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

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Summary

NEDD4 family E3 ubiquitin ligases generally participate in cell signaling and trafficking pathways in eukaryotic cells and their dysregulation is thought to contribute to various human diseases. One NEDD4 family member, WWP1, has been reported to undergo genomic amplification in breast and prostate cancer yet little is known about its function or regulation in cancer cells. Here, we report that WWP1 interacts with USP9X – a deubiquitylating enzyme previously reported to interact with other NEDD4 family members. We find that USP9X is required for activation of the canonical WNT pathway and that loss of WNT signaling in the absence of USP9X requires the activity of NEDD4 family members – including WWP1 and NEDD4L. We show that USP9X regulates WNT activation by mediating deubiquitylation of DVL2 – a key signal transducing protein in the WNT pathway. This ubiquitin switch biases DVL2 participation toward either canonical WNT or the WNT-PCP pathway, with USP9X activity towards DVL2 simultaneously promoting canonical WNT activation and guarding against WNT-PCP activation. Thus, the USP9X-DVL2-WWP1 axis effectively sets up a zero sum game with respect to canonical WNT and WNT-PCP pathway activation. These findings elucidate an important function of USP9X in cancer cells and have implications for therapeutic targeting of USP9X in human disease.

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 movement and tissue polarity. Dysregulation of the WNT-PCP pathway has been linked to cancer progression, invasion, metastasis, and angiogenesis (Wang, 2009). While the canonical WNT β-catenin pathway and the noncanonical WNT-PCP pathway use distinct effector mechanisms and regulate distinct cellular functions, these pathways share membrane receptor components and the cytoplasmic WNT transducer protein disheveled (DVL). However, 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 reglator 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 proteins, adaptors, signaling factors, and trafficking proteins (**FIG 1A**). One of the top hits from this analysis was USP9X, a DUB that contains four PY motifs which could potentially interact with the WW domains of Nedd4 family Ub ligases. We used coimmunoprecipitation (coIP) to verify some WWP1 interactors – including USP9X and the WNT signal transducing protein DVL2 – while other putative interactors (like ARRDC1) were not detected by coIP (**FIG 1B**). Importantly, other studies have reported interactions between USP9X and other NEDD4 family E3 ubiquitin ligases – including SMURF1 (Xie et al., 2013) and ITCH (Mouchantaf et al., 2006). These interactions are perhaps not surprising given that USP9X contains four PY motifs capable of binding the WW domains of NEDD4 family E3 ubiquitin ligases.

USP9X promotes canonical WNT activation

Since we identified USP9X and DVL2 as WWP1 interactors and since NEDD4 family members have been shown to negatively regulate WNT signaling (Tanksley et al., 2013) potentially via ubiquitylation of DVL2 (Ding et al., 2013), we decided to test if USP9X regulates WNT signaling. We found that USP9X knockdown or knockout attenuated WNT activation (using a TopFlash reporter) and β–catenin stabilization in STF293 and MDA-MB-231 cells (**FIG 2A-B** and data not shown, see Materials and Methods for cell line descriptions). As an alternative to depletion of USP9X we used a chemical inhibitor, WP1130, known to inhibit the deubiquitylase activity of USP9x and other DUBs (Kapuria et al., 2010) and found that inhibition of USP9X deubiquitylase activity attenuated WNT activation (**FIG 2C**) suggesting the Usp9x

deubiquitylase activity is required for canonical WNT activation. Importantly, we found that the loss of function in USP9X knockout (or knockdown) cells was suppressed by coordinate siRNA knockdown of NEDD4L or WWP1 (but not WWP2) (**FIG 2D**) suggesting that hyper-activation of multiple NEDD4 family members, including NEDD4L and WWP1, may contribute to loss of WNT signaling in the absence of USP9X. We also found that overexpression of WT USP9X sensitized MDA-MB-231 breast cancer cells to hyper-activation when stimulated with WNT3A whereas overexpression of catalytic dead USP9X did not (**FIG 2E**). Taken together, our findings indicate that ***(i)*** USP9X is required for canonical WNT activation and promotes WNT pathway activation in a titratable manner and ***(ii)*** decreased WNT activation in the absence of USP9X requires WWP1 and NEDD4L.

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, 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 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) bypasses the requirement for USP9X (**FIG 3D**) indicating that USP9X regulates WNT signaling at (or in very close proximity to) the level of DVL2. To identify post-translational modifications (PTMs) on DVL2 regulated by USP9X we performed a SILAC-MS and resolved many PTM-modified peptides derived from DVL2 but only four internal lysines exhibited

significantly elevated ubiquitylation in the absence of USP9X (**FIG 3E**). Ubiquitin modification of these lysine residues has not been previously reported, although ubiquitylation at N-terminal lysines on DVL2 has been reported (Madrzak et al., 2015; Tauriello et al., 2010). These results indicate that USP9X reverses ubiquitylation at multiple lysine residues in DVL2, and this dequbiquitylation reaction is critical for activation of WNT signaling.

DVL2 Deubiquitylation by USP9X Promotes Interaction with AP-2

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 (**FIG 4A**). These experiments revealed two near-stoichiometric interactions with the AP-2 clathrin adaptor complex (about 50% of DVL2 bound) and with VANGL1 and cofilin (about 30% of DVL2 bound) which are important factors 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 (**FIG 4A**). Many of these interactions had been previously reported (Angers et al., 2006; Yu et al., 2007). Several interactions were confirmed by co-IP (**FIG 4B** and data not shown) and co-localization by immunofluorescence microscopy (**FIG 4C** and data not shown). We also found that knockdown of AP-2 complex subunits inhibited canonical WNT activation in a TOPFLASH assay (data not shown), consistent with previous reports (Yu et al., 2007; Yu et al., 2010).

To quantify how DVL2 interactions are affected by ubiquitylation, we performed SILAC proteomic analysis comparing the interactome of catalytic active (light) or catalytic dead (heavy) DUB-DVL2 fusions (also used in **FIG 3C**). This revealed that the catalytic dead DUB-DVL2 fusion (which can be 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 4D**). 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 4E**). These results suggest 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

Previously reported studies suggesting 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 relocalization 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 both the presence and absence of USP9X. In MDA-MB-231 cells treated with control, non-targeting siRNA DVL2 exhibited a punctate distribution throughout the cell body, reflective of previous descriptions of DVL2 localization (**FIG 5A, left panel**) (Schwarz-Romond et al., 2005). Whereas MDA-MB-231 cells treated with USP9X-targeting siRNA displayed a redistribution of DVL2 to structures that appear to be projections (**FIG 5A, 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 5B**). Altogether, our findings indicate that the USP9x-WWP1 axis regulates a ubiquitin switch on DVL2 that bias interaction preferences 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 noncanonical WNT-PCP pathway (**FIG 6**).

Discussion

Dishevelled has long been known to play a key role in the transduction of WNT receptor signaling, yet how it is regulated to function in both canonical and 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. Additional biochemical and genetic studies

will be required to fully understand how USP9X interacts with various ubiquitylation events on DVL2 that may be mediated by multiple different E3 ubiquitin ligases.

Previous studies have reported conflicting roles for USP9X with respect to WNT activation. Specifically, UP9X 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. Ultimately, we believe that investigation of such context-dependent regulation of USP9X and its substrates will contribute to the overall understanding of how USP9X appears to function as a tumor suppressor in some cancer contexts and a tumor promoter in other cancer contexts.

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). It is important to note that USP9X has also been proposed to have tumor suppressor activity in other cancers such as pancreatic ductal adenocarcinoma(Pérez-Mancera et al., 2012) and colorectal adenocarcinoma (Khan et al., 2018). Additionally, USP9X exhibits tumor suppressor activity in the EGFR pathway as it has been shown to be required for the attenuation of EGFR signaling (Savio et al., 2016). Taken together, these reports create a complex picture of the role of USP9X in cancer progression and we believe that our findings may help to resolve some of the apparent disparity. Our findings suggest that USP9X functions as part of a ubiquitin rheostat on DVL2 to bias its participation in either the canonical or non-canonical WNT pathways. Based on these findings we hypothesize that in some cellular contexts increased levels of USP9X facilitate hyperactivation of the canonical WNT pathway, 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 could either suppress or promote cancer progression, depending on the context.

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 result in several possible outcomes, including E3 antagonization, E3 deubiquitylation, or polyubiquitin chain editing on the substrate. E3 antagonization has the potential to establish a futile cycle but could also create a rheostat on a shared substrate. For example, the DUB CYLD antagonizes the activity of TRAF2, an E3 ubiquitin ligase, in order to regulate NFKB signaling (Wu et al., 2014). In contrast, E3 deubiqitylation would have the potential to protect the E3 from degradation. For example, the DUB USP19 drives 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 polyubiqiutin 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, and in turn inhibiting the NFkB pathway. We have identified a novel E3-DUB interaction that functions to provide layered regulation of the WNT pathway. Based on our findings it is possible that the USP9X-WWP1 regulatory axis could fit into more than one of the above categories. First, it is possible that USP9X antagonizes WWP1 activity towards DVL2 by removing ubiquitin modifications to promote its participation in the canonical WNT pathway. On the other hand, it is also possible that USP9X and WWP1 function as part of a complex editing mechanism in which an additional E3 ubiquitin ligase - perhaps other NEDD4 family members - participate. In such an editing mechanism, certain DVL2 ubiquitylation events might promote interactions with VANGL1/cofilin and participation in the non-canonical WNT-PCP pathway while other ubiquitylation events might promote proteasomal degradation. Ultimately, our findings suggest that the balance of different ubiquitylation and deubiquitylation activities toward DVL2 is a critical factor in the regulation of both canonical and noncanonical WNT signaling.

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).

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A. WWP1 Hits from SILAC-MS Interactome

category	hits
adaptors	ARRDC1, TXNIP
putative substrates	AXL, DVL2, GLUT1, FNDC3B, ADAM9
signaling molecules	PTPN14, PTPN21, TMEM55B
deubiquitylases	USP9X, USP24, BRISC complex
trafficking	Spartin, SCAMP3, Rab14

B.

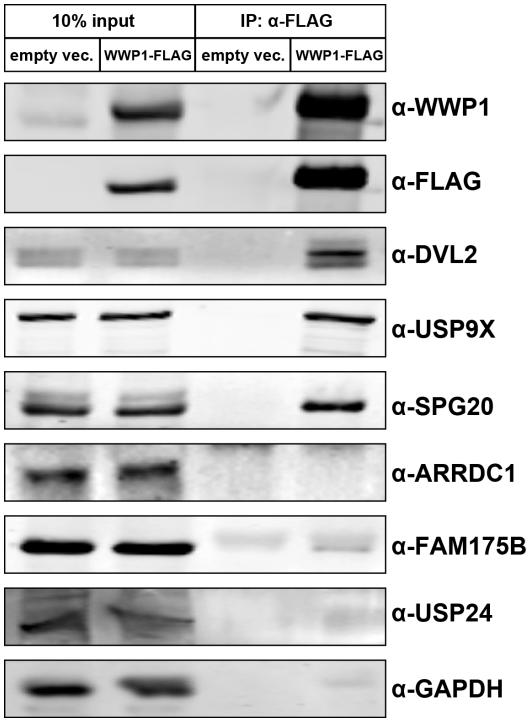

Figure 1. WWP1 interacting proteins in MDA-MB-231 human breast cancer cells. (**A**) Table summarizing results from a SILAC-MS analysis using FLAG-WWP1 as bait. (**B**) Co-immunoprecipitation analysis testing hits from SILAC-MS analysis in (A). FLAG-WWP1 was affinity purified from MDA-MB-231 cell lysates. Input and immunoprecipitated (IP) fractions were blotted for the indicated species.

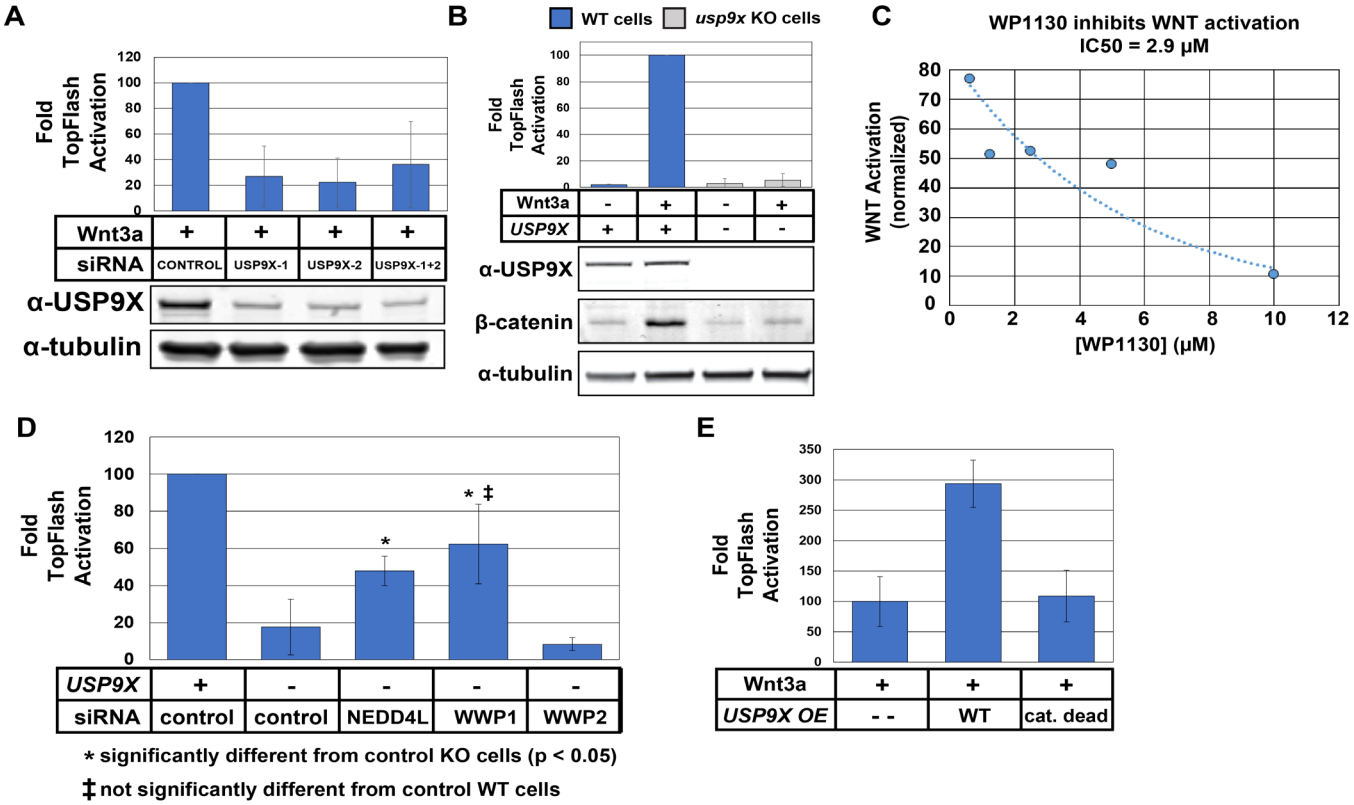

Figure 2. USP9X promotes canonical WNT activation. **(A)** Analysis of ligand-stimulated WNT activation in the presence of control siRNA or siRNAs targeting USP9X knockdown. TopFLASH luciferase assays were used to measure WNT activation (top panel) and immunoblotting was performed to assess knockdown efficiency (bottom panel). **(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. **(C)** Ligand-stimulated WNT activation was measured in the presence of different concentrations of WP1130, a known USP9X inhibitor. Based on the data an IC_{50} was estimated. **(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.

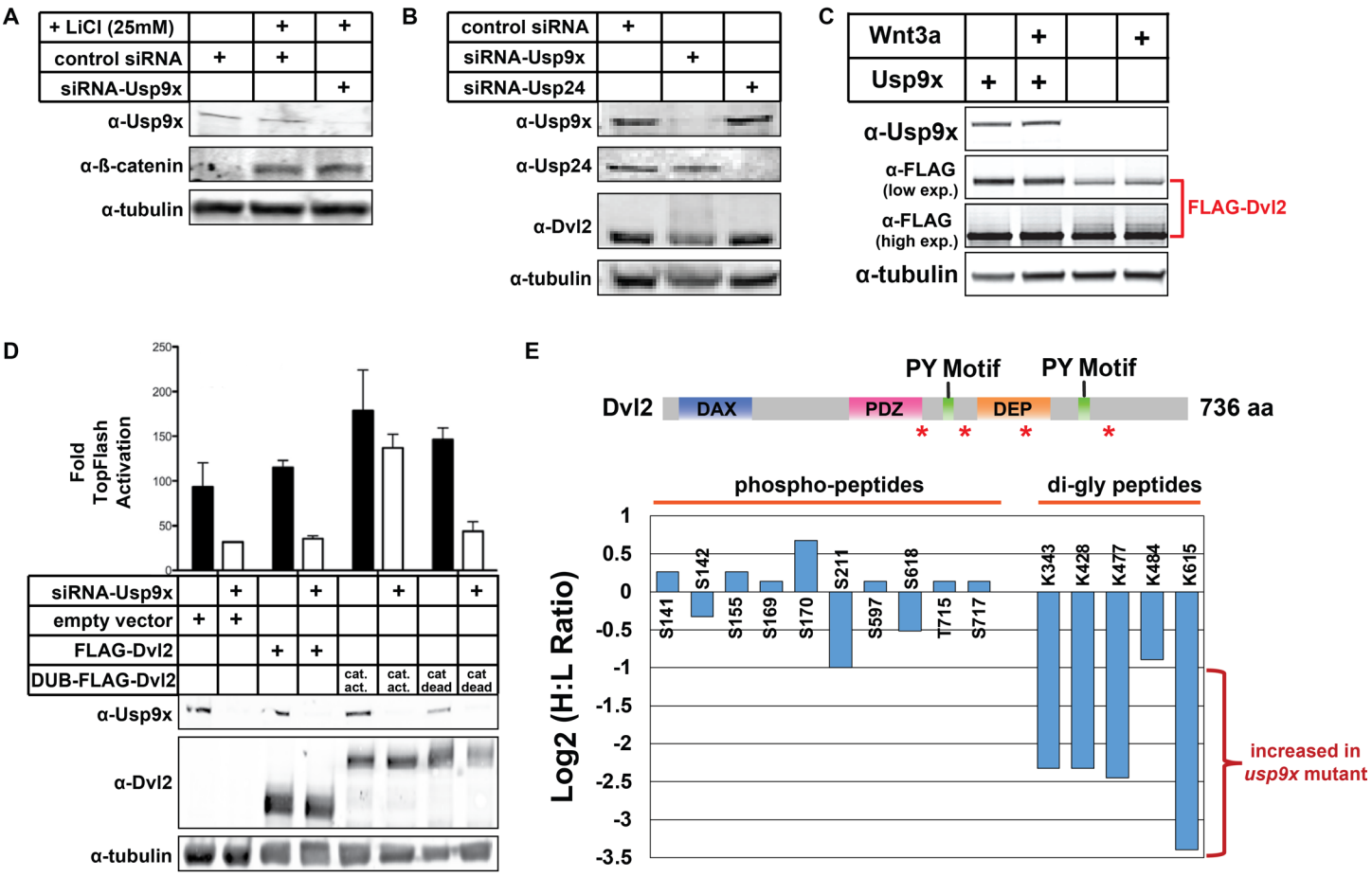

Figure 3. USP9X promotes canonical WNT activation. **(A)** Analysis of LiCl stimulation of WNT activation following transfection with control siRNA or siRNAs targeting USP9X knockdown. Immunoblotting was performed to assess knockdown efficiency and stabilization of nuclear β–catenin. **(B)** Immunoblotting 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. **(D)** 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. (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.

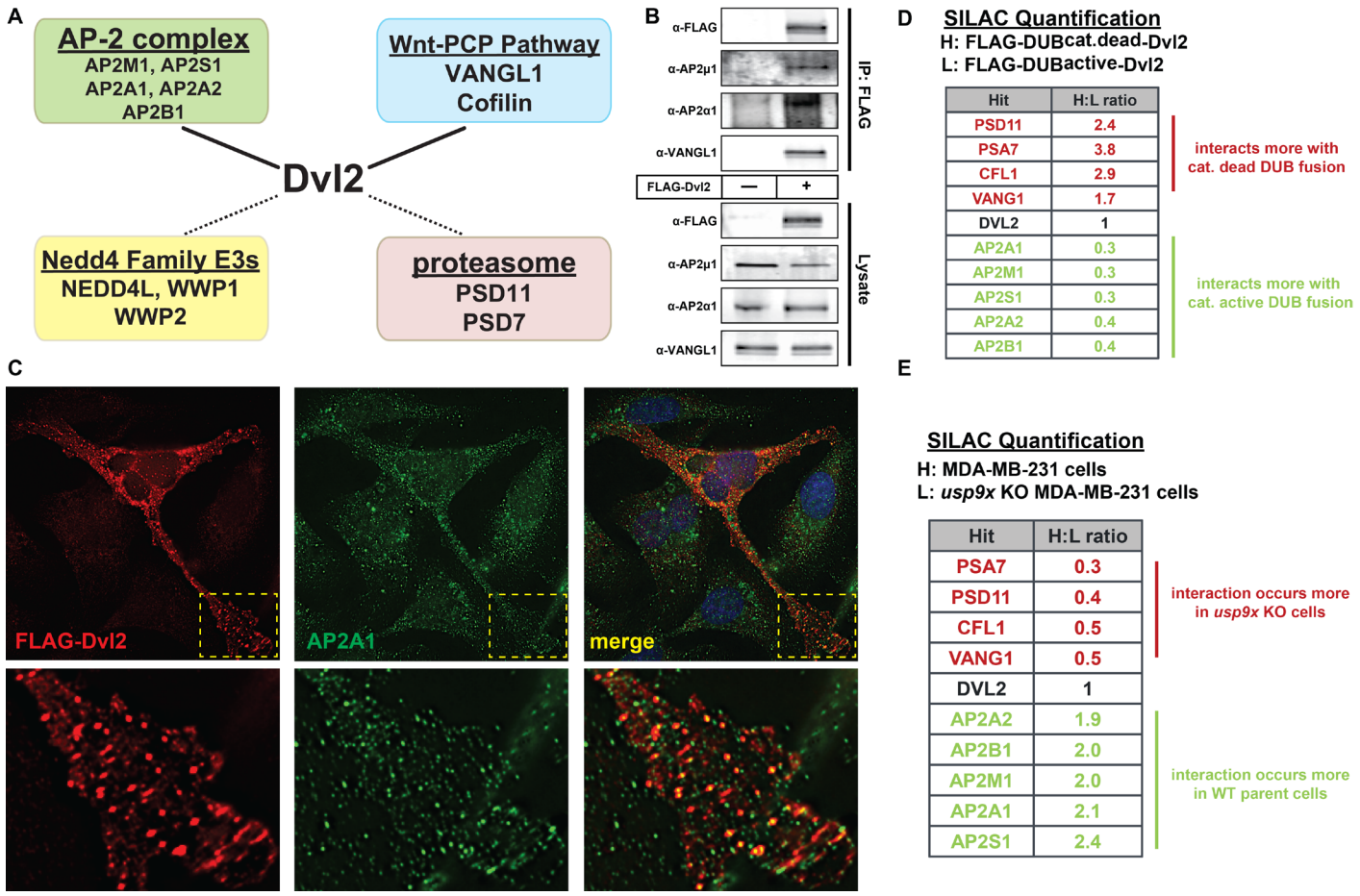

Figure 4. A ubiquitin switch on DVL2 specifies association with canonical WNT or WNT-PCP pathway factors. **(A)** SILAC-MS was used to resolve the DVL2 interaction network in MDA-MB-231 human breast cancer cells. Near-stoichiometric interactions were detected with the AP-2 clathrin adaptor complex and VANGL1-cofilin, which comprise a subcomplex of the WNT-PCP pathway. **(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.

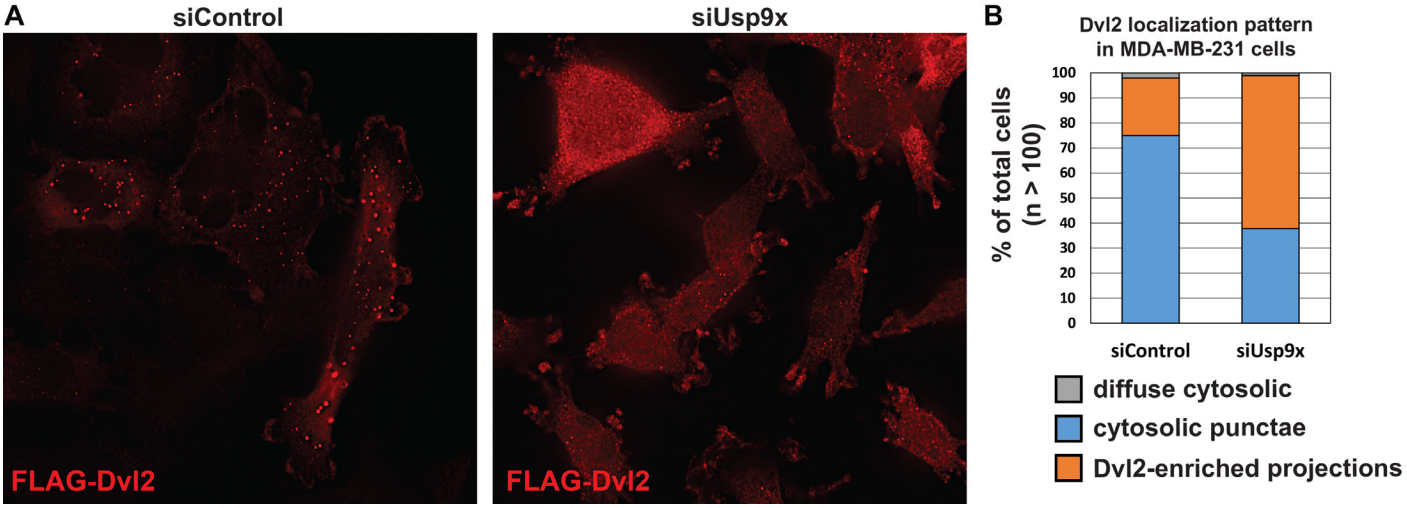

Figure 5. USP9X regulates cellular distribution of DVL2. **(A)** Immunofluorescence imaging of FLAG-DVL2 in MDA-MB-231 human breast cancer cells transfected with control siRNA (left) or siRNA targeting USP9X (right). **(B)** Quantification of DVL2 cellular distribution over a population of MDA-MB-231 cells (n > 100).

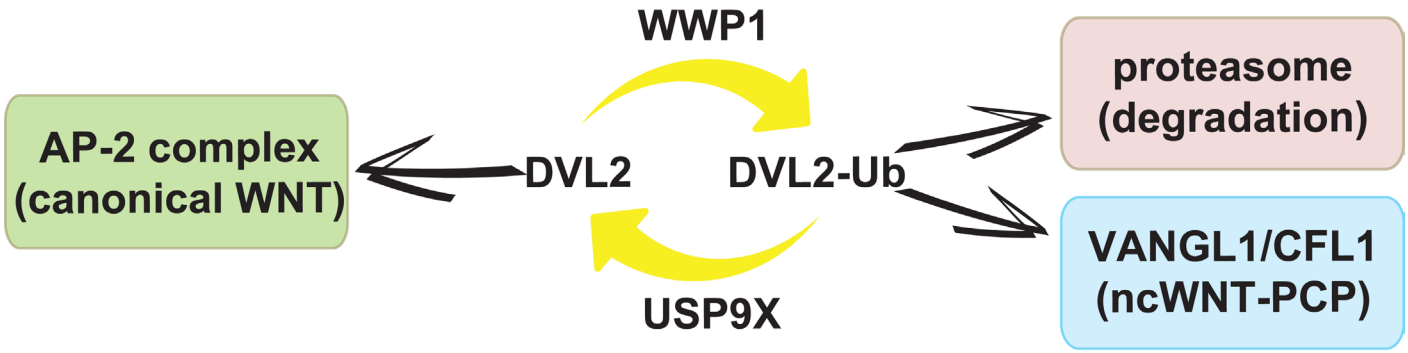

Figure 6. Activity of USP9X and NEDD4 family E3 ubiquitin ligases like WWP1 and NEDD4L regulate a ubiquitin switch on DVL2 that regulate interaction preference with either the clathrin adaptor AP-2 complex, which is important for canonical WNT signaling, or the VANGl1-cofilin complex, which is important for the WNT-PCP pathway. Thus, the USP9X-WWP1 axis biases DVL2 participation in canonical or non-canonical WNT pathways.