Regulation of DVL2 ubiquitylation by USP9X biases participation in canonical or non-canonical WNT signaling

Casey P. Nielsen[1], Kristin K. Jernigan[1] and Jason A. MacGurn [1 *]

[1] Department of Cell and Developmental Biology, Vanderbilt University, Nashville, TN, United States

*Address correspondence to Jason A. MacGurn; email: jason.a.macgurn@vanderbilt.edu

Running title: Regulation of WNT signalling by USP9X

Current Character Count: 40,656 (with spaces).

Summary

NEDD4 family E3 ubiquitin ligases regulate cell signaling and trafficking events in eukaryotic cells and their dysregulation is often observed in various human diseases. One NEDD4 family member, WWP1, has been reported to undergo genomic amplification in breast and prostate cancer yet its function in cancer progression is unclear. Proteomic analysis in breast cancer cells revealed physical association of WWP1 with the deubiquitylase USP9X and the WNT signaling protein DVL2. We report that USP9X is required for canonical WNT activation in a WWP1- and NEDD4L-dependent manner. Our findings indicate that USP9X regulates WNT activation via deubiquitylation of DVL2 and the ubiquitylation-deubiquitylation cycle of DVL2 biases its participation in either canonical WNT or WNT-PCP pathways. The finding of a WWP1/NEDD4L-USP9X regulatory axis that specifies DVL2 participation in canonical and non-canonical WNT signaling pathways has important implications for therapeutic targeting of USP9X in human cancer.

Introduction

The canonical WNT β-catenin signaling pathway is involved in regulating many cellular processes such as cell fate determination during embryonic development, cell proliferation, and adult tissue homeostasis. Thus, it is not surprising that aberrant activation of the canonical Wnt pathway is known to occur in many types of cancer (Saito-Diaz et al., 2013). There are also several noncanonical WNT signaling pathways including the WNT-Planar Cell Polarity (PCP) pathway which controls cell migration and tissue polarity. Dysregulation of the WNT-PCP pathway has been linked to cancer invasion and metastasis (Luga et al., 2012; Wang, 2009). While the canonical WNT β-catenin pathway and the noncanonical WNT-PCP pathway use divergent effector mechanisms to regulate distinct cellular functions, these pathways share membrane receptor components and the cytoplasmic WNT transducer protein disheveled (DVL). Despite its key role in both pathways, the mechanisms regulating DVL participation in canonical or noncanonical WNT signaling are yet to be elucidated.

Initiation of the canonical WNT β-catenin pathway occurs when extracellular WNT ligand binds to the co-receptors Frizzled (FZD) and low-density lipoprotein receptor-related protein 5/6 (LRP5/6) leading to recruitment of DVL and AXIN to the WNT ligand receptor complex. This ultimately results in the inhibition of β-catenin ubiquitylation and degradation such that stabilized β-catenin can enter the nucleus to initiate a transcriptional program (Saito-Diaz et al., 2013). Ubiquitylation is known to be involved in key regulatory steps of the WNT pathway. Ubiquitin-mediated regulation of cytoplasmic β-catenin stability is well characterized (Marikawa and Elinson, 1998). In addition, other steps of the WNT pathway upstream of β-catenin stabilization undergo regulation by the ubiquitin system. Notably, several members of the NEDD4 family of E3 ubiquitin ligases (SMURF1, ITCH, and NEDD4L) have been found to negatively regulate stability of WNT pathway components. SMURF1 interacts with and ubiquitylates AXIN, inhibiting its interaction with the WNT co-receptor LRP5/6 (Fei et al., 2014; Fei et al., 2013). Both ITCH

and NEDD4L promote degradation of DVL2 (Cadavid et al., 2000; Ding et al., 2013; Fei et al., 2014; Fei et al., 2013; Wei et al., 2012). Furthermore, DVL2 is also known to undergo positive regulation by the ubiquitin system. For example, K63-linked ubiquitylation of the N-terminal DAX domain, which is known to mediate dynamic polymerization of DVL2, has been implicated as a positive regulator of DVL2 signal transduction (Schwarz-Romond et al., 2007; Tauriello et al., 2010). These examples highlight the complex regulation of WNT by ubiquitylation machinery.

The NEDD4 family of E3 ubiquitin ligases is conserved from yeast to humans and has been implicated in the ubiquitin-mediated endocytosis of many plasma membrane proteins including surface receptors, ion channels, and nutrient transporters (David et al., 2013; He et al., 2008; Hryciw et al., 2004; Kuratomi et al., 2005; Lee et al., 2011; Lin et al., 2011; MacGurn et al., 2012). There are 9 NEDD4 family E3 ubiquitin ligases encoded in the human genome, each with distinct physiological functions (Rotin and Kumar, 2009). While not all NEDD4 family E3 ubiquitin ligases have been characterized extensively, several have a strong preference for catalyzing K63-linked polyubiquitin chains *in vitro (Ingham et al., 2004; Kim and Huibregtse, 2009)*. NEDD4 family ligases have a conserved domain structure including an N-terminal C2 domain that binds lipids, a C-terminal HECT ubiquitin ligase domain, and 2-4 internal WW domains. The WW domains have a high affinity for PY motifs (Pro-Pro-x-Tyr) and mediate interactions with substrates, adaptors, and regulatory factors (Ingham et al., 2004). Given the broad role of NEDD4 family members in the regulation of membrane trafficking and cell signaling, it is not surprising that they are often found to be dysregulated in human diseases including cancer (Bernassola et al., 2008). Here, we report that the NEDD4 family member WWP1 interacts with USP9X and that this interaction regulates canonical WNT activation by controlling the ubiquitylation status of DVL2. This ubiquitin switch on DVL2 biases its participation in either the canonical WNT or WNT-PCP pathways. Thus, we find that this

USP9X-DVL2-WWP1 axis is a key regulatory node in the WNT pathway with the potential to influence both canonical and noncanonical WNT signaling in the context of human cancer.

Results

WWP1 interacts with USP9X and DVL2

In an attempt to identify factors that regulate WWP1 function we used SILAC-based quantitative proteomics to generate a WWP1 interaction profile in MDA-MB-231 cells (a triple negative breast cancer cell line). This experiment identified interactions with various plasma membrane (PM) and PM-associated proteins, factors associated with membrane trafficking, signaling factors, and elements of the ubiquitin-proteasome system (**Table S1** and **FIG 1A**). To validate results obtained by quantitative proteomics, we tested for co-purification of a subset of putative WWP1 interacting proteins from MDA-MB-231 cells stably expressing FLAG-WWP1 (**FIG 1B**). This approach confirmed FLAG-WWP1 interactions with several putative interactors - including USP9X, DVL2 and SPG20 – although a few putative interactors such as ARRDC1 and USP24 could not be confirmed by this approach. Considering the possibility that some putative interacting proteins may artificially result from stable overexpression of FLAG-WWP1 we performed native co-immunoprecipitation of WWP1 and identified interactions with USP9X and DVL2 at endogenous expression levels (**FIG 1C**). Importantly, other studies have reported interactions between USP9X and NEDD4 family E3 ubiquitin ligases – including SMURF1 (Xie et al., 2013) and ITCH (Mouchantaf et al., 2006). Similarly, DVL2 was previously reported to interact with other NEDD4 family E3 ubiquitin ligases – including WWP2 (Mund et al., 2015) and NEDD4L (Ding et al., 2013; Wei et al., 2012). Notably, both USP9X and DVL2 contain multiple PY motifs capable of binding the WW domains of NEDD4 family E3 ubiquitin ligases. Based on our findings and previous reports we decided to investigate the regulatory significance of the interactions between WWP1, USP9X, and DVL2.

1 ***USP9X promotes canonical WNT activation***

2 Given the established function of DVL2 as a key signal transducing protein in the WNT
3 pathway and given that NEDD4 family members have been reported to negatively regulate
4 WNT signaling (Tanksley et al., 2013) potentially via ubiquitylation of DVL2 (Ding et al., 2013),
5 we decided to test if USP9X regulates WNT signaling. We found that knockdown or knockout of
6 USP9X attenuated WNT activation as measured by a TopFLASH reporter assay (**FIG 2A** and
7 **FIG 2B**) and by β–catenin stabilization (**FIG 2B**). The WNT signaling defect observed in *usp9x*
8 knockout cells was complemented by transient expression of wildtype FLAG-USP9X but not a
9 catalytic dead (C1556V) variant (**FIG 2C**), indicating that catalytic activity of USP9X is required
10 for canonical WNT activation. As an alternative to depletion of USP9X we treated cells with
11 WP1130 – a small molecule known to inhibit the deubiquitylase activity of USP9X (Kapuria et
12 al., 2010) – and found that this attenuated WNT activation with an IC_{50} of 2.9μM (**FIG S1A-B**),
13 consistent with a role for USP9X catalytic activity in canonical WNT activation. Importantly, we
14 found that the loss of function in *usp9x* knockout (or knockdown) cells was suppressed by
15 coordinate knockdown of WWP1 or NEDD4L (but not WWP2) (**FIG 2D**) suggesting that hyper-
16 activation of multiple NEDD4 family members, including NEDD4L and WWP1, may attenuate
17 WNT signaling in the absence of USP9X. To further explore the possibility that hyper-activation
18 of NEDD4 family members can inhibit WNT activation, we treated cells with GSK650394 – a
19 small molecule known to inhibit the SGK1 kinase and thus indirectly hyper-activate NEDD4L
20 (Sherk et al., 2008) – and found that this attenuated WNT activation with an IC_{50} of 3.6μM (**FIG**
21 **S1A** and **S1C**). Taken together, our findings indicate that loss of USP9X leads to hyper-
22 activation of a subset of NEDD4 family E3 ubiquitin ligases – specifically WWP1 and NEDD4L –
23 resulting in attenuation of WNT activation.

24 To further explore the possibility that USP9X activity is sufficient to promote WNT activation
25 we transiently transfected expression vectors for FLAG-USP9X (wildtype or catalytic dead) and

measured WNT activation in the context of WNT3a stimulation. Importantly, we found that transient expression of wildtype FLAG-USP9X, but not a catalytic-dead variant, resulted in increased activation of the canonical WNT pathway (**FIG 2E**). Thus, or results indicate that increased USP9X activity can sensitize cells to WNT hyper-activation.

USP9X regulation of WNT requires DVL2 deubiquitylation

To address the mechanism of WNT regulation by USP9X, we first tested if USP9X is required for β-catenin stabilization by LiCl – an inhibitor of GSK3. Importantly, LiCl-mediated stabilization did not require USP9X (**FIG 3A**), indicating USP9X functions upstream of the β-catenin destruction complex. Since DVL2 was reported to be a substrate of NEDD4L (Ding et al., 2013) we tested if USP9X regulates DVL2 ubiquitylation and found that knockdown of USP9X, but not USP24 (a paralog that is 30% identical and 45% similar to USP9X), resulted in DVL2 mobility shifts by SDS-PAGE consistent with hyper-ubiquitylation (**FIG 3B**). In *usp9x* knockout cells (MDA-MB-231) we observed lower steady-state DVL2 protein levels and increased DVL2 laddering by SDS-PAGE (**FIG 3C**). To identify post-translational modifications on DVL2 regulated by USP9X we performed a SILAC-MS on FLAG-DVL2 affinity purified from MDA-MB-231 cells and usp9x knockout equivalents. This analysis resolved many phospho-peptides and ubiquitin remnant (diGly) peptides derived from DVL2 with four internal lysines exhibiting elevated ubiquitylation in the absence of USP9X (**FIG 3D**). Although ubiquitylation at N-terminal lysines on DVL2 has been reported (Madrzak et al., 2015; Tauriello et al., 2010) ubiquitin modification of these residues (K343, K428, K477 and K615) has not previously been reported.

To test if USP9X-mediated regulation of WNT signaling requires DVL2 ubiquitylation we expressed DVL2 fused to the UL36 DUB domain (from human herpes virus) and found that this (but not a catalytic dead DUB fusion) suppresses the WNT activation defect in the absence of

USP9X (**FIG 4A**). These results indicate that USP9X regulates WNT signaling at (or in very close proximity to) the level of DVL2. To explore the possibility that USP9X-mediated deubiquitylation of DVL2 at specific sites is required for WNT activation we tested a panel of DVL2 lysine mutants (K343R, K429R, K477R and K615R) but found expression of these mutants was insufficient to suppress the WNT signaling defect observed in the absence of USP9X (**FIG S2**). We next considered the possibility that USP9X may regulate multiple ubiquitylation events on DVL2. Importantly, we found that expression of a "lysine-less" DVL2 variant with all encoded lysine residues switched to arginine (DVL2-K0) is sufficient to restore WNT activation in the absence of USP9X (**FIG 4B**). Taken together, these results indicate that increased ubiquitylation of DVL2 is required for the attenuation of WNT signaling observed in the absence of USP9X. Thus, the USP9X-mediated deubiquitylation of DVL2 is critical for activation of canonical WNT signaling.

DVL2 Deubiquitylation by USP9X Promotes Interaction with the AP-2 adaptor complex

We hypothesized that WWP1-USP9X interaction controls a ubiquitin switch (or rheostat) on DVL2 that regulates its function as a signal transducing protein. To test this hypothesis, we first characterized the DVL2 interaction network in MDA-MB-231 cells using SILAC-MS (**TABLE S2, TABLE S3,** and **FIG 5A**). These experiments revealed two near-stoichiometric interactions with the AP-2 clathrin adaptor complex and with VANGL1 and cofilin which are important factors known to participate in the non-canonical WNT PCP pathway (Luga et al., 2012). Additionally, we identified several sub-stoichiometric interactions with NEDD4 family E3 Ub ligases and proteasome subunits (**TABLE S2, TABLE S3,** and **FIG 5A**). Many of these interactions had been previously reported (Angers et al., 2006; Yu et al., 2007). Several interactions were confirmed by co-IP (**FIG 5B** and data not shown) and co-localization by immunofluorescence microscopy (**FIG 5C** and data not shown). Consistent with previous reports (Yu et al., 2007; Yu

et al., 2010) we also found that knockdown of AP-2 complex subunits inhibited canonical WNT activation in a TOPFLASH assay (data not shown).

To quantify how DVL2 interactions are affected by ubiquitylation, we performed SILAC-MS proteomic analysis comparing the interactome of catalytic active (light) or catalytic dead (heavy) DUB-DVL2 fusions (also used in **FIG 4A**). This revealed that the catalytic dead DUB-DVL2 fusion (which can be stably ubiquitylated) interacts more with proteasome subunits and VANGL1/cofilin while the catalytic active DUB-DVL2 fusion (which is not stably ubiquitylated) interacts more with the AP-2 complex (**FIG 5D**). We also used SILAC-MS to quantify how DVL2 interactions are altered in *usp9x* knockout cells and found similar results: DVL2 interacted more with VANGL1 and cofilin in *usp9x* knockout cells (where DVL2 ubiquitylation is elevated) but interacted more with AP-2 in the presence of USP9X (where DVL2 is less ubiquitylated) (**FIG 5E**). These results indicate ubiquitylated DVL2 interacts more with VANGL1/cofilin – components of the WNT-PCP pathway – while deubiquitylated DVL2 interacts more with the AP-2 complex, which is critical for canonical WNT activation.

USP9X regulates cellular distribution of DVL2

Previous studies have reported that VANGL1 and cofilin localize to F-actin rich projections in migrating MDA-MB-231 cells (Hatakeyama et al., 2014). Based on our findings that loss of USP9X results in increased ubiquitylation of DVL2 and increased interactions between DVL2 and VANGL1/cofilin we hypothesized that loss of USP9X may result in re-localization of DVL2 to projections in MDA-MB-231 cells. To test this we used immunofluorescence microscopy to characterize FLAG-Dvl2 distribution in MDA-MB-231 cells in the presence or absence of USP9X. In MDA-MB-231 cells treated with control siRNAs, DVL2 exhibited a punctate distribution throughout the cell body, reflective of previous descriptions of DVL2 localization (**FIG 6A, left panel**) (Schwarz-Romond et al., 2005) whereas MDA-MB-231

cells treated with siRNAs targeting USP9X knockdown displayed a redistribution of DVL2 to structures that appear to be projections (**FIG 6A, right panel**). Quantification of the cellular distribution of DVL2 in the presence and absence of USP9X reveals a significant increase in DVL2 in cellular projections upon loss of USP9X (**FIG 6B**). Taken together, our findings indicate that interactions between USP9X and WWP1/NEDD4L regulate a ubiquitin switch on DVL2 that biases its interaction with either the clathrin adaptor AP-2 complex, which is critical for the canonical WNT signaling pathway, or the VANGL1/cofilin complex, which is critical for the non-canonical WNT-PCP pathway (**FIG 6C**).

Discussion

Dishevelled has long been known to play a key role in the transduction of WNT receptor signaling, yet how it is regulated to specify participation in either canonical or non-canonical signaling relays is not well understood. Here, we report that NEDD4 family E3 ubiquitin ligases and their interaction with the deubiquitylase USP9X regulate DVL2 ubiquitylation, and that the deubiquitylation of DVL2 by USP9X is critical for canonical WNT activation. Importantly, we find that USP9X and WWP1 mediate a ubiquitin switch on DVL2 that determines whether DVL2 will preferentially interact with AP-2, a canonical signaling factor, or VANGL1 and cofilin which are critical for the WNT-PCP pathway. Thus, the USP9X-WWP1 axis biases DVL2 participation in either the canonical WNT pathway or the noncanonical WNT-PCP pathway.

A Novel Regulatory Axis in the WNT Pathway

DVL2 has been reported to undergo both positive and negative regulation by the ubiquitin system through the action of multiple different proteins. For example, both ITCH and NEDD4L have been shown to negatively regulate DVL2 stability by conjugating K48-linked polyubiquitin chains on DVL2, targeting it to the proteasome for degradation (Ding et al., 2013; Liu et al., 2014; Marikawa and Elinson, 1998; Mukai et al., 2010; Tauriello et al., 2010; Wei et al., 2012). On the other hand, K63-linked polyubiquitin modifications of the DVL2 DIX domain were reported to enhance canonical WNT activation (Tauriello et al., 2010). The apparent regulation of DVL2 by multiple members of the NEDD4 family could result from functional redundancy, but could also reflect differential regulation based on expression in different contexts or different cell types. Our finding that siRNA targeting of NEDD4L or WWP1 partially rescued WNT activation in the absence of USP9X is consistent with a redundancy model, but it does not exclude the possibility that USP9X antagonizes multiple exclusive ubiquitylation events on DVL2 mediated by different E3 ubiquitin ligases. Indeed, our data suggests that functional regulation of DVL2 by USP9X may involve reversal of multiple distinct ubiquitylation events –

possibly mediated by multiple E3 ubiquitin ligases - since no single K→R mutation was capable of suppressing the loss of WNT activation phenotype observed in the absence of USP9X (**FIG S2**). Additional biochemical and genetic studies will be required to fully understand how USP9X affects various distinct ubiquitylation events on DVL2 that may be mediated by different E3 ubiquitin ligases.

Previous studies have reported conflicting roles for USP9X with respect to WNT activation. Specifically, USP9X has previously been reported to positively regulate the stability of β-catenin in glioma cells (Yang et al., 2016). However, USP9X was also reported to negatively regulate the stability of β-catenin in mouse neural progenitor cells (Premarathne et al., 2017). Our studies are consistent with a positive role for USP9X in canonical WNT pathway activation and a negative role for USP9X in non-canonical WNT-PCP pathway activation. To determine where USP9X functions relative to the β-catenin destruction complex we performed an experiment to test the USP9X requirement for LiCl-mediated activation of the canonical WNT pathway suggesting that USP9X functions upstream of the destruction complex. Although our findings are consistent with a role for USP9X in the regulation of WNT at the level of DVL2 we cannot exclude the possibility of a context-dependent role for USP9X at other tiers of the WNT pathway. Furthermore, it is possible that expression of different NEDD4 family members, other USP9X-interactors, or substrate adaptors and targeting factors could modify USP9X function in a context-specific manner.

USP9X and Cancer Biology

Given that USP9X has been implicated in the regulation of multiple receptor signaling and trafficking pathways, it is not surprising that in recent years USP9X dysregulation has been linked to many different types of cancers. USP9X has been suggested to exhibit oncogenic behavior in some cancer contexts including multiple myeloma, melanoma, lung cancer, and

breast cancer (Kushwaha et al., 2015; Li et al., 2017; Potu et al., 2017; Schwickart et al., 2010). In contrast, USP9X has also been proposed to have tumor suppressor activity in other cancers including pancreatic ductal adenocarcinoma (Pérez-Mancera et al., 2012) and colorectal adenocarcinoma (Khan et al., 2018). Recently, USP9X has been shown to be required for the attenuation of EGFR signaling (Savio et al., 2016) which is suggestive of a tumor suppressor function. Taken together, these reports suggest a complex role for USP9X in cancer progression that may be highly context-dependent. Our findings suggest that USP9X functions along with WWP1 and NEDD4L to regulate a ubiquitylation-deubiquitylation cycle of DVL2. The ubiquitylation status of DVL2 determines its engagement with either canonical or non-canonical WNT factors. Based on these findings we hypothesize that in some cellular contexts increased levels of USP9X may sensitize cells to activation of the canonical WNT pathway (**FIG 2E**), which promotes proliferation and tumor growth, while simultaneously antagonizing the non-canonical WNT-PCP pathway which is involved in cell motility and metastasis. These results indicate that dysregulation of USP9X can simultaneously function to promote canonical WNT-driven proliferation while simultaneously guarding against migration and metastasis mediated by the WNT-PCP pathway. Based on these findings, we propose that small molecule inhibitors of USP9X may be effective as antagonists of WNT-based proliferation but they may also ultimately promote migration and metastasis.

Regulation of cell signaling pathways by DUB-E3 interactions

The ubiquitin proteasome system contributes to the tight spatial and temporal regulation of protein trafficking, localization, and stability. Recently, several examples of interactions between E3 ubiquitin ligases and DUBs have been reported, raising questions about the regulatory significance and design logic of such interactions. In principle, DUB-E3 interactions could facilitate a number of useful design features, including E3 antagonization, E3 deubiquitylation, or polyubiquitin chain editing on the substrate. E3 antagonization has the

potential to result in a futile cycle, but coordinated regulation of ligase and DUB activities could also establish a ubiquitin rheostat on a shared substrate. For example, the DUB USP7 interacts with the MAGE-L2-TRIM27 E3 ubiquitin ligase to regulate the ubiquitylation status of WASH, a shared substrate, on the endosome (Wu et al., 2014). In contrast, E3 deubiqitylation by interacting DUBs could protect the E3 ubiquitin ligase from degradation, stabilizing it and thus promoting its activity. For example, the DUB USP19 promotes cellular proliferation by stabilizing KPC1 (also known as RNF123), a ubiquitin ligase for the cyclin-dependent kinase inhibitor p27Kip1 (also known as CDKN1B) (Lu et al., 2009). A striking example of polyubiquitin chain editing occurs with the protein A20, a potent inhibitor of the NFkB signaling pathway that contains both a DUB domain and an E3 ubiquitin ligase domain (Wertz et al., 2004). The DUB domain of A20 removes a K63-linked polyubiquitin chain from the Receptor Interacting Protein (RIP), a protein essential for NFkB signaling, and subsequently, the E3 ubiquitin ligase domain of A20 conjugates a K48-linked polyubiquitin chain to RIP targeting it for degradation by the proteasome - thus inhibiting the NFkB pathway. We have identified a novel E3-DUB interaction that functions to provide layered regulation of the WNT pathway. Our results indicate that the interaction between WWP1 and USP9X plays an important role in the regulation of WNT signaling by incorporating the above-described design features. Functionally, USP9X does seem to antagonize WWP1 and NEDD4L on DVL2 (a shared substrate), and our data also suggests that WWP1 is destabilized in the absence of USP9X (**FIG 2D**). Furthermore, our data cannot exclude the possibility that WWP1-USP9x interactions function in a chain-editing capacity on polyubiquitin chains of DVL2. Certainly, it is possible that DUB-E3 interactions facilitate combinations of the above-described design features. Ultimately, rigorous biochemical analysis dissecting mechanisms that regulate and balance of different ubiquitylation and deubiquitylation activities toward DVL2 will be critical to understand its specification in either canonical and noncanonical WNT signaling pathways.

Experimental Procedures

Assays for measuring WNT activation

Topflash reporter assays in HEK293 STF cells were performed as follows: HEK293 STF cells were seeded on 24-well plates at ~50% confluency. Cells were treated with 100ng/mL purified recombinant WNT3a (R&D Systems) and 100ng/mL purified recombinant R-spondin (R&D systems) 24 hours prior to lysis with 1X Passive Lysis buffer (Promega). Luciferase activity was measured using the Steady-Glo luciferase Assay (Promega). Luciferase signal was normalized to viable cell number using Cell-Titer Glo Assay (Promega). For siRNA knockdown studies, cells were treated for 72 hours prior to lysis, for inhibitor studies cells were treated for 24 hours prior to lysis, and for overexpression studies cells were transfected with plasmid DNA for 48 hours prior to lysis.

Topflash reporter assays in MDA-MB-231 cells were performed as follows: Cells were plated, co-transfected with TOPflash and Renilla expression plasmids at 24 hours and lysed at 48 hours using the Dual-Glo Luciferase Assay system (Promega). Luciferase signal was normalized to co-transfected Renilla expression.Data were normalized to a positive control (set at 100% activation). Assays were performed in triplicate and repeated at least 3 times.

B-catenin stabilization assay: HEK293 STF cells were cultured in Dulbecco's modified Eagle's Medium (DMEM) and activated with WNT3A ligand or LiCl for 24 hours. Then cells were lysed and nuclear/cytoplasmic fractionation was performed as previously described (Thorne et al., 2010).

SILAC-based Quantitative Proteomic Analysis

Quantitative mass spectrometry analysis by SILAC was performed on MDA-MB-231 cells stably expressing the indicated FLAG-tagged substrates. These cells were cultured in the

presence of either heavy or light isotopes (lysine and arginine) for at least 6 doublings to ensure incorporation of these isotopes into 100% of the proteome. Affinity purification was performed as previously described (Lee et al., 2017). Eluted proteins were digested with 1µg Trypsin Gold (Promega) at 37 F overnight. Digested peptides were cleaned-up on a Sep-Pak C18 column. Purified peptides were then dried, reconstituted in 0.1% trifluoroacetic acid, and analysed by LC-MS/MS using an Orbitrap XL mass spectrometer. Database search and SILAC quantitation were performed using MaxQuant software.

Co-Immunoprecipitation Studies

For coimmunoprecipitation assays, cells were washed with cold PBS and lysed with a nondenaturing lysis buffer (NDLB): 50mM tris (pH 7.4), 300mM NaCl, 5mM EDTA, 1% Triton X-100, cOmplete protease inhibitor cocktail (Roche), Phosphatase inhibitor cocktail (Roche), 1mM PMSF, 1mM Phenanthroline, 10mM Iodoacetamide, and 20µM MG132. Lysates were diluted to 1mg/mL with NDLB and incubated with Sigma FLAG EZ-view beads for 2 hours with rotation at 4°C. Beads were washed 3 times with cold NDLB and once with cold PBS. Proteins were then eluted from beads using sample buffer and samples were processed for SDS-PAGE followed by immunoblotting.

Fluorescence Microscopy

MDA-MB-231 cells were seeded on coverslips at 50% confluency. At 24 hours after seeding, cells were transfected with FLAG-Dvl2 using LipoJet Transfection Reagent (Signagen). 72 hours after seeding cells were fixed and permeabilized using 4% paraformaldehyde (PFA) and immunofluorescence block buffer (PBS + 0.1% Triton-x + 10% FBS). Cells were then labeled with primary antibodies for 1 hour followed by a 1 hour incubation with alexa-fluor conjugated secondary antibodies. Coverslips were mounted on slides using ProLong AntiFade

mountant (Thermo). Microscopy images were acquired using a DeltaVision Elite system (GE Healthcare) and processed using SoftWoRx software (GE Healthcare, Chicago, IL).

Acknowledgements

We are very grateful to K. Rose for helpful advice regarding technical aspects and analysis of quantitative proteomic data. This research was supported by NIH grant R00 GM101077 (to J.A.M.) and GI SPORE grant in GI Cancer (P50 CA095103).

References

Angers, S., Thorpe, C.J., Biechele, T.L., Goldenberg, S.J., Zheng, N., MacCoss, M.J., and Moon, R.T. (2006). The KLHL12-Cullin-3 ubiquitin ligase negatively regulates the Wnt-beta-catenin pathway by targeting Dishevelled for degradation. Nat Cell Biol *8*, 348-357.

Bernassola, F., Karin, M., Ciechanover, A., and Melino, G. (2008). The HECT family of E3 ubiquitin ligases: multiple players in cancer development. Cancer Cell *14*, 10-21.

Cadavid, A.L., Ginzel, A., and Fischer, J.A. (2000). The function of the Drosophila fat facets deubiquitinating enzyme in limiting photoreceptor cell number is intimately associated with endocytosis. Development *127*, 1727-1736.

David, D., Nair, S.A., and Pillai, M.R. (2013). Smurf E3 ubiquitin ligases at the cross roads of oncogenesis and tumor suppression. Biochim Biophys Acta *1835*, 119-128.

Ding, Y., Zhang, Y., Xu, C., Tao, Q.H., and Chen, Y.G. (2013). HECT domain-containing E3 ubiquitin ligase NEDD4L negatively regulates Wnt signaling by targeting dishevelled for proteasomal degradation. J Biol Chem *288*, 8289-8298.

Fei, C., He, X., Xie, S., Miao, H., Zhou, Z., and Li, L. (2014). Smurf1-mediated axin ubiquitination requires Smurf1 C2 domain and is cell cycle-dependent. J Biol Chem *289*, 14170-14177.

Fei, C., Li, Z., Li, C., Chen, Y., Chen, Z., He, X., Mao, L., Wang, X., Zeng, R., and Li, L. (2013). Smurf1-mediated Lys29-linked nonproteolytic polyubiquitination of axin negatively regulates Wnt/β-catenin signaling. Mol Cell Biol *33*, 4095-4105.

Hatakeyama, J., Wald, J.H., Printsev, I., Ho, H.Y., and Carraway, K.L. (2014). Vangl1 and Vangl2: planar cell polarity components with a developing role in cancer. Endocr Relat Cancer *21*, R345-356.

He, Y., Hryciw, D.H., Carroll, M.L., Myers, S.A., Whitbread, A.K., Kumar, S., Poronnik, P., and Hooper, J.D. (2008). The ubiquitin-protein ligase Nedd4-2 differentially interacts with and regulates members of the Tweety family of chloride ion channels. J Biol Chem *283*, 24000-24010.

Hryciw, D.H., Ekberg, J., Lee, A., Lensink, I.L., Kumar, S., Guggino, W.B., Cook, D.I., Pollock, C.A., and Poronnik, P. (2004). Nedd4-2 functionally interacts with ClC-5: involvement in constitutive albumin endocytosis in proximal tubule cells. J Biol Chem *279*, 54996-55007.

Ingham, R.J., Gish, G., and Pawson, T. (2004). The Nedd4 family of E3 ubiquitin ligases: functional diversity within a common modular architecture. Oncogene *23*, 1972-1984.

Kapuria, V., Peterson, L.F., Fang, D., Bornmann, W.G., Talpaz, M., and Donato, N.J. (2010). Deubiquitinase inhibition by small-molecule WP1130 triggers aggresome formation and tumor cell apoptosis. Cancer Res *70*, 9265-9276.

Khan, O.M., Carvalho, J., Spencer-Dene, B., Mitter, R., Frith, D., Snijders, A.P., Wood, S.A., and Behrens, A. (2018). The deubiquitinase USP9X regulates FBW7 stability and suppresses colorectal cancer. J Clin Invest.

Kim, H.C., and Huibregtse, J.M. (2009). Polyubiquitination by HECT E3s and the determinants of chain type specificity. Mol Cell Biol *29*, 3307-3318.

Kuratomi, G., Komuro, A., Goto, K., Shinozaki, M., Miyazawa, K., Miyazono, K., and Imamura, T. (2005). NEDD4-2 (neural precursor cell expressed, developmentally down-regulated 4-2) negatively regulates TGF-beta (transforming growth factor-beta) signalling by inducing ubiquitin-mediated degradation of Smad2 and TGF-beta type I receptor. Biochem J *386*, 461-470.

Kushwaha, D., O'Leary, C., Cron, K.R., Deraska, P., Zhu, K., D'Andrea, A.D., and Kozono, D. (2015). USP9X inhibition promotes radiation-induced apoptosis in non-small cell lung cancer cells expressing mid-to-high MCL1. Cancer Biol Ther *16*, 392-401.

Lee, S., Tumolo, J.M., Ehlinger, A.C., Jernigan, K.K., Qualls-Histed, S.J., Hsu, P.C., McDonald, W.H., Chazin, W.J., and MacGurn, J.A. (2017). Ubiquitin turnover and endocytic trafficking in yeast are regulated by Ser57 phosphorylation of ubiquitin. Elife *6*.

Lee, Y.S., Park, J.S., Kim, J.H., Jung, S.M., Lee, J.Y., Kim, S.J., and Park, S.H. (2011). Smad6-specific recruitment of Smurf E3 ligases mediates TGF-β1-induced degradation of MyD88 in TLR4 signalling. Nat Commun *2*, 460.

Li, X., Song, N., Liu, L., Liu, X., Ding, X., Song, X., Yang, S., Shan, L., Zhou, X., Su, D.*, et al.* (2017). USP9X regulates centrosome duplication and promotes breast carcinogenesis. Nat Commun *8*, 14866.

Lin, A., Hou, Q., Jarzylo, L., Amato, S., Gilbert, J., Shang, F., and Man, H.Y. (2011). Nedd4-mediated AMPA receptor ubiquitination regulates receptor turnover and trafficking. J Neurochem *119*, 27-39.

Liu, C.C., Kanekiyo, T., Roth, B., and Bu, G. (2014). Tyrosine-based signal mediates LRP6 receptor endocytosis and desensitization of Wnt/β-catenin pathway signaling. J Biol Chem *289*, 27562-27570.

Lu, Y., Adegoke, O.A., Nepveu, A., Nakayama, K.I., Bedard, N., Cheng, D., Peng, J., and Wing, S.S. (2009). USP19 deubiquitinating enzyme supports cell proliferation by stabilizing KPC1, a ubiquitin ligase for p27Kip1. Mol Cell Biol *29*, 547-558.

Luga, V., Zhang, L., Viloria-Petit, A.M., Ogunjimi, A.A., Inanlou, M.R., Chiu, E., Buchanan, M., Hosein, A.N., Basik, M., and Wrana, J.L. (2012). Exosomes mediate stromal mobilization of autocrine Wnt-PCP signaling in breast cancer cell migration. Cell *151*, 1542-1556.

MacGurn, J.A., Hsu, P.C., and Emr, S.D. (2012). Ubiquitin and membrane protein turnover: from cradle to grave. Annu Rev Biochem *81*, 231-259.

Madrzak, J., Fiedler, M., Johnson, C.M., Ewan, R., Knebel, A., Bienz, M., and Chin, J.W. (2015). Ubiquitination of the Dishevelled DIX domain blocks its head-to-tail polymerization. Nat Commun *6*, 6718.

Marikawa, Y., and Elinson, R.P. (1998). beta-TrCP is a negative regulator of Wnt/beta-catenin signaling pathway and dorsal axis formation in Xenopus embryos. Mech Dev *77*, 75-80.

Mouchantaf, R., Azakir, B.A., McPherson, P.S., Millard, S.M., Wood, S.A., and Angers, A. (2006). The ubiquitin ligase itch is auto-ubiquitylated in vivo and in vitro but is protected from degradation by interacting with the deubiquitylating enzyme FAM/USP9X. J Biol Chem *281*, 38738-38747.

Mukai, A., Yamamoto-Hino, M., Awano, W., Watanabe, W., Komada, M., and Goto, S. (2010). Balanced ubiquitylation and deubiquitylation of Frizzled regulate cellular responsiveness to Wg/Wnt. EMBO J *29*, 2114-2125.

Mund, T., Graeb, M., Mieszczanek, J., Gammons, M., Pelham, H.R., and Bienz, M. (2015). Disinhibition of the HECT E3 ubiquitin ligase WWP2 by polymerized Dishevelled. Open Biol *5*, 150185.

Potu, H., Peterson, L.F., Kandarpa, M., Pal, A., Sun, H., Durham, A., Harms, P.W., Hollenhorst, P.C., Eskiocak, U., Talpaz, M.*, et al.* (2017). Usp9x regulates Ets-1 ubiquitination and stability to control NRAS expression and tumorigenicity in melanoma. Nat Commun *8*, 14449.

Premarathne, S., Murtaza, M., Matigian, N., Jolly, L.A., and Wood, S.A. (2017). Loss of Usp9x disrupts cell adhesion, and components of the Wnt and Notch signaling pathways in neural progenitors. Sci Rep *7*, 8109.

Pérez-Mancera, P.A., Rust, A.G., van der Weyden, L., Kristiansen, G., Li, A., Sarver, A.L., Silverstein, K.A., Grützmann, R., Aust, D., Rümmele, P.*, et al.* (2012). The deubiquitinase USP9X suppresses pancreatic ductal adenocarcinoma. Nature *486*, 266-270.

Rotin, D., and Kumar, S. (2009). Physiological functions of the HECT family of ubiquitin ligases. Nat Rev Mol Cell Biol *10*, 398-409.

Saito-Diaz, K., Chen, T.W., Wang, X., Thorne, C.A., Wallace, H.A., Page-McCaw, A., and Lee, E. (2013). The way Wnt works: components and mechanism. Growth Factors *31*, 1-31.

Savio, M.G., Wollscheid, N., Cavallaro, E., Algisi, V., Di Fiore, P.P., Sigismund, S., Maspero, E., and Polo, S. (2016). USP9X Controls EGFR Fate by Deubiquitinating the Endocytic Adaptor Eps15. Curr Biol *26*, 173-183.

Schwarz-Romond, T., Fiedler, M., Shibata, N., Butler, P.J., Kikuchi, A., Higuchi, Y., and Bienz, M. (2007). The DIX domain of Dishevelled confers Wnt signaling by dynamic polymerization. Nat Struct Mol Biol *14*, 484-492.

Schwarz-Romond, T., Merrifield, C., Nichols, B.J., and Bienz, M. (2005). The Wnt signalling effector Dishevelled forms dynamic protein assemblies rather than stable associations with cytoplasmic vesicles. J Cell Sci *118*, 5269-5277.

Schwickart, M., Huang, X., Lill, J.R., Liu, J., Ferrando, R., French, D.M., Maecker, H., O'Rourke, K., Bazan, F., Eastham-Anderson, J.*, et al.* (2010). Deubiquitinase USP9X stabilizes MCL1 and promotes tumour cell survival. Nature *463*, 103-107.

Sherk, A.B., Frigo, D.E., Schnackenberg, C.G., Bray, J.D., Laping, N.J., Trizna, W., Hammond, M., Patterson, J.R., Thompson, S.K., Kazmin, D.*, et al.* (2008). Development of a small-molecule serum- and glucocorticoid-regulated kinase-1 antagonist and its evaluation as a prostate cancer therapeutic. Cancer Res *68*, 7475-7483.

Tanksley, J.P., Chen, X., and Coffey, R.J. (2013). NEDD4L is downregulated in colorectal cancer and inhibits canonical WNT signaling. PLoS One *8*, e81514.

Tauriello, D.V., Haegebarth, A., Kuper, I., Edelmann, M.J., Henraat, M., Canninga-van Dijk, M.R., Kessler, B.M., Clevers, H., and Maurice, M.M. (2010). Loss of the tumor suppressor CYLD enhances Wnt/beta-catenin signaling through K63-linked ubiquitination of Dvl. Mol Cell *37*, 607-619.

Thorne, C.A., Hanson, A.J., Schneider, J., Tahinci, E., Orton, D., Cselenyi, C.S., Jernigan, K.K., Meyers, K.C., Hang, B.I., Waterson, A.G.*, et al.* (2010). Small-molecule inhibition of Wnt signaling through activation of casein kinase 1α. Nat Chem Biol *6*, 829-836.

Wang, Y. (2009). Wnt/Planar cell polarity signaling: a new paradigm for cancer therapy. Mol Cancer Ther *8*, 2103-2109.

Wei, W., Li, M., Wang, J., Nie, F., and Li, L. (2012). The E3 ubiquitin ligase ITCH negatively regulates canonical Wnt signaling by targeting dishevelled protein. Mol Cell Biol *32*, 3903-3912.

Wertz, I.E., O'Rourke, K.M., Zhou, H., Eby, M., Aravind, L., Seshagiri, S., Wu, P., Wiesmann, C., Baker, R., Boone, D.L.*, et al.* (2004). De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-kappaB signalling. Nature *430*, 694-699.

Wu, X., Fukushima, H., North, B.J., Nagaoka, Y., Nagashima, K., Deng, F., Okabe, K., Inuzuka, H., and Wei, W. (2014). SCFβ-TRCP regulates osteoclastogenesis via promoting CYLD ubiquitination. Oncotarget *5*, 4211-4221.

Xie, Y., Avello, M., Schirle, M., McWhinnie, E., Feng, Y., Bric-Furlong, E., Wilson, C., Nathans, R., Zhang, J., Kirschner, M.W.*, et al.* (2013). Deubiquitinase FAM/USP9X interacts with the E3 ubiquitin ligase SMURF1 protein and protects it from ligase activity-dependent self-degradation. J Biol Chem *288*, 2976-2985.

Yang, B., Zhang, S., Wang, Z., Yang, C., Ouyang, W., Zhou, F., Zhou, Y., and Xie, C. (2016). Deubiquitinase USP9X deubiquitinates β-catenin and promotes high grade glioma cell growth. Oncotarget *7*, 79515-79525.

Yu, A., Rual, J.F., Tamai, K., Harada, Y., Vidal, M., He, X., and Kirchhausen, T. (2007). Association of Dishevelled with the clathrin AP-2 adaptor is required for Frizzled endocytosis and planar cell polarity signaling. Dev Cell *12*, 129-141.

Yu, A., Xing, Y., Harrison, S.C., and Kirchhausen, T. (2010). Structural analysis of the interaction between Dishevelled2 and clathrin AP-2 adaptor, a critical step in noncanonical Wnt signaling. Structure *18*, 1311-1320.

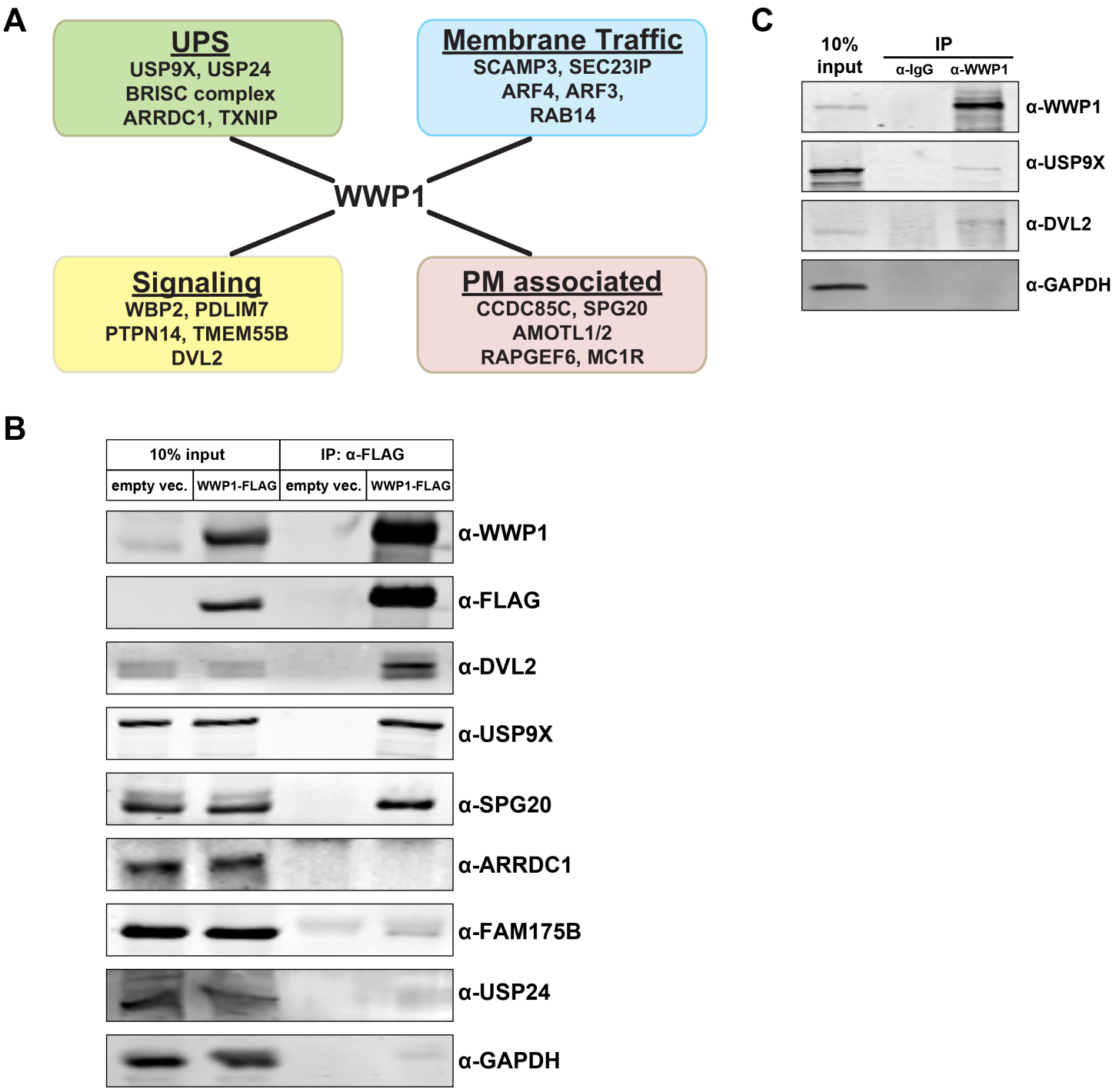


Figure 1. WWP1 interacting proteins in MDA-MB-231 human breast cancer cells. (**A**) Schematic summarizing putative WWP1 interacting proteins identified from SILAC-MS. Complete data set for this experiment is reported in Table S1. (**B**) Co-immunoprecipitation analysis testing hits from SILAC-MS analysis in (A). FLAG-WWP1 was affinity purified from MDA-MB-231 cell lysates. Input and immunoprecipitated (IP) fractions were blotted for the indicated species. **(C)** Endogenous WWP1 was immunoprecipitated from MDA-MB-231 cells. Input (10%) and immunoprecipitates were resolved by SDS-PAGE and immunoblotted for the indicated species.

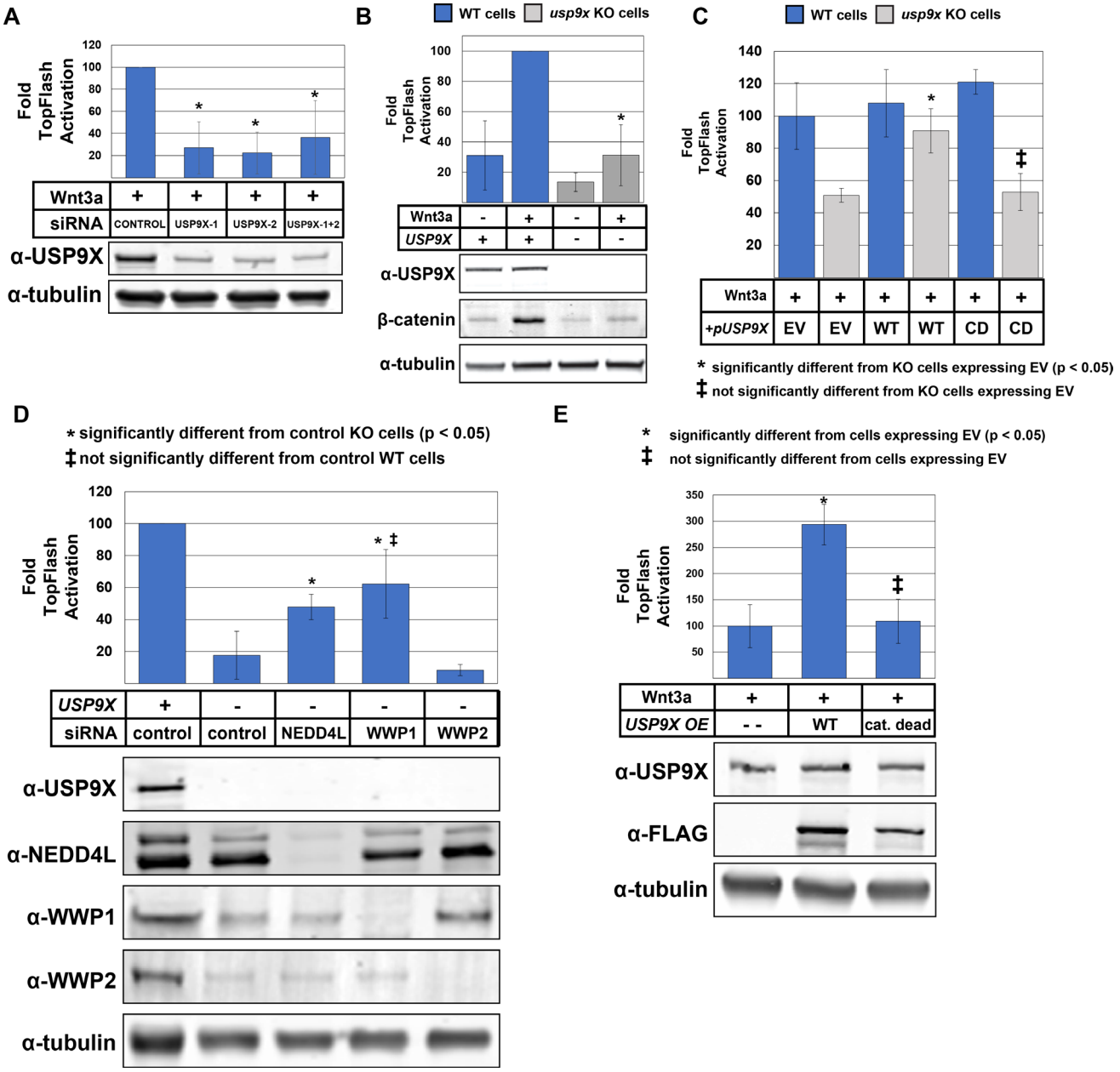


Figure 2. USP9X promotes canonical WNT activation. **(A)** Analysis of ligand-stimulated WNT activation in the presence of control siRNA or siRNAs targeting USP9X knockdown. TopFLASH luciferase assays were used to measure WNT activation (top panel) and immunoblotting was performed to assess knockdown efficiency (bottom panel). * indicates significant difference compared to control siRNA treatment. **(B)** Analysis of ligand-stimulated WNT activation in MDA-MB-231 cells (blue bars) and *usp9x* knockout equivalents (gray bars). TopFLASH luciferase assays were used to measure WNT activation (top panel) and immunoblotting was performed to assess stabilization of nuclear β-catenin. * indicates WNT activation in *usp9x* knockout cells is significantly decreased compared to sibling MDA-MB-231 cells. **(C)** MDA-MB-231 cells (wildtype and *usp9x* knockout) were stimulated with WNT3a ligand and R-spondin and WNT activation was measured by TopFLASH luciferase assays. Prior to activation, cells were transiently transfected with empty vector (EV) or vector expression USP9X (wildtype (WT) or catalytic dead (CD; C1566V)). **(D)** Ligand-stimulated WNT activation was measured using TopFLASH luciferase assays for MDA-MB-231 cells and *usp9x* knockout equivalents. Cells were transfected with control siRNA or siRNA targeting knockdown of NEDD4L, WWP1 or WWP2. (E) MDA-MB-231 cells were transfected with empty vector or plasmids for CMV-driven expression of wildtype (WT) or catalytic dead USP9X.

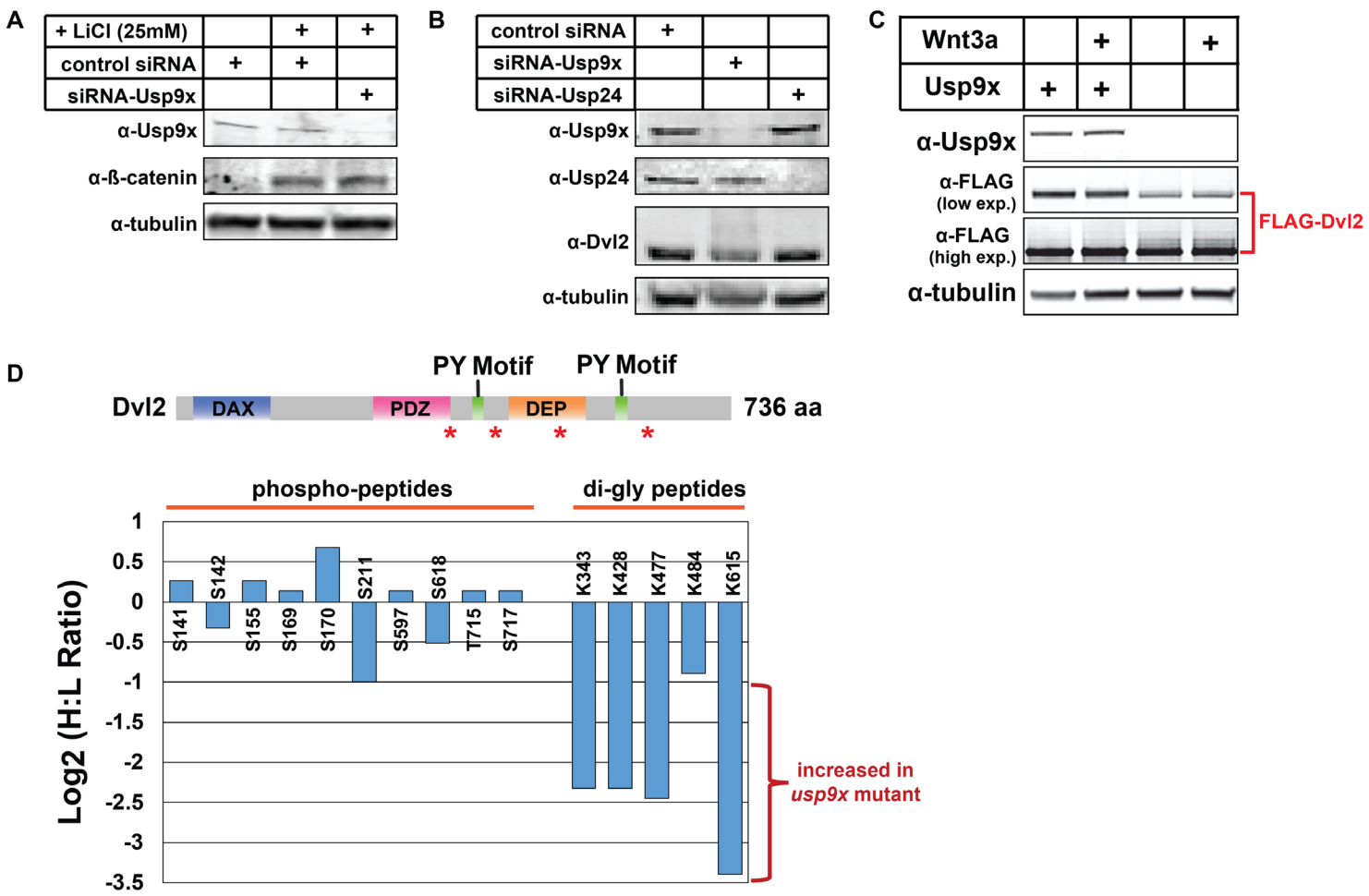


Figure 3. USP9X promotes canonical WNT activation. **(A)** Analysis of LiCl stimulation of WNT activation following transfection with control siRNA or siRNAs targeting USP9X knockdown. Immunoblotting was performed to assess knockdown efficiency and stabilization of nuclear β–catenin. **(B)** Immunoblotting analysis of DVL2 mobility by SDS-PAGE following transfection with control siRNA or siRNAs targeting USP9X or USP24 knockdown. **(C)** Immunoblotting analysis of FLAG-DVL2 mobility by SDS-PAGE from lysates of MDA-MB-231 cells or *usp9x* knockout equivalents. **(E)** SILAC-MS profiling of post-translational modification sites (phosphorylation and ubiquitylation) on DVL2. In this experiment, FLAG-DVL2 purified from MDA-MB-231 cells (heavy) was compared to *usp9x* knockout equivalents (light). A schematic illustrating the domain structure of DVL2 is shown at the top with asterisks to indicate the position of detected ubiquitylation events.

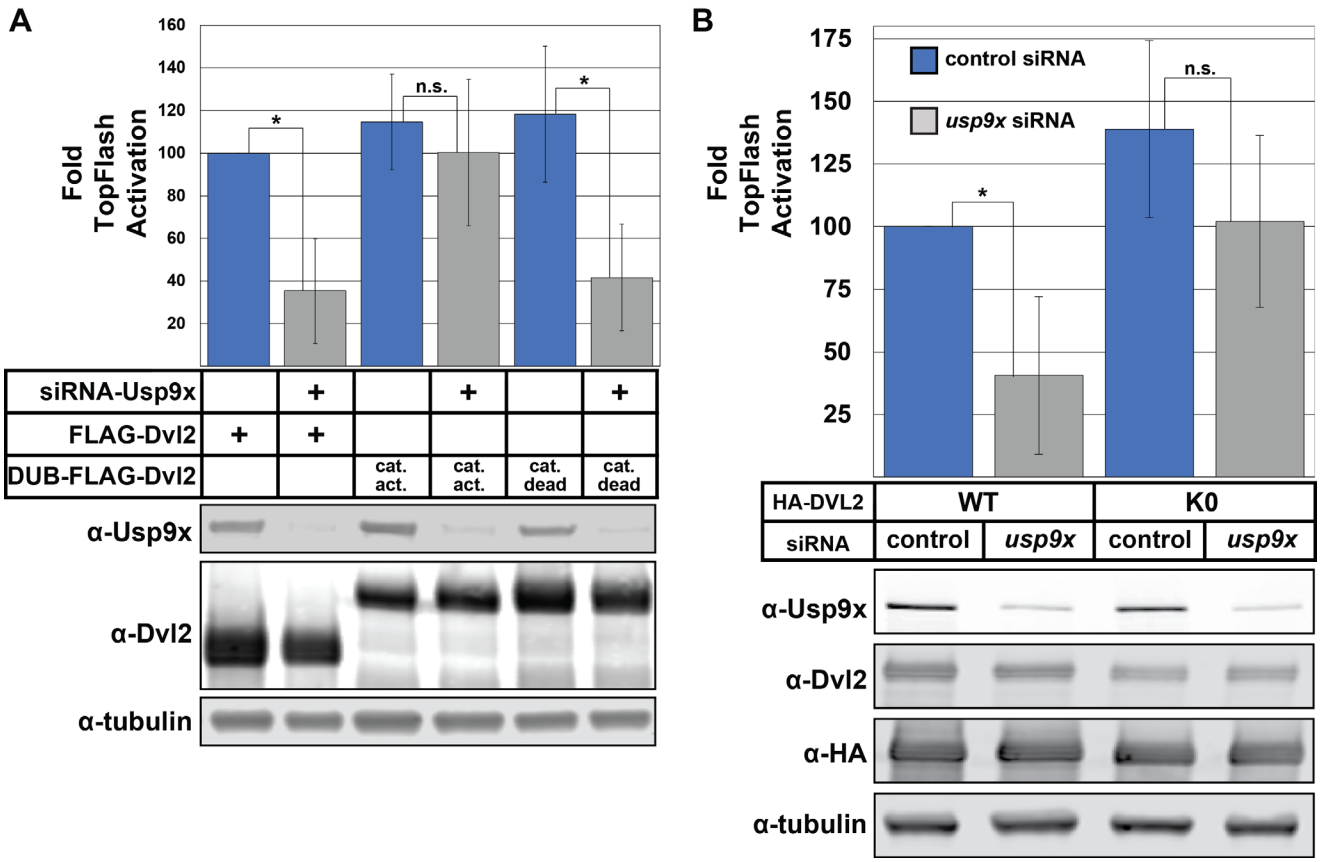


Figure 4. Ubiquitylation of DVL2 is required for *usp9x* loss of function phenotypes. **(A)** Analysis of ligand-stimulated WNT activation was measured using TopFlash reporter assays of MDA-MB-231 cells transfected with the indicated expression vectors. UL36 is the DUB domain fused to DVL2 in these experiments. **(B)** Following siRNA knockdown of USP9X, ligand-stimulated WNT activation was measured using TopFlash reporter assays of MDA-MB-231 cells transfected with the indicated HA-DVL2 expression vectors. DVL2-K0 is a variant where all encoded Lys residues are substituted with Arg residues.

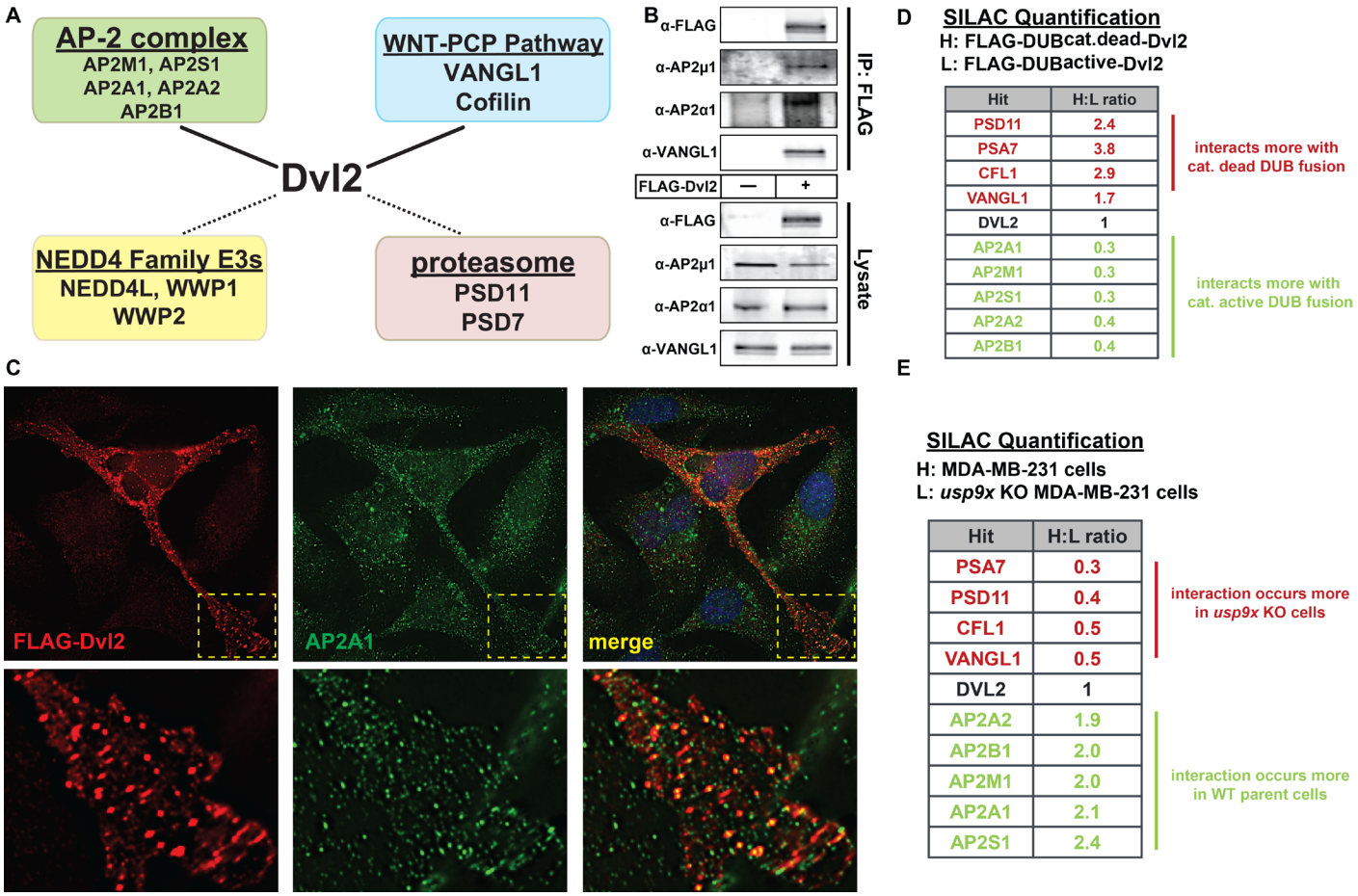


Figure 5. A ubiquitin switch on DVL2 specifies association with canonical WNT or WNT-PCP pathway factors. **(A)** SILAC-MS was used to resolve the DVL2 interaction network in MDA-MB-231 human breast cancer cells. Near-stoichiometric interactions (solid lines) were detected with the AP-2 clathrin adaptor complex and VANGL1-cofilin, which comprise a subcomplex of the WNT-PCP pathway. Sub-stoichiometric interactions (dotted lines) were identified for NEDD4 family E3 ubiquitin ligases and proteasome subunits. **(B)** Interactions detected by SILAC-MS were confirmed by coimmunoprecipitation. **(C)** Immunofluorescence microscopy analysis was performed on MDA-MB-231 breast cancer cells expressing FLAG-DVL2. **(D and E)** SILAC-MS analysis quantifying changes in the DVL2 interaction profile.

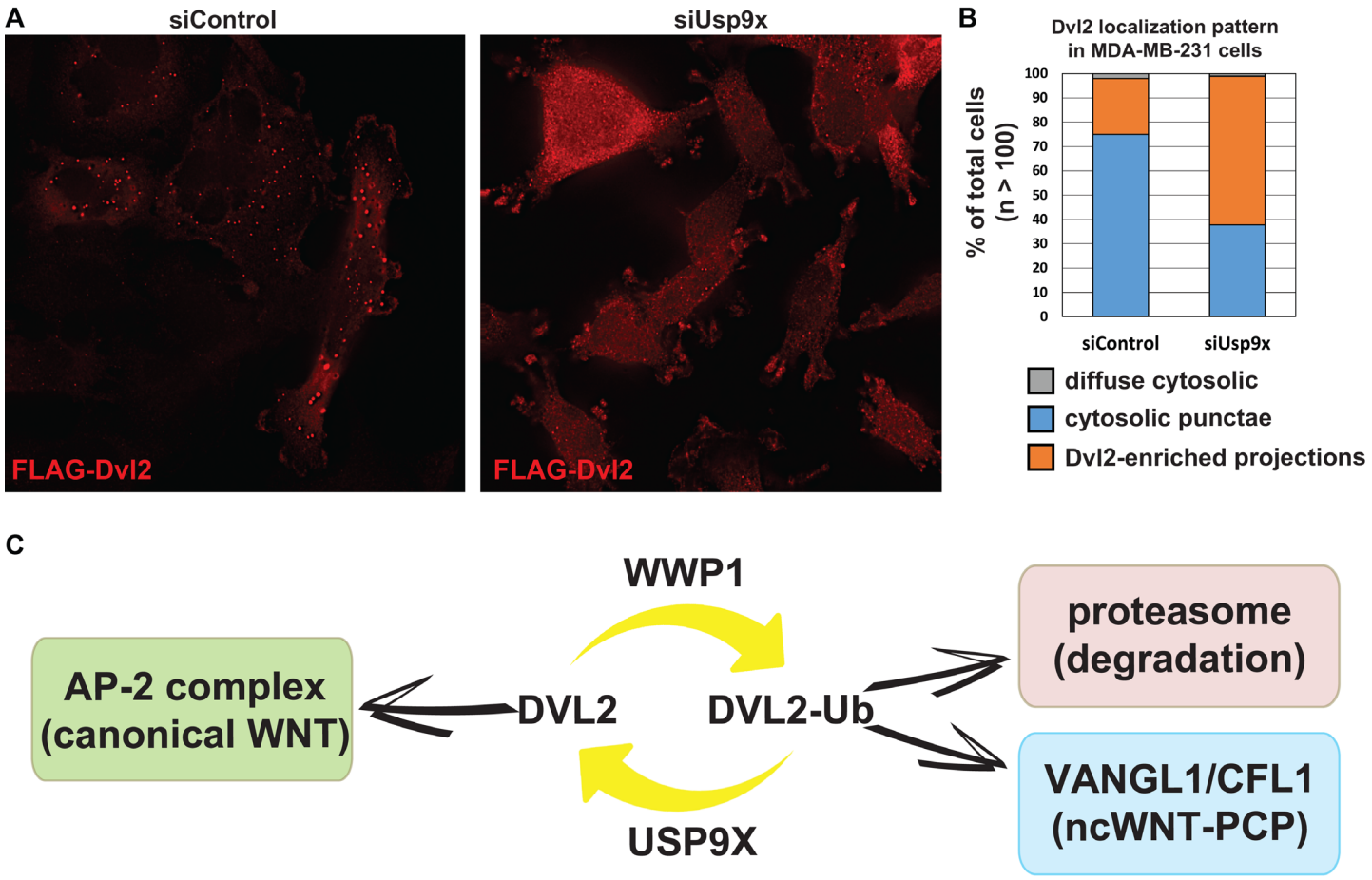
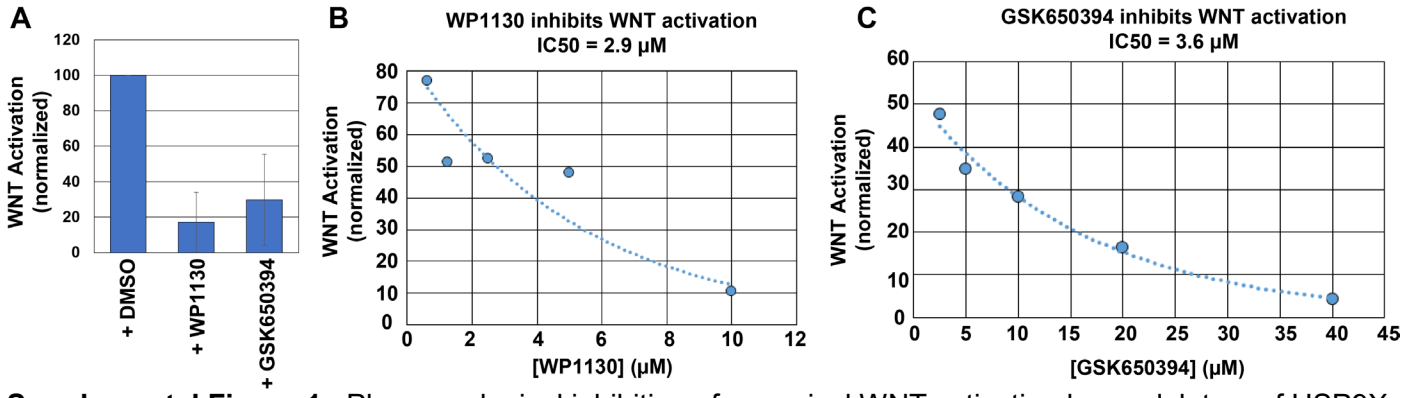
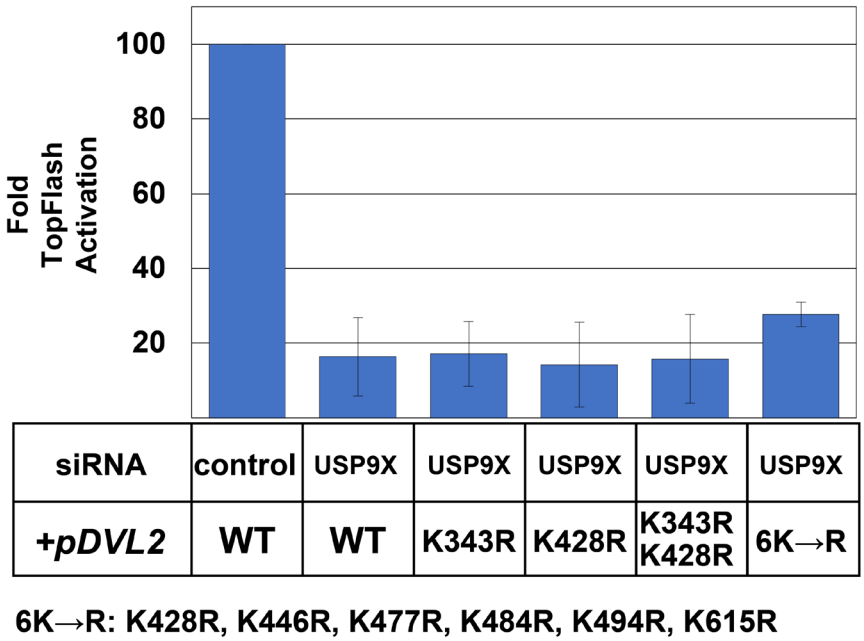


Figure 6. USP9X regulates cellular distribution of DVL2. **(A)** Immunofluorescence imaging of FLAG-DVL2 in MDA-MB-231 human breast cancer cells transfected with control siRNA (left) or siRNA targeting USP9X (right). **(B)** Quantification of DVL2 cellular distribution over a population of MDA-MB-231 cells (n > 100). **(C)** Model illustrating how the ubiquitylation status of DVL2 – as regulated by USP9X and WWP1 – biases engagement of either canonical or non-canonical WNT factors.



Supplemental Figure 1. Pharmacological inhibition of canonical WNT activation by modulators of USP9X and NEDD4L. **(A)** Analysis of ligand-stimulated WNT activation in the presence of a known USP9X inhibitor (WP1130) and an inhibitor of SGK1 kinase (GSK650394) which indirectly hyper-activates NEDD4L. TopFLASH luciferase assays were used to measure WNT activation. **(B** and **C)** Ligand-stimulated WNT activation was measured in the presence of different concentrations of WP1130 **(B)** or GSK650394 **(C)**. Based on the data an IC_{50} was estimated.



6K→R: K428R, K446R, K477R, K484R, K494R, K615R

Supplemental Figure 2. Functional analysis of DLV2 lysine mutants. STF-293 reporter cells were transiently transfected with the indicated FLAG-DVL2 plasmids and siRNAs and analyzed for WNT activation using the TopFLASH luciferase assay.

Table S1. WWP1 Interaction Profile using SILAC-MS

category	protein	known function / description	pept. count	H:L ratio	stoich. (norm.)
Bait	WWP1	NEDD4 family E3 ubiquitin ligase (bait)	289	36.01	1.00
membrane traffic	SCAMP3	endosomal recycling	18	37.43	0.16
	ARF4	polarized exocytosis	8	7.13	0.14
	RAB14	regulation of Golgi-to-endosome transport	5	9.22	0.07
	ARF5	endocytosis of integrins	3	12.40	0.05
	SEC23IP	regulates ER exit	9	5.47	0.03
plasma membrane / membrane associated	CCDC85C	function unknown	21	17.47	0.16
	SPG20	function unknown	33	47.71	0.16
	AMOTL2	regulation of cell junctions; Hippo signaling	22	38.39	0.09
	AMOTL1	regulation of cell junctions; Hippo signaling	21	33.19	0.07
	RAPGEF6	function unknown	31	28.00	0.06
	MC1R	melanocortin receptor	3	26.71	0.03
	AXL	receptor tyrosine kinase	8	5.18	0.03
	ERBB2IP	binds and stabilizes ERBB2 receptor	12	9.36	0.03
	FNDC3B	function unknown	10	5.19	0.03
	SLC2A1	facilitative glucose transporter	3	6.67	0.02
	LRP10	putative receptor; function unknown	4	12.62	0.02
	EGFR	epidermal growth factor receptor	6	8.66	0.02
	ADAM9	cell surface metalloproteinase	4	6.04	0.02
	NPC1	intracellular cholesterol transporter	4	8.53	0.01
signaling	WBP2	Hippo pathway and hormone signaling	26	17.07	0.32
	PDLIM7	bone formation and BMP signaling pathways	30	22.18	0.21
	PTPN14	protein tyrosine phosphatase	75	22.11	0.20
	TMEM55B	phosphatidylinositol 4,5-bisphosphate 4-phosphatase	4	6.14	0.05
	DVL2	signal transducer, WNT pathway	9	25.67	0.04
	PTPN21	protein tyrosine phosphatase	4	10.24	0.01
ubiquitin proteasome system	USP9X	deubiquitylase	94	25.37	0.12
	FAM175B	member of the BRISC complex; deubiquitylase	9	13.22	0.07
	TXNIP	thioredoxin interacting protein; may function as E3 adaptor	8	8.86	0.06
	BRCC3	member of the BRISC complex; deubiquitylase	5	21.72	0.05
	ARRDC1	thioredoxin interacting protein; may function as E3 adaptor	6	20.14	0.04
	C19ORF62	member of the BRISC complex; deubiquitylase	3	9.28	0.03
	USP24	deubiquitylase	20	23.66	0.02

Light: MDA-MB-231 cells stably expressing pQCXIP (empty vector)
Heavy: MDA-MB-231 cells stably expressing FLAG-WWP1
Selection Criteria: (1) number of peptides quantified ≥ 3; (2) H:L ratio > 5

Table S2. DVL2 Interaction Profile using SILAC-MS

category	protein	known function / description	pept. count	H:L ratio	stoich. (norm.)
Bait	DVL2	WNT pathway signaling protein	29	28.70	1.00
AAA ATPase	ATAD3A	mitochondria-localized AAA ATPase of unknown function	18	8.58	0.72
	ATAD3B		14	**	0.55
endocytosis	AP2M1	AP2 clathrin adpator complex	10	19.79	0.58
	AP2B1		19	8.83	0.51
	AP2A1		18	37.89	0.47
	AP2S1		2	10.99	0.36
	AP2A2		8	**	0.22
	EPS15L1	endocytic adaptor proteins; sort ubiquitylated cargo for endocytosis	3	**	0.09
	EPS15		2	**	0.06
signaling	VANGL1	WNT-PCP signaling protein	5	**	0.24
	DVL3	WNT pathway signaling protein	5	**	0.18
	DVL1		4	**	0.15
	CK1delta	kinase; regulates canonical WNT activation	2	**	0.12
	WDR26	signal transduction protein	2	13.00	0.08
	RANBP9	signal transduction protein	2	**	0.07
ubiquitin proteasome system	NEDD4L	NEDD4 family E3 ubiquitin ligase	4	22.38	0.10
	PSD11	proteasome subunits	2	5.58	0.12
	PSA7		2	36.59	0.20
cytoskeleton	Cofilin-1	regulator of actin dynamics	3	5.70	0.46

Light: MDA-MB-231 cells stably expressing pQCXIP (empty vector)
Heavy: MDA-MB-231 cells stably expressing FLAG-DVL2
Selection Criteria: (1) number of peptides quantified ≥ 2; (2) H:L ratio > 5
** indicates signal in heavy channel but no signal in light channel

Table S3. DVL2 Interaction Profile in WNT-activated cells using SILAC-MS

category	Protein	known function / description	pept. count	H:L ratio	stoich. (norm.)
bait	DVL2	WNT pathway signaling protein	43	107.20	1.00
AAA ATPase	ATAD3B	mitochondria-localized AAA ATPase of unknown function	15	**	0.40
	ATAD3A		25	62.37	0.67
endocytosis and trafficking	AP2B1	AP2 clathrin adaptor complex	49	52.27	0.90
	AP2M1		17	43.41	0.67
	AP2A1		38	60.82	0.67
	AP2S1		5	71.38	0.60
	AP2A2		30	27.94	0.55
	CAVIN1	caveolae formation and organization	10	18.36	0.44
	CAVIN2		8	**	0.32
	SQSTM1	autophagy receptor	10	10.15	0.39
	EPS15L1	endocytic adaptor proteins; sort ubiquitylated cargo for endocytosis	7	6.31	0.14
	EPS15		2	**	0.04
signaling	VANGL1	WNT-PCP signaling protein	13	**	0.42
	CK1 delta	casein kinase isoforms; regulate canonical WNT activation	17	**	0.70
	CKII beta		7	**	0.56
	CK1 epsilon		12	**	0.49
	CKII alpha		9	**	0.39
	PLK1	polo-like kinase-1	16	**	0.45
	DVL3	WNT pathway signaling protein	15	**	0.36
	RANBP9	signal transduction protein	15	**	0.35
	WDR26	signal transduction protein	13	74.44	0.34
ubiquitin proteasome system	NEDD4L	NEDD4 family E3 ubiquitin ligase	29	35.38	0.51
	WWP2		12	**	0.24
	WWP1		3	**	0.06
	PSA7	proteasome subunits	2	**	0.14
	PRS10		3	**	0.13
	PSA1		2	**	0.13
	PSD11		2	**	0.08
transcription	FOXK2	transcriptional regulators	18	**	0.47
	FOXK1		13	**	0.30
protein folding	BAG3	co-factor for HSP70	14	**	0.42

Light: MDA-MB-231 cells stably expressing pQCXIP (empty vector) stimulated with 200ng/mL mouse-Wnt3a and 100ng/mL mouse-Rspondin

Heavy: MDA-MB-231 cells stably expressing FLAG-DVL2 stimulated with 200ng/mL mouse-Wnt3a and 100ng/mL mouse-Rspondin

Selection Criteria: (1) number of peptides quantified ≥ 2; (2) H:L ratio > 5

****** indicates signal in heavy channel but no signal in light channel