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Suppression of planar cell polarity signaling and migration in glioblastoma by Nrdp1-mediated Dvl polyubiquitination

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Abstract

The lethality of the aggressive brain tumor glioblastoma multiforme (GBM) results in part from its strong propensity to invade surrounding normal brain tissue. While oncogenic drivers such as EGFR activation and PTEN loss are thought to promote the motility and invasiveness of GBM cells via PI3K activation, other unexplored mechanisms may also contribute to malignancy. Here we demonstrate that several components of the planar cell polarity (PCP) arm of non-canonical Wnt signaling including *VANGL1*, *VANGL2*, and *FZD7* are transcriptionally upregulated in glioma and correlate with poorer patient outcome. Knockdown of the core PCP pathway protein Vangl1 suppresses the motility of GBM cell lines, pointing to an important mechanistic role for this pathway in glioblastoma malignancy. We further observe that restoration of Nrdp1, a RING finger type E3 ubiquitin ligase whose suppression in GBM also correlates with poor prognosis, reduces GBM cell migration and invasiveness by suppressing PCP signaling. Our observations indicate that Nrdp1 physically interacts with the Vangl1 and Vangl2 proteins to mediate the K63-linked polyubiquitination of the DEP domain of the Wnt pathway protein Dishevelled (Dvl). Ubiquitination hinders Dvl binding to phosphatidic acid, an interaction necessary for efficient Dvl recruitment to the plasma membrane upon Wnt stimulation of Fzd receptor and for the propagation of downstream signals. We conclude that the PCP pathway contributes significantly to the motility and hence the invasiveness of glioblastoma cells, and that Nrdp1 acts as a negative regulator of PCP signaling in GBM cells by inhibiting Dvl through a novel polyubiquitination mechanism. We propose that the upregulation of core PCP components, together with the loss of the key negative regulator Nrdp1, act coordinately to promote GBM invasiveness and malignancy.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

planar cell polarity; non-canonical Wnt; glioblastoma; motility; ubiquitination

Introduction

Glioblastoma multiforme (GBM) is the most lethal brain cancer, representing 45% of malignant primary CNS tumors[1]. Despite a standard of care involving surgical resection of the primary tumor followed by radiation and chemotherapy, median survival time is about one year, and only 5% of patients survive five years past diagnosis[2]. Tumors are heterogeneous, and subpopulations of cells often exhibit the highly invasive properties responsible for surgical evasion and recurrence. These cells, whose migratory ability is unhindered by radiation therapy, invade vital parts of the patient's brain leading to inevitable death[3,4].

While GBM is a heterogeneous disease, common molecular drivers include overexpression or mutation of EGFR, elevation of PDGF ligands and receptors, and loss of the tumor suppressor PTEN[5]. Downstream signaling through the phosphatidylinostitol 3-kinase (PI3K)/Akt pathway promotes migration, and disruption of PI3K/Akt signaling in GBM cells can partially suppress cellular invasion[6]. However, alterations in other molecular pathways such as Wnt signaling also promote invasive processes[7], and it is likely that dysregulation of multiple signaling pathways contributes to GBM aggressiveness.

A common theme in tumorigenesis concerns the reactivation of embryonic developmental pathways to promote tumor growth and malignancy. Planar cell polarity (PCP) is a well characterized pathway in *Drosophila* development essential for generating epithelial cell polarity in the planar axis orthogonal to the apical-basal axis. Signaling via the tetraspanin-like scaffolds Vangl1 and Vangl2 in mammals comprises a branch of non-canonical Wnt signaling associated with developmental PCP, and dysregulation of this pathway is associated with various disease states[8,9]. In Vangl-dependent non-canonical Wnt signaling, the Frizzled (Fzd) receptor is activated by Wnt ligand binding, followed by Dishevelled (Dvl) recruitment to the plasma membrane. These events give rise to downstream activation of c-Jun N-terminal kinase (JNK) and the small GTPases Rac1 and RhoA, resulting in AP1 transcriptional activation and cytoskeletal rearrangements[8]. Signaling through this pathway can be localized to specific subcellular regions by the presence of Vangl to promote directed cell movements[9,10].

While PCP signaling is essential for development, its role in maintenance of adult tissues is not well studied. Vangl proteins localize to the leading edge of lamellapodia and to the base and arms of actin protrusions of migrating breast cancer cells, and *VANGL1* knockdown in these cells reduces motility[10,11]. Although the mechanism by which Vangl-dependent non-canonical Wnt signaling regulates cell migration remains unknown, mounting evidence suggests that signaling mediated by Vangl proteins is hijacked by tumors to modulate cell invasiveness. Vangl1 and/or Vangl2 dysregulation has been reported in several cancer types, including GBM[12,13]. Higher Vangl1 transcript and protein is associated with increased tumor grade and reduced survival in glioma patients. Further, Vangl1 overexpression in a

murine glioma line increases cell migration and reduces survival in mice with orthotopic xenografts, while Vangl1 loss suppresses U251 cell motility and prolongs survival in a similar xenograft model[12]. These observations suggest that Vangl-dependent non-canonical Wnt signaling contributes to GBM progression.

Nrdp1 is a RING finger E3 ubiquitin ligase that mediates the ubiquitination of several protein targets involved in cancer progression, including the inhibitor of apoptosis protein BRUCE[14] and the growth factor receptors ErbB3 and ErbB4[15]. Loss of Nrdp1 has been associated with breast cancer[16,17], prostate cancer[18], colon cancer[19] and recently GBM. In glioma cell lines, re-expression of Nrdp1 has been reported to reduce BRUCE levels and increase apoptosis in response to temozolomide treatment[20], and may reduce cell migration in a subset of gliomas via ErbB3 suppression[21]. However, while increased signaling through PI3K/Akt is a hallmark of glioma, ErbB3 is not commonly dysregulated in GBM[22], suggesting that other Nrdp1-mediated pathways regulate migration in brain tumors.

Here we report that *VANGL1* and *VANGL2* are overexpressed and *NRDP1* is suppressed in brain tumors relative to normal brain tissue, and that restoration of Nrdp1 to GBM cell lines reduces cellular motility and invasiveness. We further demonstrate that Nrdp1 interacts with Vangl1 and Vangl2 to mediate the ubiquitination of Dvl proteins, downregulating planar cell polarity signaling by suppressing Dvl recruitment to activated Fzd receptor. These observations point to a novel role for Nrdp1 in suppressing Vangl-dependent non-canonical Wnt signaling, and highlight an unappreciated role for this pathway in regulating the motility of GBM cells.

Results

Nrdp1 inhibits GBM cell invasiveness

Using publicly available microarray data, we observed that *NRDP1* transcript is significantly lower in brain tumor samples than non-tumor brain samples in the REMBRANDT dataset (Figs. 1A and S1A), regardless of molecular subtype (Fig. S1B)[23]. Moreover, low *NRDP1* transcript levels are associated with decreased patient survival (Figs. 1B and S1C), raising the possibility that *NRDP1* suppression contributes to disease progression and mortality.

To assess the impact of Nrdp1 protein on GBM biology, we restored its cDNA to PTEN-mutated GBM cell lines A1207, T98G and U87-MG. We observed that Nrdp1 expression alters cellular morphology, favoring flatter cells with fewer protrusions (Fig. 2A), and suppresses motility as well as invasiveness in Matrigel-coated Boyden chambers (Figs. 2B and 2C). Conversely, shRNA-mediated suppression of the low endogenous levels of *NRDP1* in T98G (Figs. S2A and 2B) and U87-MG cells (Fig. S2D) augments their migration (Figs. S2C and S2E) and favors a more spindly U87-MG morphology (Fig. S2F). However, Nrdp1 restoration does not alter the proliferation of GBM lines (Fig. S2G), suggesting that Nrdp1 is a specific negative regulator of GBM migration and invasiveness.

To assess the impact of Nrdp1 restoration on GBM invasiveness in brain tissue, we examined the dissemination of highly invasive GFP-labeled U251 cells into cultured slices of mouse brain. Like the other glioma lines, Nrdp1 restoration to U251 cells (Fig. S3A)

induces a more flattened morphology (Fig. S3B) and suppresses their migration (Fig. S3D), but does not impact their proliferation (Fig. S3C). We observed that when seeded on brain slices, U251 cells with restored Nrdp1 disseminated less efficiently than control cells, as determined histologically and by anti-GFP staining (Figs. 2D and 2E), providing strong evidence that Nrdp1 is a potent negative regulator of brain tumor invasiveness.

Although Nrdp1 is most well characterized as a negative regulator of the ErbB3 and ErbB4 receptor tyrosine kinases[15], previous studies indicate that neither receptor is significantly dysregulated in GBM[22]. Likewise, levels of EGFR and phosphorylation of signaling intermediates Akt and Erk are not altered upon Nrdp1 restoration (Fig. S4A), consistent with its inability to influence GBM cell proliferation. β-catenin phosphorylation is also not affected by Nrdp1 (Figs. S4B and S4C), suggesting that canonical Wnt signaling is not targeted. While we observed no reproducible alterations in the activation state of the motility-associated GTPase Rac1 (Figs. S4D and S4E), Nrdp1 restoration significantly decreases active RhoA (Figs. S4F and S4G) and phospho-JNK (Figs. S4H and S4I), suggesting that Nrdp1 might regulate non-canonical Wnt signaling[8,24].

Nrdp1 interacts with Vangl1 and Vangl2

Wnt signaling is broadly separated into two categories. Canonical Wnt signaling results in stabilized β-catenin, most often driving proliferation and differentiation. Non-canonical Wnt signaling consists of several interconnected pathways defined by β-catenin independence, and activation of these pathways often results in AP1-dependent transcription through phospho-JNK and increased cell motility through Rac and/or Rho[8,24]. Nrdp1 may regulate non-canonical Wnt signaling and GBM migration by interacting with the non-canonical Wnt components Vangl1 and Vangl2, interactions first identified in a published Master's thesis[25]. The Vangl proteins are critical for PCP, and loss-of-function Vangl mutants lead to developmental defects in multiple tissues. Because Vangl1 and Vangl2 have nearly identical protein structures and biochemical properties, variation in their developmental roles[26] may arise from differential tissue or temporal expression. Still, Vangl proteins have been linked to migration and invasion in several carcinomas[13] and expression of both *VANGL1* and *VANGL2* is increased in GBM (Fig. 3A). Indeed, aberrant expression of core Vangl-dependent non-canonical Wnt signaling components appears to be a common feature of glial-derived brain tumors across molecular subtypes, as transcripts of the Wnt ligand *WNT5A* and the transmembrane Wnt receptor *FZD7* are also elevated compared to non-tumor brain tissue (Figs. S5A and S5B). Dysregulation of this pathway is also associated with decreased patient survival times (Fig. S5C–S5F), underscoring the important role of PCP signaling in promoting dissemination.

We confirmed the interaction of Vangl1 and Vangl2 with Nrdp1 through co-immunoprecipitation assays in HEK293T cells (Figs. S6A and S6B). Importantly, endogenous Vangl2 and Nrdp1 could be co-immunoprecipitated from mouse brain lysates, demonstrating the physiological relevance of this interaction (Fig. S6C). Vangl proteins contain an intracellular N-terminal region with multiple phosphorylation sites[27], four transmembrane domains, and an intracellular C-terminal region with a coiled-coil domain and PDZ-binding motif[28]. To determine the Vangl2 region responsible for interacting with

Nrdp1, we employed deletion mutants (Fig. S6D). Only the Vangl2ΔC mutant fails to precipitate with Nrdp1 (Fig. S6E), indicating that Nrdp1 interacts with the Vangl2 C-terminus. The Vangl2ΔCC mutant may have a diminished interaction with Nrdp1, of interest because Nrdp1 itself has a coiled-coil domain and these domains often self-associate[29]. Indeed, Nrdp1 lacking this domain (Nrdp1ΔCC in Fig. S6F) does not interact with Vangl2 (Fig. S6G), suggesting that the Nrdp1 coiled-coil domain is necessary for the Nrdp1-Vangl2 interaction while the Vangl2 coiled-coil domain supports but is dispensable for the interaction. We have demonstrated that the Nrdp1 coiled-coil domain mediates Nrdp1 oligomerization[29], and it is possible that Vangl2 interacts with Nrdp1 oligomers or disrupts Nrdp1 oligomerization. Surprisingly, neither augmented ubiquitination of Vangl1 or Vangl2 (Figs. S7A and S7B) nor suppressed Vangl protein levels (Fig. S7C) is observed with Nrdp1 expression, implying that Vangl1 and Vangl2 could as scaffolds for Nrdp1 as they do in previously characterized modes of PCP[30,31].

Dvl is subject to Nrdp1-mediated ubiquitination

While the precise molecular complexes involved in each branch of non-canonical Wnt signaling are not clearly defined, most branches engage the scaffolding protein family Dvl[24]. Because Dvl proteins are regulated by ubiquitination[32], we tested whether they are substrates for Nrdp1-mediated ubiquitination. Each of the three Dvl family members exhibits increased ubiquitination when co-expressed with Vangl2 and Nrdp1 (Figs. 3B and S7D). Interestingly, Nrdp1-mediated Dvl ubiquitination occurs only in the presence of Vangl2, demonstrating that Nrdp1 alone is insufficient to promote Dvl ubiquitination, and suggesting that Vangl2 acts as a scaffold to bring Nrdp1 and Dvl into proximity. Both Vangl1 and Vangl2 promote Nrdp1-mediated Dvl ubiquitination (Figs. 3B and S7D–F), providing another example of the biochemical interchangeability of these proteins. Vangl1 or Vangl2 expression alone is sufficient to promote Dvl ubiquitination, possibly due to endogenous Nrdp1 or other interacting E3 ubiquitin ligases. Dvl ubiquitination is suppressed by Nrdp1 knockdown (Fig. 3C), and a double point-mutant of the Nrdp1 RING domain (Nrdp1-CHSQ-FLAG) that cannot catalyze transfer of ubiquitin from the E2 to the substrate[33] cannot promote ubiquitination of Dvl2 (Figs. S7E and S7F), indicating that the ubiquitin ligase activity of Nrdp1 is necessary for Dvl ubiquitination. Because Nrdp1 promotes ubiquitination of endogenous Dvl2 in T98G cells (Fig. 3D) but does not alter expression of *WNT5A* or *VANGL1* (Fig. S8), Nrdp1-mediated regulation of non-canonical Wnt signaling via Dvl ubiquitination may be responsible for the decreased motility in these cells. However, Nrdp1 does not promote Dvl2 degradation, as its expression in GBM cell lines does not decrease endogenous Dvl2 protein (Fig. S7G).

Polyubiquitination consists of ubiquitin monomers chained through any of seven lysines or the N-terminal amino group, most commonly utilizing K48, K63 or K11[ref. 34]. To determine the ubiquitin linkage on Dvl2 promoted by Nrdp1, we conducted ubiquitination assays using ubiquitin mutants where K48, K63, or K11 is mutated to arginine, thus preventing polyubiquitination through the mutated lysine. We observed that only the K63R mutant cannot form polyubiquitin chains on Dvl2 (Fig. 3E), indicating that Nrdp1 promotes K63-linked polyubiquitination of Dvl2. This complements our observation that Nrdp1 does not promote endogenous Dvl2 degradation; unlike K48 and K11-linked ubiquitin chains, K63-

linked poyubiquitination is not typically a degradation marker, but promotes context-dependent regulation of protein activity and complexes[34]. These observations suggest that Nrdp1-mediated K63-linked polyubiquitination of Dvl proteins alters Dvl function to suppress GBM cell motility.

Nrdp1-mediated ubiquitination prevents Dvl binding to phosphatidic acid

We next explored the mechanism whereby Nrdp1-mediated Dvl ubiquitination impairs PCP signaling. Dvl proteins have three domains: the DEP and PDZ domains, required for canonical and non-canonical Wnt signaling, and the DIX domain, required for canonical Wnt signaling[24]. Using Dvl1 mutants with the lysines in each domain mutated to arginines (Fig. 4A), we determined that the Dvl1-DEP-6KR mutant is resistant to Nrdp1-mediated ubiquitination (Figs. 4B and 4C), mapping the predominant Nrdp1 polyubiquitination sites to the DEP domain. Because the Dvl1-DEP-6KR mutant interacts normally with Vangl2 (Fig. S9A), this decreased ubiquitination is likely not the result of impaired Dvl-DEP-6KR folding or function.

The Dvl DEP domain interacts with phosphatidic acid (PA) within plasma membrane inner leaflet through an electrostatic interaction between negatively charged PA and positively charged lysine and arginine residues on the DEP membrane-binding face. Recruitment to the plasma membrane by these residues is necessary for efficient formation of complexes between Dvl and the Wnt receptor Fzd[35]. Five mutated lysines in the Dvl1-DEP-6KR mutant are on this membrane-binding face (Fig. S9B), suggesting that ubiquitination of these residues could reduce the affinity of the DEP domain for PA by steric hindrance. To test this, we overlayed purified Dvl1 onto phospholipid strips to assess alterations in the avidity of Dvl1 for PA upon Nrdp1-mediated ubiquitination (Figs. 4D and 4E). Vangl2 increases the amount of Dvl1 bound to PA, consistent with its pro-migratory role[10,11]. However, in the presence of Vangl2 and Nrdp1, Dvl1 binding to PA is significantly decreased, suggesting that Nrdp1-mediated ubiquitination inhibits Dvl function by preventing its association with the plasma membrane. To confirm that Nrdp1 abrogates the Dvl-PA interaction by promoting DEP ubiquitination, we overlaid Dvl1 wild-type (Dvl1-WT) or Dvl1-DEP-6KR onto lipid strips (Fig. S9C). While Nrdp1 reduces the affinity of Dvl1-WT for PA, the Dvl1-DEP-6KR mutant resists the effects of Nrdp1 and retains PA-binding potential. We conclude that Nrdp1 inhibits signaling through Dvl by mediating the ubiquitination of the DEP domain region necessary for interaction with the plasma membrane.

Nrdp1 promotes Dvl segregation into a Vangl complex

A recurring theme in Vangl-dependent non-canonical Wnt signaling is intracellular segregation of protein complexes to limit active signaling to specific subcellular areas to promote directed movement. Non-canonical Wnt ligand binds to Fzd receptors, which drives formation of active Fzd-Dvl complexes at the tips of migratory protrusions, while Vangl localizes to the arms of these protrusions[9,10]. Because Fzd and Vangl display mutually exclusive localization yet both interact with Dvl, we hypothesized that they compete for Dvl and that Nrdp1 influences this competition to suppress Dvl activation. In the presence of Wnt5a, increasing amounts of Fzd7 results in decreased amounts of Dvl1 co-

immunoprecipitated with Vangl2 (Fig. 5A), consistent with a model where ligand-bound Fzd competes with Vangl2 for available Dvl1. Nrdp1 expression favors Dvl1-Vangl2 complex maintenance, decreasing the ability of Fzd7 to inhibit this interaction (Fig. 5A and 5B) and suppressing the accumulation of active, phosphorylated Dvl1[ref. 36] (Fig. 5C and 5D), suggesting that polyubiquitinated Dvl cannot form the active Fzd-Dvl complexes necessary for directed migration. Likewise, restoration of Nrdp1 to T98G cells suppresses Wnt5a-induced accumulation of phospho-Dvl2, implying that Nrdp1 interferes with Fzd-induced Dvl signaling in GBM cells (Fig. 5E and 5F).

To demonstrate that Nrdp1-mediated Vangl-dependent ubiquitination of Dvl is relevant in GBM cell lines, we sought to knockdown Vangl. A1207 and T98G cells do not express *VANGL2* (Fig. S10A), and *VANGL1* expression in these cell lines can be decreased with two independent shRNAs (Fig. S10B). In T98G cells, *VANGL1* knockdown significantly decreases Nrdp1-mediated Dvl2 ubiquitination (Figs. 6A and 6B), again demonstrating that Vangl proteins are a critical link between Nrdp1 and its Dvl substrate. *VANGL1* knockdown is sufficient to inhibit migration in A1207 and T98G cells (Fig. 6C), indicating that Vangl-dependent non-canonical Wnt signaling promotes GBM migration, and mirroring results observed in breast cancer cells[10,11]. However, in the presence of Nrdp1, *VANGL1* knockdown has no additional effect on cell migration, implying that Nrdp1 and Vangl1 regulate GBM migration through the same pathway.

Discussion

Collectively, our observations underscore the importance of the PCP pathway in glioma malignancy, and establish Nrdp1 as a central regulator of PCP signaling. Although dysregulation of *NRDP1*, *VANGL1* and *WNT5A*[12,20,37] in GBM has been reported in small sample sizes, our study finds that in large, publicly available microarray datasets the collective dysregulation of Vangl-dependent non-canonical Wnt signaling components *NRDP1*, *VANGL1*, *VANGL2*, *WNT5A* and *FZD7* is a nearly ubiquitous event in GBMs of all molecular subtypes and lower grade astrocytomas and oligodendrogliomas. The correlation of aberrant expression of these components with decreased patient survival is strong evidence that aberrant PCP activation promotes glioma cell invasiveness, the primary causes of relapse in GBM patients.

We hypothesize that Nrdp1 promotes K63-linked polyubiquitination of Dvl to suppress Vangl-dependent non-canonical Wnt signaling (Fig. 7). Upon pathway activation, Dvl binds to the negatively charged plasma membrane phospholipid PA via positively charged residues (K409, K456, R461, R464, K465, K472, K482) in the DEP domain[38]; mutation of these lysine residues to glutamate reduces the PA binding ability of Dvl and Fzd-dependent membrane recruitment[35]. We demonstrate that Nrdp1-mediated ubiquitination modifies the DEP domain of Dvl and abrogates its ability to bind PA using the Dvl1-DEP-6KR mutant. While Nrdp1 suppresses the interaction between Dvl1-WT and PA, Dvl1-DEP-6KR largely lacks polyubiquitination and retains PA binding in the presence of Nrdp1. These results imply that the critical Nrdp1-mediated polyubiquitination sites are on this polybasic face of the DEP domain, where ubiquitination disrupts membrane binding by sterically hindering the interaction. The mutated lysine in the Dvl1-DEP-6KR mutant not in the polybasic face,

K434, participates in canonical Wnt/β-catenin signaling and likely does not contribute to Nrdp1-mediated negative regulation of Dvl[39]. We also found that Nrdp1 inhibits Dvl phosphorylation and promotes maintenance of Dvl-Vangl complexes in the presence of Fzd and Wnt5a. In this model, Nrdp1 favors the Dvl-Vangl complex by decreasing the affinity of Dvl for PA, which is necessary for formation of the Dvl-Fzd complex and downstream signaling.

Ubiquitination is a common mechanism of Dvl regulation in both arms of Wnt signaling. Several E3 ubiquitin ligases promote Dvl turnover through proteasomal degradation[32], and several E3 ubiquitin ligases have been shown to promote K63-linked polyubiquitination of Dvl. The HECT E3 ligase Huwe1 inhibits canonical Wnt signaling by preventing formation of Dvl oligomers via K63-linked polyubiquitination of Dvl[40], while the RING E3 ligase PDZRN3 promotes non-canonical Wnt signaling by K63-linked polyubiquitination of the DIX domain to regulate Dvl3-Fzd4 complex endocytosis[41]. Nrdp1-mediated Dvl ubiquitination inhibits Dvl membrane binding, a mechanism distinct from Huwe1 or PDZRN3, demonstrating the context-dependent versatility of K63-linked polyubiquitination of a single protein.

Nrdp1 has been previously linked to cell polarity through its interaction with the Ser/Thr kinase MARK2 (Par-1b) in breast epithelial cells, where disrupting MARK2-dependent phosphorylation of Nrdp1 prevents proper apical-basal polarity[42]. As Vangl-dependent non-canonical Wnt signaling is required for PCP establishment in development, Nrdp1 may regulate polarity in two axes. There is precedent for interplay between these polarity pathways; Scribble, a *Drosophila* PCP effector that interacts with Vang/Vangl in *Drosophila* and mammalian cells[11], is a well-characterized component of the basolateral complex that maintains apical-basal polarity[43]. This suggests that the two polarity modes may be more closely linked than previously appreciated, and future studies are needed to establish the extent to which Nrdp1 regulates polarity both in development and disease.

Although Nrdp1 levels are decreased in many cancer types, the role and regulation of Nrdp1 seem to vary dramatically. In breast cancer, aberrant ErbB3 signaling is associated with loss of Nrdp1 protein despite unchanged mRNA levels[17]. However, *NRDP1* transcript is decreased in GBM tumors, implying that different regulatory mechanisms govern *NRDP1* expression in these tumors. To date, the only known transcriptional regulator of *NRDP1* is the androgen receptor in prostate cancer[18], which is not known to have a significant role in GBM. Just as signaling through the ErbB3-Akt axis stabilizes Nrdp1 protein in a negative feedback loop[44], it is possible that non-canonical Wnt signaling regulates *NRDP1* transcript. However, *VANGL1* knockdown does not change *NRDP1* transcript levels in GMB cell lines (Fig. S10C), suggesting that a feedback loop between Vangl-dependent non-canonical Wnt signaling and *NRDP1* gene regulation does not exist. The mechanism governing *NRDP1* transcript loss in GBM should be the subject of further study, as its restoration may decrease GBM invasiveness.

In summary, this study is the first to demonstrate that Nrdp1 regulates Wnt signaling, and we propose that loss of Nrdp1 in aggressive GBM tumors contributes to aberrant activation of a Vangl-dependent non-canonical Wnt pathway to promote tumor invasion. Our findings

contribute significantly to the understanding of Dvl regulation, highlighting another avenue through which non-canonical Wnt signaling is disrupted in tumor biology. Moreover, our findings underscore the role of PCP signaling in GBM motility and invasiveness, suggesting that targeting the PI3K pathway may not be sufficient to suppress malignancy.

Materials and Methods

Cell culture and reagents

HEK293T cells were from ATCC, and T98G, U87-MG, A1207, and U251 cells were gifts from Dr. Paul Knoepfler. T98G, U87-MG and U251 cells were authenticated by Arizona Research Labs (http://uagc.arl.arizona.edu/). Antibodies employed were anti-Vangl2 N-13, anti-Muc4 P-20, anti-Rac1, anti-EGFR (1005) from Santa Cruz, anti-FLAG M2, anti-tubulin, anti-actin AC-15 from Sigma, anti-HA 12CA5 for Ub-HA from Roche, anti-Myc 9E10 from Calbiochem, anti-V5 from Invitrogen, anti-RhoA from BD Biosciences, anti-FLRF/RNF41/Nrdp1 from Bethyl Laboratories, anti-HA C29F4 for Fzd7-HA western blots, anti-Dvl2, anti-phospho-JNK (T183/Y185), anti-phospho-Erk (T202/Y204), anti-phospho-Akt (S473), anti-phospho-β-catenin (Ser33/37/Thr41), anti-phospho-β-catenin (Ser552), and anti-β-catenin from Cell Signaling.

Transfection and transduction

HEK293T cells were transfected using 4μg polyethylenimine (Sigma) per μg DNA, and T98G cells were transfected with Lipofectamine 3000 (Life Technologies). HA-tagged ubiquitin (#17608), HA-tagged K48R and K63R ubiquitin mutants (#17604 and #17606), Wnt5a (#35911), and mouse Fzd7 (#42259) were from Addgene. K11 in HA-tagged ubiquitin was replaced with arginine using site-directed mutagenesis to generate the K11R mutant. Fzd7 was subcloned into pcDNA3.1(+) with a C-terminal HA-tag. Vangl1 and Vangl2 plasmids were from the Harvard PlasmID repository (HsCD00339551 and HsCD00294893) and subcloned into pcDNA3.1(+) with N-terminal FLAG and V5 epitope tags, respectively. Nrdp1-CHSQ-FLAG, Nrdp1-ΔCC (FLAG-tag removed by site-directed mutagenesis), and wild-type Nrdp1-V5 and Nrdp1-FLAG were described[15,29,33]. Plasmids encoding human Myc-Dvl2 and Myc-Dvl3 were gifts from Dr. Hiroaki Miki[45]. Wild-type mouse FLAG-Dvl1, FLAG-Dvl1-DIX-7KR and FLAG-Dvl1-PDZ-3KR were gifts from Dr. Madelon Maurice[46]. K409, K434, K458, K465, K472, and K482 in FLAG-Dvl1 were replaced with arginines using site-directed mutagenesis to generate FLAG-Dvl1-DEP-6KR.

The pMXpie-Nrdp1-FLAG, pMXpie-Nrdp1 (FLAG-tag removed by site-directed mutagenesis), pMXpie control, pSuper-shNrdp1-KD1 and -KD4 knockdown plasmids, and pSuper-scramble control have been described[16,47]. Plasmids were transfected into HEK-293-GPG cells to produce VSVG-pseudotyped retrovirus as described[16]. Stable cell lines were transduced with 12μg/mL protamine sulfate (MP Biomedicals), followed by drug selection with 1μg/mL puromycin (Sigma) for pMXpie or 200μg/mL neomycin (G418, VWR) for pSuper.

Human Nrdp1 shRNA pLKO.1 (#51, ID:TRCN0000034251; #53, ID:TRCN0000034253) and human Vangl1 shRNA pLKO.1 (#90, ID: TRCN0000062090; #92, ID:

TRCN0000062092) lentiviral constructs were from Open Biosystems, and scramble control pLKO.1 was from Addgene (#1864). VSVG-pseudotyped lentivirus was generated by transfecting HEK293T cells with psPax2 packaging vector. Cells were transduced with 10μg/mL polybrene (Millipore), followed by drug selection with 1μg/mL puromycin.

Gene expression and outcome analyses

Expression analysis was conducted using the REMBRANDT dataset (https://gdoc.georgetown.edu/gdoc/, access date: 07-06-2016) (X201962_s_at *NRDP1*, X229997_at *VANGL1*, X226029_at *VANGL2*, X213425_at *WNT5A* and X203706_s_at *FZD7*)[48]. Analysis of tumors of known molecular subtype was carried out using the TCGA subset included in Verhaak et al[23]. *P*-values were determined by Mann-Whitney-Wilcoxon test and box-plots were created using GraphPad Prism. Kaplan-Meier analysis was conducted using the REMBRANDT dataset and *P*-values were determined by logrank test using Prism.

PCR

Total RNA was isolated using the RNeasy Mini Kit (Qiagen) and DNaseI treated (Roche) according to manufacturer's protocol. The High-Capacity cDNA reverse transcription kit (Applied Biosystems) was used to make cDNA, and PCR analysis was performed using the following primer sets: Vangl1 (5′-GGACTCAAGCCACAACGAGTTGTAT-3′) and (5′-ACTACGAGGCTGAAGTCCAAGC-3′), and Vangl2 (5′-ATGAGCGGGATGACAACTGG-3′ and 5′-ACCTTGAGCGTGAACTGAGG-3′). Quantitative real time PCR was performed in a Bio-Rad iCycler CFX-96 real-time PCR machine using SsoAdvanced Universal Probes Supermix (BioRad) and TaqMan gene-specific primer/probe sets (Applied Biosystems). Levels for *VANGL1* (Hs01572998_m1), *WNT5A* (Hs00998537_m1), and *NRDP1/RNF41* (Hs00195064_m1) were normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*, 4352934E) levels for each sample.

Motility and invasion assays

Cells were plated on chambers with 8μm-pore polycarbonate membranes (Corning) and seeded in DMEM with 0.01% FCS, migrating overnight toward the lower chamber containing DMEM with 10% FCS. Migrated cells were fixed and stained with Diff-Quick staining solution (Dade Behring). For cell invasion assays, chambers were pre-coated with Matrigel (BD Bioscience). Cells that migrated or invaded were imaged and counted in five microscopic fields per filter on an Olympus IX81 microscope with cellSens Entry software. Results were normalized to proliferation rates and averaged among at least three independent experiments.

Organotypic brain slice invasion assays

Animal studies were conducted according to protocols approved by the IACUC of the University of California, Davis. Fresh brain tissue sections from wild-type FvB mice 12–15 weeks of age were prepared under sterile conditions immediately after sacrifice using a precision brain slicer (Braintree Scientific, Inc). High quality 1mm coronal tissue slices were cut in half (sagittal plane) in dissection media (minimum essential medium with glutamine, 1% penicillin/streptomycin, 50μg/mL gentamycin (Thermo), and 4.5mg/mL D-glucose

(Sigma)). The matching left and right hemisphere sections were then placed on sterile porous (0.4μm) membranes in a 12-well Transwell plate (Corning) and co-cultured with 250,000 U251 cells either expressing control pMX-GFP or pMX-Nrdp1-FLAG-IRES-GFP in brain culture media (50% dissection media, 25% Hanks' balanced salt solution, 25% horse serum, 1% penicillin/streptomycin, 50μg/mL gentamycin, 1x non-essential amino acids (Thermo)). Media was replaced in both the upper and lower chambers of the transwell plate after 2–3 days. At 5 days, the tissue was gently washed with PBS, fixed in 10% neutral buffered formalin, and embedded in 1% agarose for stability during histological processing and paraffin-embedding. Representative 5μm thick sections about 50, 100, and 200μm deep were processed for both H&E and anti-GFP (Clontech) IHC.

Co-immunoprecipitation, ubiquitination and blotting

Cells were lysed in RIPA buffer for ubiquitination and lipid strip assays or in co-immunoprecipitation buffer, and immunoprecipitations and immunoblotting were conducted as previously described[29].

Rho/Rac pulldowns

Active Rac and Rho were measured as described[49]. Briefly, cleared GBM cell lysates were incubated with glutathione beads bound with GST-Rhotekin-RBD or GST-PAK-PBD. Beads were washed with GTPase wash buffer, resuspended in SDS-DTT lysis buffer, and boiled supernatants immunoblotted.

Lipid strips

Cleared RIPA lysates from transfected HEK293T cells were incubated with anti-FLAG M2 affinity gel beads (Sigma) for 4hrs at 4°C. Beads were washed in RIPA buffer 5 times, then PBS, and FLAG-Dvl1 was eluted with 100μL FLAG-peptide (Sigma) solution (0.5mg/mL peptide, PBS, protease and phosphatase inhibitors) for 30 minutes at 4°C. Beads were removed and a fraction of eluate was saved. Purified protein was re-suspended in 2mL blocking buffer (1x PBS-T (0.1% Tween-20), 3% BSA) with inhibitors, and added to blocked membrane lipid strips (Echelon) for 1hr at room temperature. Membranes were washed in PBS-T, treated with primary and secondary antibody, and developed. The amount of FLAG-Dvl1 bound to lipid was normalized to the amount eluted.

Wnt5a stimulation

Conditioned media was produced by transfecting HEK293T cells with vector or Wnt5a-containing plasmid. After 72hrs, conditioned media was collected, cleared of debris, and stored at −80°C. T98G cells were serum-starved in 0.01% FCS overnight, then treated with conditioned media for 1hr to stimulate Wnt signaling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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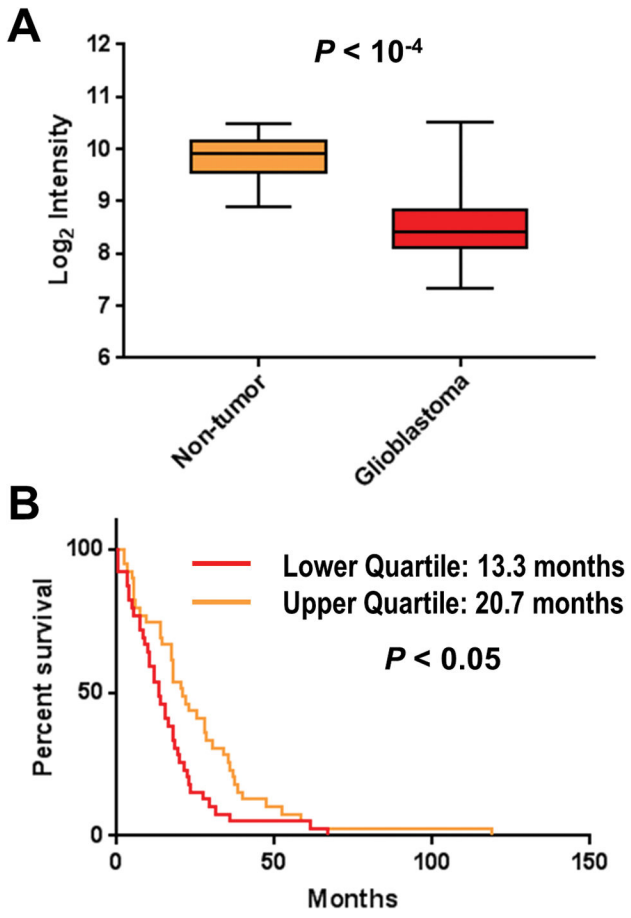

Figure 1. Nrdp1 expression is decreased in GBM

(A) Microarray analysis of *NRDP1* transcript levels in non-tumor brain and GBM tissues from REMBRANDT (n = 28 non-tumor and 188 GBM samples; $P < 10^{-4}$ by Mann-Whitney test). Boxes represent the 2nd and 3rd quartiles, while whiskers extend to the maximum and minimum values. Low numbers of normal patient samples are likely related to difficulties in obtaining healthy human brain tissue. **(B)** Kaplan-Meier plot of GBM patient survival in the upper and lower quartiles of *NRDP1* expression from REMBRANDT (n = 155 GBM samples, $P < 0.05$ by logrank test). Median survival times were 20.7 months and 13.3 months, respectively, for upper and lower quartile patients.

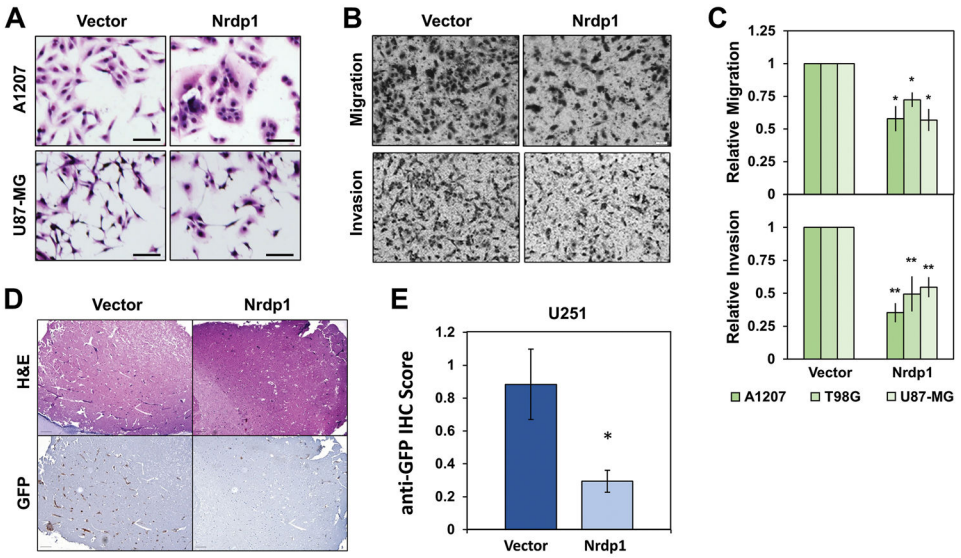

Figure 2. Nrdp1 restoration inhibits GBM cell migration

(A) A1207 and U87-MG cells transduced with either vector or Nrdp1 stained with Diff-Quick and visualized microscopically (scale bar = 100 μm). **(B)** Representative images of migrated (top) or invaded (bottom) vector- or Nrdp1-transduced T98G cells stained with Diff-Quick in Boyden chambers. **(C)** Relative migration in Boyden chamber assays (top) and invasion in Matrigel-coated Boyden chamber assays (bottom) is quantified over three or four independent experiments for vector- or Nrdp1-transduced cells. **(D)** Dissemination of U251 cells transduced with vector or Nrdp1 through cultured mouse brain slices, analyzed histologically (upper) and by immunohistochemical staining for GFP (lower). **(E)** Quantification of U251 tumor cell invasion by blinded scoring of the anti-GFP sections, analyzed by paired Mann-Whitney test.* $P < 0.05$, ** $P < 0.005$ by student's t-test.

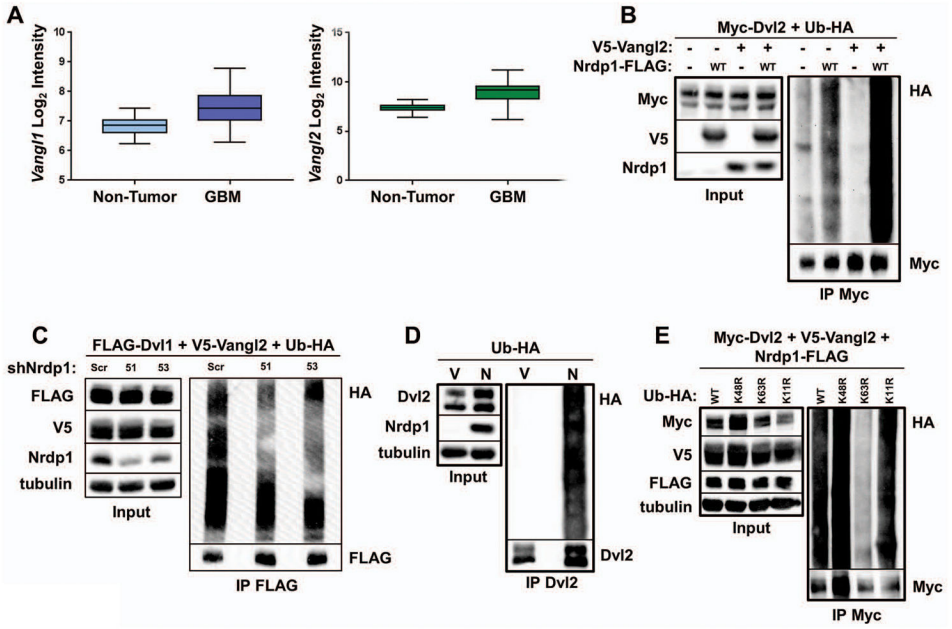

Figure 3. Nrdp1 promotes Vangl-dependent K63-linked polyubiquitination of Dvl proteins

(A) Microarray analyses of *VANGL1* (left panel) and *VANGL2* (right panel) transcript levels in REMBRANDT (n = 28 non-tumor and 188 GBM samples). $P < 10^{-4}$ by Mann-Whitney test, boxes represent the 2nd and 3rd quartiles and whiskers extend to the maximum and minimum values. **(B)** HEK293T cells were transfected as indicated, and anti-Myc precipitates were blotted for ubiquitinated Myc-Dvl2. **(C)** Anti-FLAG precipitates from HEK293T cells transfected as indicated were blotted for ubiquitinated FLAG-Dvl1. **(D)** T98G cells were transfected with Ub-HA along with either vector (V) or Nrdp1 (N), and anti-Dvl2 precipitates were blotted for ubiquitinated Dvl2. **(E)** Anti-Myc precipitates from transfected HEK293T cells were blotted for ubiquitinated Myc-Dvl2.

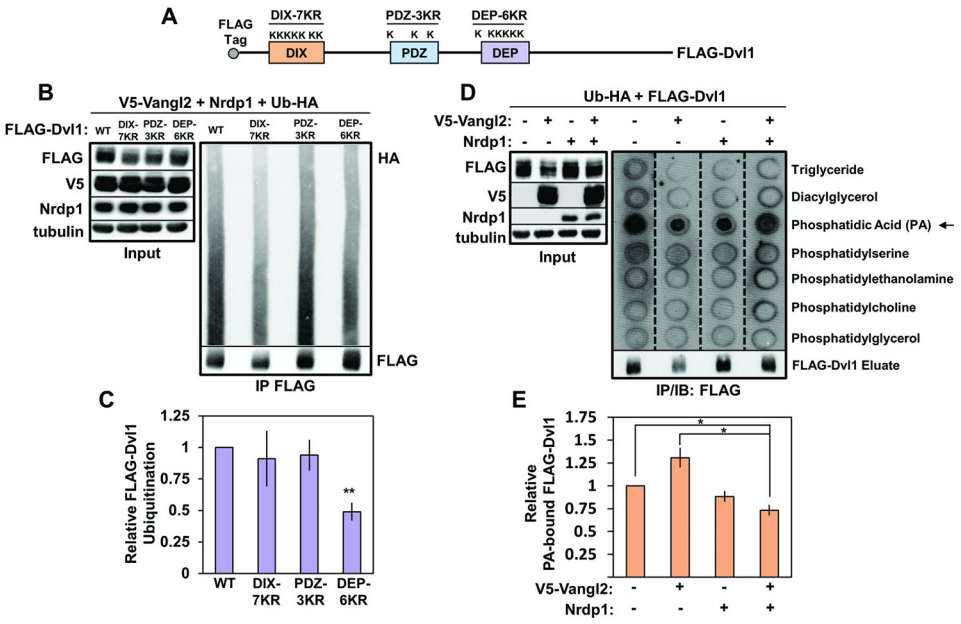

Figure 4. Nrdp1-mediated ubiquitination of the Dvl1 DEP domain inhibits its binding to phosphatidic acid

(A) The domain structure of Dvl proteins, highlighting the mutated Dvl1 lysine residues employed in experiments. **(B)** Anti-FLAG precipitates from HEK293T cells transfected as indicated were blotted for ubiquitinated Dvl1. **(C)** Results from six independent experiments such as that in (B) were quantified. **(D)** FLAG-Dvl1 affinity purified from HEK293T cells transfected as indicated was overlaid onto lipid strips and blotted with anti-FLAG to reveal lipid binding. Dashed lines separate images from a single exposure. **(E)** Three independent experiments such as that in (D) were quantified. Relative binding refers to the quantity of PA-bound FLAG immunoreactivity relative to the amount of FLAG-Dvl1 in the eluate. Error bars represent SEM. *, $P < 0.05$; **, $P < 0.005$ by student's t-test.

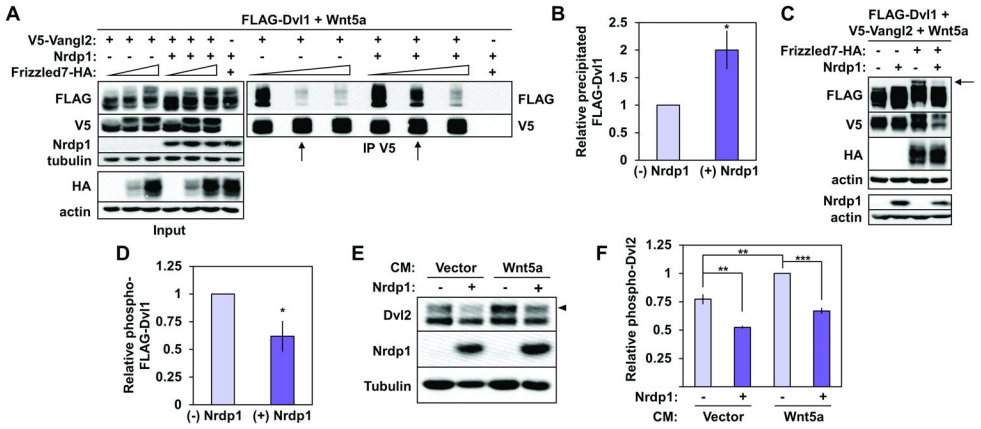

Figure 5. Nrdp1 suppresses Wnt5a/Fzd7-mediated Dvl1 activation by promoting Dvl1/Vangl2 complex formation

(A) Anti-V5 precipitates from HEK293T cells transfected with increasing levels of Fzd7-HA were blotted as indicated. **(B)** The amount of FLAG-Dvl1 associated with V5-Vangl2 under the conditions marked by arrows (A) was quantified over four independent experiments. **(C)** Lysates from HEK293Ts transfected as indicated. The band marked with the arrow represents active phosphorylated FLAG-Dvl1. **(D)** Phosphorylated FLAG-Dvl1 was quantified over five independent experiments such as that in (D). **(E)** Lysates from vector- or Nrdp1-transduced T98G cells treated with Wnt5a conditioned media. The arrow indicates phosphorylated Dvl2. **(F)** Phosphorylated Dvl2 from four independent experiments such as that in (E) was quantified. Error bars represent SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$ by student's t-test.

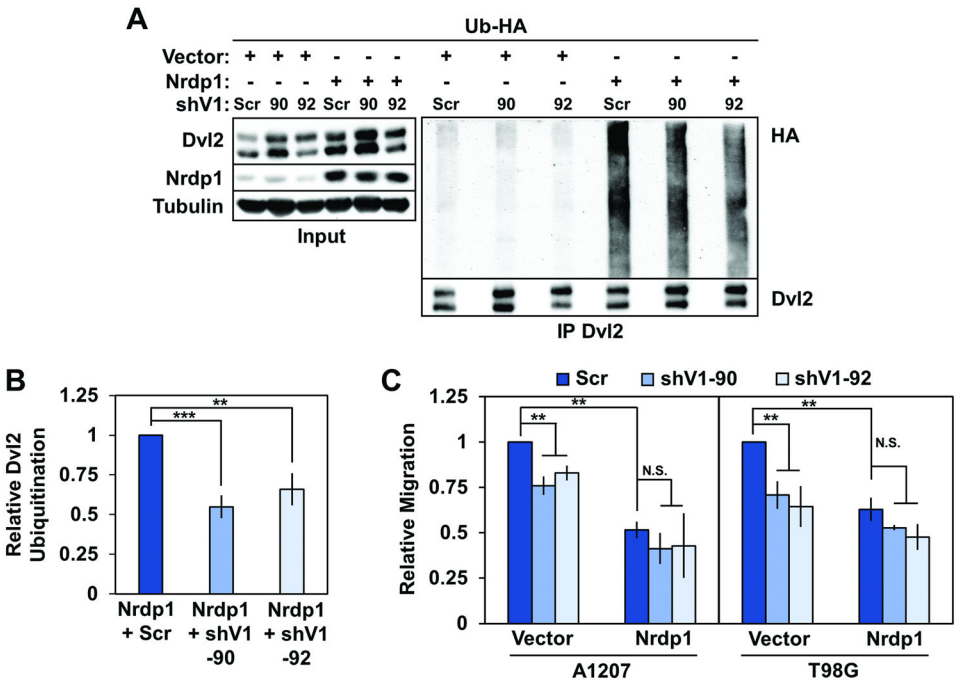

Figure 6. Nrdp1-mediated Dvl ubiquitination and inhibition of GBM migration are Vangl-dependent

(A) T98G cells transduced with scrambled (Scr) or two *VANGL1*-targeting shRNAs (90, 92) were transfected as indicated. Anti-Dvl2 precipitates were blotted for ubiquitinated endogenous Dvl2. **(B)** Dvl2 ubiquitination in the presence of Nrdp1 was quantified across four (90) or six (92) independent experiments such as that in (A). **(C)** The migration of glioma cells was assessed with and without restored Nrdp1, as well as the presence and absence of *VANGL1*-targeting shRNAs, and quantified over three to five independent determinations. Error bars represent SEM. **, $P < 0.01$; ***, $P < 0.005$, by student's t-test.

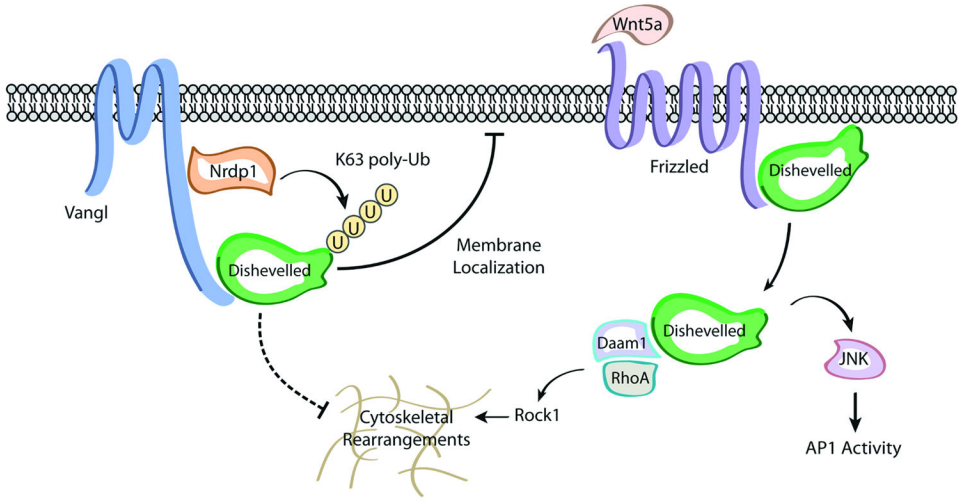

Figure 7. Model of Vangl-dependent ubiquitination of Dvl by Nrdp1

Wnt5a ligand engagement of Fzd receptor causes the recruitment, phosphorylation and activation of Dvl to initiate cytoskeletal rearrangements via Rho GTPase, and transcriptional regulation through phospho-JNK and AP1. The presence of Nrdp1 leads to the Vangl-dependent K63-polyubiquitination of Dvl, inhibiting Dvl recruitment to Fzd and the plasma membrane, suppressing downstream signaling.