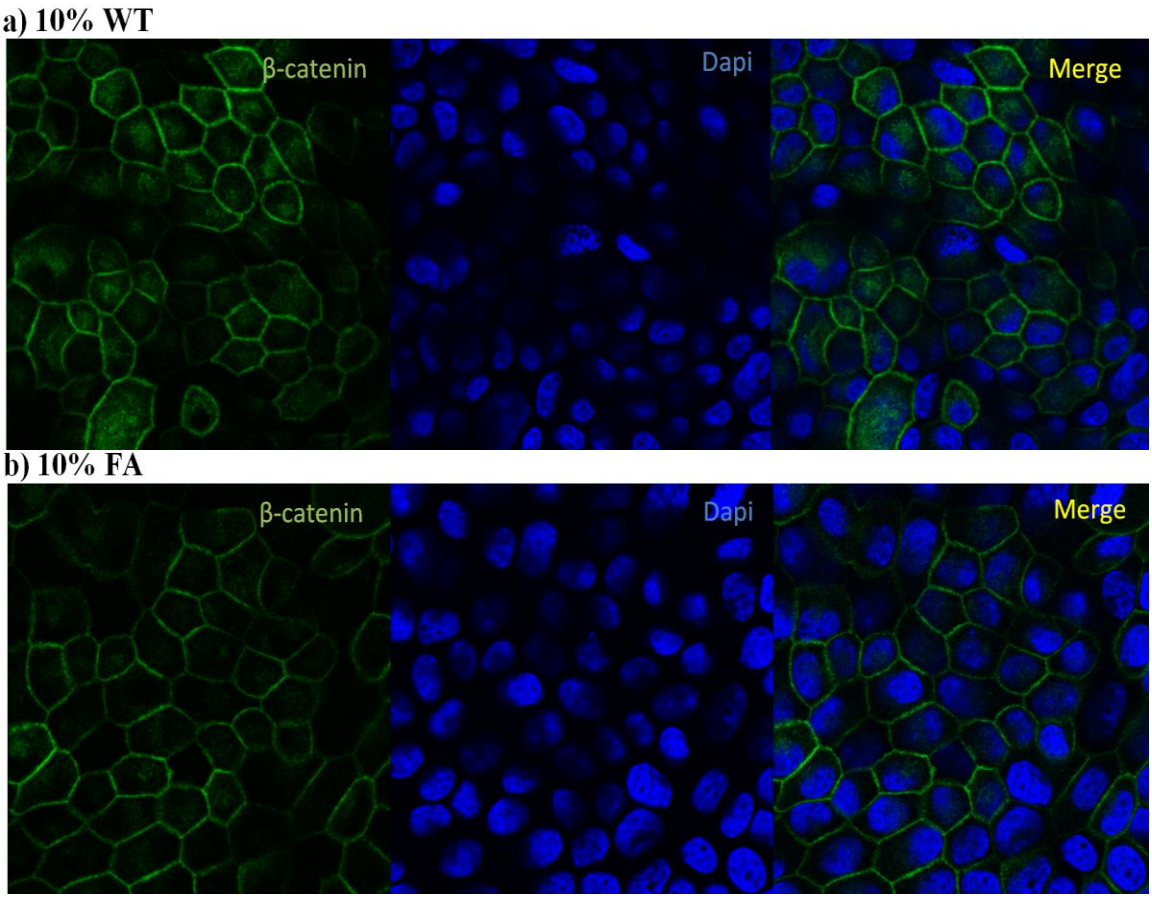

D
HeLa cells
DAPLE-WT
DAPLE-FA
–
+
–
+
KDa
37-
37-
Wnt 5a
Gαi3:GTP
Total Gαi3
1 2 3 4

Supplementary 2. **Identification of Critical Determinants for Effective Interaction between Gαi and Daple's GBA Motif**

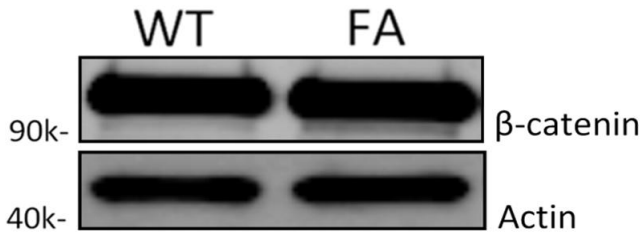
(A) A homology model of the interaction between Daple's GBA domain and Gαi3 reveals a hydrophobic cleft between the Switch II (SwII) region and the Ras-like region on the G-protein which can bind to Daple's conserved phenylalanine on residue 1675. **(B)** Purified, recombinant GST-Gαi3 proteins were incubated with purified His-Daple-CT (aa 1650-2028), WT and FA. Resin-bound proteins were eluted, separated by SDS-PAGE and analyzed by Ponceau S-staining. No binding to GST-Gαi3 was detected in the FA mutant. **(C)** Gαi3 proteins pre-loaded with [γ-^{32}P]GTP were incubated with either WT or FA His-Daple-CT at various concentrations, and measured for [γ-^{32}Pi] release by scintillation counting. Daple was able to accelerate the rate of G-protein exchange release by ~3 fold. **(D)** HeLa cells transfected with Myc-Daple WT or FA were stimulated with and without Wnt 5a for 5 minutes before lysis and pulldown with the Gαi3:GTP antibody. A binding response between Daple WT and Gαi3 was identified under Wnt 5a stimulation.



Supplementary 3. Daple enhances PI3K-Akt signaling via release of free Gβγ subunits
(A) Crystal structure comparative analysis of Gαi's binding surface to Daple and Gβγ shows overlapping sites, suggesting that Daple binding to Gαi would displace Gβγ from the heterotrimic G-protein. **(B)** Recombinant purified GST-Gαi3:Gβ proteins were added to increasing concentrations of His-Daple WT or His-Daple F1675A. There was rising displacement of Gβ as higher concentrations of Daple-WT were added. Note also that the FA mutation did not result in any Gβ displacement. **(C)** HeLa cells transfected with Myc-Daple WT or FA constructs under Wnt 5a stimulation show an increase in Akt activation (demonstrated by an increase in Akt's S473 phosphorylation) in a GEF dependent manner.

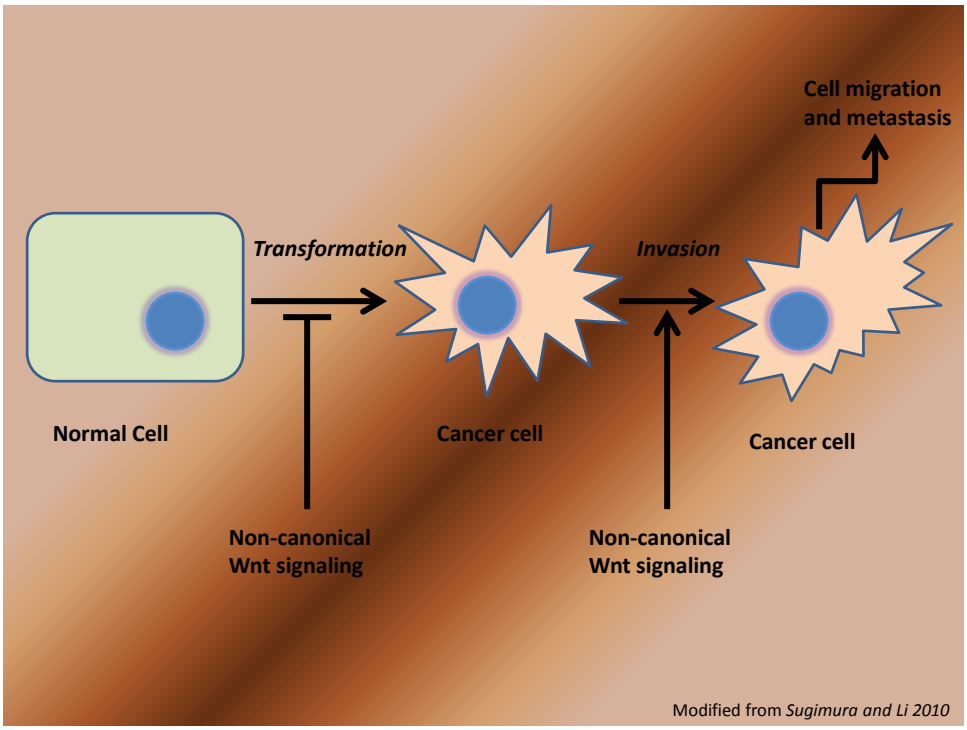


Supplementary 4. Immunofluorescence of MDCK cells under 10% Serum
MDCK stable cell lines with MYC-Daple WT and FA plasmid constructs were produced and grown in 10% FBS before fixation, and probed for β-catenin along with the nucleus. No significant difference in phenotype outcomes were observed for either of the MDCK stable cell lines at 10% FBS serum.



Supplementary 5. Consistent β-catenin Concentrations for MDCK cell lines under 2% FBS serum.

A whole cell lysate of MDCK stable cell lines of MYC-Daple WT and FA was performed, after a 24 hour serum deprivation in 2% FBS. An SDS-PAGE was performed on the lysate and probed for β-catenin to check relative concentrations among the cell lines. Actin was used as a loading control. We see even β-catenin expression across both the WT and FA cell lines.



Supplementary 6. The Dual Functionality of Non-canonical Wnt Signaling in Cancer Progression.
Current research proposes a dual role in non-canonical Wnt signaling. Initially, the pathway works to suppress cell transformation and maintain proper growth signals. However, after cell transformation has occurred, it could act as a driving force for cell invasion and metastasis.

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