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PLEKHA4 Promotes Wnt/β-catenin Signaling-Mediated G1/S Transition and Proliferation in Melanoma

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Abstract

Despite recent promising advances in targeted therapies and immunotherapies, melanoma patients incur substantial mortality. In particular, inhibitors targeting BRAF-mutant melanoma can lead to resistance, and no targeted therapies exist for NRAS-mutant melanoma, motivating the search for additional therapeutic targets and vulnerable pathways. Here we identify a regulator of Wnt/β-catenin signaling, PLEKHA4, as a factor required for melanoma proliferation and survival. PLEKHA4 knockdown in vitro decreased Dishevelled levels, attenuated Wnt/β-catenin signaling, and blocked progression through the G1/S cell cycle transition. In mouse xenograft and allograft models, inducible PLEKHA4 knockdown attenuated tumor growth in BRAF- and NRAS-mutant melanomas and exhibited an additive effect with the clinically used inhibitor encorafenib in a BRAF-mutant model. As an E3 ubiquitin ligase regulator with both lipid and protein binding partners, PLEKHA4 presents several opportunities for targeting with small molecules. Our work identifies PLEKHA4 as a promising drug target for melanoma and clarifies a controversial role for Wnt/β-catenin signaling in the control of melanoma proliferation.

INTRODUCTION

Melanoma is the most aggressive and deadliest form of skin cancer. The root cause of most melanomas is somatic mutations in a relatively small number of genes (1). Roughly 65% of melanoma cases feature a V600D/E mutation in the Ser/Thr kinase BRAF, and an additional 10% feature a Q61K/R mutation in the GTPase NRAS (2). These genetic alterations cause phenotypic changes, including elevated signaling through MAP kinase, PI 3-kinase, and other related pathways, which lead to increased cell proliferation, differentiation, and ultimately tumorigenesis and malignancy (3).

Inhibitors of BRAF or the downstream kinase MEK heralded an era of targeted therapies for BRAF-mutant melanomas (4,5). Nonetheless, resistance typically occurs in roughly one year, leading to relapse, and no targeted therapies exist for NRAS-mutant melanomas (6). Further, immunotherapies, such as checkpoint inhibitors, have more long-lasting effects but

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are only successful in a subset of patients (7). Combinations of BRAF targeted therapies and anti-PD1 immunotherapies are promising avenues but are still not universally effective (8). Thus, new therapeutic strategies are needed to prevent melanomagenesis and progression.

Wnt/β-catenin signaling, which regulates proliferation, is aberrantly hyperactive in several cancers, including melanoma (9). In the canonical, β-catenin-dependent form of this pathway, secreted Wnt ligands engage a receptor from the Frizzled family in the plasma membrane of the Wnt-receiving cell (10). This binding event causes recruitment of Dishevelled (DVL), which mediates disassembly of a multicomponent β-catenin destruction complex, resulting in β-catenin stabilization, nuclear translocation, and altered gene expression at several loci, most notably those associated with the TCF/LEF transcription factor family. In cancer, aberrant Wnt/β-catenin signaling leads to increased expression of Wnt/β-catenin target genes including Cyclin D1 and c-Myc, which regulate progression through the G1/S transition of the cell cycle, helping to promote proliferation, tumorigenesis, and malignancy (9).

Wnt signaling pathways have been linked to melanoma, but their exact roles remain controversial (9,11–13). Wnt/β-catenin signaling has been shown to promote melanoma tumor initiation and growth in both BRAF and NRAS mutant backgrounds (14–17). Further, a recent study using a new engineered mouse model implicated Wnt signaling in the transformation of healthy melanocyte stem cells to melanoma in a BRAF and PTEN mutant background (18). As well, BRAF inhibition is more effective in settings with lower levels of β-catenin (19). Yet, elevated levels of nuclear (active) β-catenin have correlated with diverging patient survival, depending on the study (12,20–23). Beyond the controversial roles of Wnt/β-catenin signaling in melanoma, β-catenin-independent non-canonical Wnt signaling controls actin cytoskeletal dynamics and cell migration and has been implicated in melanoma metastasis (24,25). In fact, melanoma progression has been proposed to involve a phenotype switching model wherein the canonical and non-canonical pathways alternate to allow cells to switch between proliferative and migratory phenotypes (9,26). Thus, Wnt signaling pathways appear to be important players in melanoma progression in most contexts and are thus a potential point of therapeutic intervention.

Numerous efforts have been made to drug Wnt signaling in cancer (23,27). These efforts have largely focused on inhibiting core Wnt components (e.g., PORCN, FZD, β-catenin/CBP) (28). Though efficacious in model systems, they have seen limited success in vivo due, in part, to undesirable side effects on homeostatic Wnt signaling in non-diseased tissues (29). Fortunately, Wnt/β-catenin signaling is subject to many levels of regulation, and though core Wnt components are typically essential due to important roles in development and tissue homeostasis, many modulators, or tuners, of Wnt signaling strength may not be required for viability (10,27,30). Thus, it is a high priority to identify modulators of Wnt signaling, whose inhibition downregulates but does not completely eliminate Wnt signaling, as potential therapeutic targets.

Among the many factors involved in Wnt signaling, DVL has emerged as a major point of regulation (31). Several different E3 ubiquitin ligases act on DVL, modulating its levels and thus changing the strength of the Wnt signal in Wnt-receiving cells (32–35). To this end, we

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recently discovered that the phosphoinositide-binding protein PLEKHA4 (pleckstrin homology containing family A, number 4) modulates the activity of the CUL3–KLHL12 E3 ligase that polyubiquitinates DVL (36,37). PLEKHA4 acts to sequester the substrate-specific adaptor KLHL12 within plasma membrane-associated clusters, thus reducing DVL ubiquitination, increasing DVL levels, and enhancing Wnt/β-catenin signaling in mammalian cells. Thus, PLEKHA4 acts as a tuner for DVL levels and Wnt signaling strength, as near-complete elimination of PLEKHA4 resulted in only partial DVL depletion and attenuation of Wnt signaling.

Intriguingly, PLEKHA4 expression is high in melanoma but its levels are low in healthy melanocytes (2,38). We were thus motivated to test whether PLEKHA4 is an important factor for promoting pathological Wnt signaling in melanoma, as a step toward both validating Wnt/β-catenin signaling in general, and PLEKHA4 in particular, as therapeutic targets in melanoma. Here, we report that melanoma cells from both BRAF and NRAS mutant backgrounds require PLEKHA4 for survival and proliferation in vitro and in vivo in mouse xenograft and allograft models. Depletion of PLEKHA4 by siRNA and shRNA led to attenuated Wnt signaling in these models and phenocopied inhibitors or siRNA knockdown of core Wnt components. Further, inducible PLEKHA4 knockdown in the presence of the clinically used BRAF V600D/E inhibitor encorafenib (39) displayed an additive effect in a xenograft model of BRAF-mutant melanoma, suggesting the therapeutic potential of targeting PLEKHA4 in melanoma. This work highlights PLEKHA4 as a new modulator of Wnt/β-catenin signaling strength in melanoma that, by promoting the G1/S cell cycle transition, maintains cell proliferation in melanoma. Importantly, our study provides additional clarity on the pathological role of Wnt/β-catenin signaling in this disease and suggests that pharmacological inhibition of PLEKHA4 could represent a promising new avenue for targeted therapy in melanoma.

MATERIALS AND METHODS

Cell culture

All cell lines (Supplemental Methods) were obtained from ATCC except for WM266–4 and SK-MEL-2 (NCI PSOC) in 2017 and used without further authentication. Cells were tested yearly for mycoplasma (MycoSensor, Agilent) and were grown for 7 d after thawing prior to use.

Cell proliferation assays

SiRNA (50 nM) against PLEKHA4, DVL2, and DVL3 was performed overnight on WM266–4 or SK-MEL-2 cells on a 6-well plate. After 16 h, cells were lifted, and 4000 cells were seeded in each well of a low-evaporation 96-well plate. For Wnt inhibition experiments, cells were seeded in media containing either DMSO vehicle or 2.5 μM IWP-4 (Inhibitor of Wnt Production-4). Images were acquired every hour for at least 4 d in an IncuCyte incubator (20X objective).

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Anchorage-dependent colony formation assays

SiRNA (50 nM) against PLEKHA4, DVL2, and DVL3 was performed overnight on WM266–4 or SK-MEL-2 cells in a 6-well plate. After 16 h, cells were lifted, and 4000 cells were plated evenly in each well of a 6-well plate. Fresh media was changed every 3 d. For Wnt inhibition experiments, 4000 untreated cells were plated in media containing either DMSO or IWP-4 (2.5 μM). The cells were grown for two weeks until colonies were observed. Cells were washed with PBS, fixed with methanol for 1 h at room temperature, and stained overnight with 0.1% crystal violet in 95% ethanol. The lids were propped open slightly to allow the stain solution to evaporate overnight. Plates were then rinsed gently with cold water to remove excess stain and allowed to dry for 3 h. Images were acquired with a Bio-Rad ChemiDoc, and colonies were counted using ImageJ.

Anchorage-independent colony formation assays

SiRNA (50 nM) against PLEKHA4, DVL2, and DVL3 was performed overnight on WM266–4 or SK-MEL-2 cells in a 6-well plate. After 16 h, cells were lifted, and 5000 cells were plated evenly in each well of a 6-well plate. The soft agar assay was set up as described previously (40). Three weeks after the seeding, colonies were observed and stained overnight at 37 °C with nitrotetrazolium blue (1 mg/mL in PBS). Images were acquired with a Bio-Rad Chemidoc, and colonies were counted using ImageJ.

Cell cycle analysis

Unsynchronized: For cell cycle analysis in unsynchronized WM266–4 cells, siRNA (50 nM) against PLEKHA4 was performed in a 12-well plate. After 48 h, cells were lifted, fixed overnight with prechilled ethanol, and stained using propidium iodide as described previously (41), and analyzed by flow cytometry.

Synchronized: Stable cells expressing FUCCI (see Supplementary Methods) were seeded on a 15-cm dish, grown to 90% confluence, and starved with FBS-free media for 48 h. SiRNA (50 nM) against PLEKHA4 was added for the final 16 h of serum starvation. Cells were then stimulated by addition of fresh FBS-containing media for 36 h, and then lifted and fixed overnight with pre-chilled ethanol at 4 °C. Cells were washed three times with FACS buffer (0.1% FBS in PBS) and analyzed via flow cytometry.

Rescue: Media containing lentivirus encoding rescue constructs (GFP, PLEKHA4-GFP, DVL2-GFP, DVL3-GFP, or a combination of DVL2-GFP and DVL3-GFP) was generated (see Supplementary Methods). RNAi against PLEKHA4 was performed as above on a 60-mm plate, and 16 h post RNAi, cells were stimulated with rescue media (a mix of 1.5 mL of fresh media and 2.5 mL of virus-containing rescue media). After 32 h, cells were harvested and fixed overnight with prechilled ethanol at 4 °C. Cell cycle analysis by flow cytometry was performed either by propidium iodide on wild-type cells or FUCCI-expressing stable lines, quantifying fraction of cells in G1.

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Tumor xenograft and allograft studies of PLEKHA4 shRNA

Stable cell lines with doxycycline-inducible PLEKHA4 or control shRNA were generated (Supplementary Methods). One day before the cell injections, the dorsal sides of mice (4–6 week-old) were shaved to enable four injections per animal, two each near the upper and lower flanks. On injection day, cells were lifted, and resuspended in media containing 1% penicillin/streptomycin. A 1:1 mixture of cells:Matrigel was made, and 1×10^6 of shRNA-expressing WM266–4 or SK-MEL-2 cells were injected subcutaneously into NSG mice using a 28-gauge needle. The same procedure was used for shRNA-expressing YUMM1.7 cells except that 1×10^5 cells were injected subcutaneously into C57BL/6J mice. Injections were performed within 30 min of preparing the cells/Matrigel mixture. Mice were monitored every 2 d. For WM266–4 and YUMM1.7 xenografts, tumor formation appeared at day 12, whereas for SK-MEL-2, the tumor formation appeared at 1.5 months post injection. To induce shRNA expression, doxycycline (1 mg/mL in sterile water) was added to the drinking water in amber bottles and changed every 2 d (WM266–4: 12 d; YUMM1.7: 10 d; SK-MEL-2: 16 d). Tumor progression was measured every 2 d with a digital caliper, with volume calculated using $v = 0.5233*l*w^2$. All mouse studies were approved by the Cornell Institutional Animal Care and Use Committee.

Tumor xenograft studies of PLEKHA4 shRNA combined with BRAFi treatment

PLEKHA4 or control shRNA-expressing WM266–4 cells (1×10^6) were injected subcutaneously as described above. Mice were monitored every 2 d. Tumors appeared at day 12. Doxycycline (1 mg/mL in sterile water) was then added to the drinking water to induce shRNA as described above, and, concurrently, encorafenib or vehicle was administered daily via oral gavage (30 mg/kg in 0.5% carboxymethylcellulose and 0.05% Tween-80 in PBS, freshly prepared) for 12 d. Tumor progression was monitored as described above. At the end of 12 d of encorafenib treatment, the encorafenib treatment was terminated but doxycycline was continued for another 14 d. Tumor progression was monitored every 2 days, and volumes were calculated as described above.

Luciferase Wnt reporter assays

Generation of cells stably expressing Wnt reporters: WM266–4 or SK-MEL-2 cells were co-transduced with lentiviruses expressing Firefly luciferase-7TFP (Addgene #24308) and Renilla luciferase pLenti.PGK.blast-Renilla_Luciferase (Addgene #74444) (Supplementary Methods). After 48 h, puromycin dihydrochloride (2.5 μg/mL) and blasticidin S hydrochloride (2 μg/mL) selection was performed until resistant colonies appeared. These reporter cell lines were used in siRNA-based Wnt reporter luciferase assays below.

Transient knockdown: SiRNA-mediated knockdown against PLEKHA4 was performed in Wnt/β-catenin luciferase reporter-expressing WM266–4 and SK-MEL-2 cells on 6-well plates. After 30 h of cell growth post-transfection, cells were treated with sterile-filtered, Wnt3a-containing conditioned media in a 1:1 ratio with fresh media for 30 h. Cells were then lysed, and 150 μL of lysates were transferred to an opaque 96-well flat-bottom plate (Greiner) for measuring chemiluminescence. Firefly luciferin substrate (50 μL of a 470 μM

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stock solution) was added to each well, and the firefly luciferase signal was measured by a Tecan plate reader. Subsequently, Renilla luciferase substrate (50 µL of a 5.5 µM stock solution also containing 25 µM of the firefly luciferase inhibitor 4-(6-methyl-1,3-benzothiazol-2-yl)-aniline (Enamine.net)) was added to each well, and the Renilla luciferase signal was measured.

Stable knockdown: ShRNA expression against PLEKHA4 in WM266–4 and SK-MEL-2 cells was induced by addition of 2.5 µg/mL doxycycline for 10 d in 6-well plates. As a negative control, stable cells bearing inducible Renilla shRNA were treated in the absence of doxycycline. Doxycycline-containing media was exchanged for fresh media every 2 d. On day 8, cells were treated with a 1:1:1 mixture of 7TFP lentivirus-containing conditioned media:PGK-Renilla lentivirus-containing conditioned media:fresh media, and 8 µg/mL polybrene and 2.5 µg/mL doxycycline for 24 h. Spent media was exchanged for fresh 1:1:1 media mixture as described above every 12 h. On day 9, Wnt signaling was induced by adding Wnt3a-containing conditioned media in a 1:1 ratio with fresh media containing doxycycline for 30 h. Firefly and Renilla luciferase signals were then measured as described above.

Data availability statement

The authors declare that all data supporting the findings of this study are available within the paper and its supporting information files.

RESULTS

PLEKHA4 knockdown blocks proliferation and increases apoptosis in melanoma cells

In the course of earlier work in HeLa cells, we noticed that PLEKHA4 knockdown by siRNA had mild qualitative effects on cell proliferation and viability (36). We reasoned that cancer cells expressing the highest levels of PLEKHA4 might be more sensitive to its loss. Analysis of patient gene expression data in the TCGA database revealed widespread expression of PLEKHA4 in many types of cancers, but, relative to other cancers, PLEKHA4 levels were highest in melanoma (Figure 1A) (2). This high expression of PLEKHA4 in melanoma was independent of genotype, across 121 different melanoma cell lines (Figure S1A), and of melanoma subtype (e.g., cutaneous vs. non-cutaneous) in 259 primary tumor samples (Figure S1B). In healthy melanocytes, however, PLEKHA4 levels were low, as analyzed in the Genevestigator database (38). With a working hypothesis that PLEKHA4 might be an important factor in melanomagenesis and progression, we examined its requirement for proliferation and survival in two melanoma cell lines: WM266–4, a BRAF V600D mutant line, and SK-MEL-2, an NRAS Q61R mutant line.

We validated several PLEKHA4 siRNA duplexes (Figure 1B and Table S1) and examined effects of PLEKHA4 knockdown using automated, continual monitoring of cell number on IncuCyte system, wherein images were acquired every hour for 100–150 hours. We observed a strong reduction of cell proliferation upon PLEKHA4 knockdown in both cell lines, using multiple siRNA duplexes (Figure 1B–C and Table S2). Examination of the images suggested substantial cell death was occurring, and indeed, Western blot analysis of lysates from these

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cells revealed that PLEKHA4 knockdown caused increases in levels of cleaved PARP and activated caspase 3, two markers of apoptosis (Figure 1D). Interestingly, overexpression of PLEKHA4-GFP in WM266–4 cells resulted in a modest increase in proliferation relative to control (Figure S1C).

PLEKHA4 promotes Wnt/β-catenin signaling in melanoma cells

Given the role of PLEKHA4 as a positive regulator of Wnt/β-catenin signaling in other cells (36), we next investigated effects of PLEKHA4 knockdown on Wnt signaling in the context of melanoma. We found that siRNA-mediated PLEKHA4 knockdown led to reduced levels of DVL2 and DVL3, the two major DVL isoforms in both the BRAF and NRAS mutant melanoma cell lines (Figure 1E–F). To further reinforce the generality of this finding, we also determined that *Plekha4* knockdown reduces DVL2 and DVL3 levels in YUMM1.7 cells, a mouse melanoma cell line derived from a genetically engineered mouse model bearing several mutations commonly found within melanoma, including BRAF V600E, as well as inactivating mutations in PTEN and CDKN2A (Figure S2A–C) (42). We then examined effects on Wnt/β-catenin signaling using two approaches. First, PLEKHA4 knockdown led to a >50% decrease in luminescence from the two human melanoma cell lines that were engineered to stably express a β-catenin-dependent luciferase transcriptional reporter (TOPFlash) and then stimulated with Wnt3a (Figure 1G). Second, we found that PLEKHA4 knockdown in cells stimulated with Wnt3a led to reduced levels of Axin2, whose expression is induced by canonical Wnt/β-catenin signaling, by Western blot (Figure 1E–F).

To complement these studies on PLEKHA4 knockdown, we examined whether perturbing Wnt signaling via two distinct mechanisms would similarly affect viability and proliferation of these melanoma cells. First, we used a pan Wnt inhibitor (IWP-4) that targets Porcupine, an O-acyltransferase that installs a posttranslational modification that is required for their secretion from Wnt-producing cells and thus for Wnt signaling (43). We found that IWP-4 treatment led to a drastic cell proliferation defect in both the cell lines (Figure S3A–B). Second, we performed siRNA-mediated knockdown of DVL2 or DVL3, the direct mechanistic targets of PLEKHA4 action (36), and found similar effects on cell proliferation in both human melanoma cell lines (Figure S3C–D). Further, Western blot analyses on DVL2 or DVL3 knockdown samples revealed increases in the levels of cleaved PARP and activated caspase 3, suggesting increases in apoptosis similar to PLEKHA4 knockdown (Figure S3E). Together, these data indicate that PLEKHA4 acts as a positive modulator of Wnt/β-catenin signaling in melanoma and suggests that it mediates cell survival and proliferation in melanoma.

PLEKHA4 regulates the G1/S transition and melanoma cell proliferation

A major role of Wnt/β-catenin signaling is to stimulate proliferation by promoting progression through the G1/S cell cycle transition. The effects of PLEKHA4 and Wnt perturbation on cell growth curves suggested an effect on proliferation, and we next examined whether the mechanism of action of PLEKHA4 occurred via perturbing the cell cycle. First, we analyzed the cell cycle phase of asynchronous WM266–4 cells treated with either control or two different PLEKHA4 siRNA duplexes and stained fixed cells with propidium iodide to measure DNA content. We found that PLEKHA4 knockdown led to an

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accumulation of cells in the G1 phase (Figure 2A). Importantly, this PLEKHA4 knockdown-induced G1/S transition defect could be rescued by introduction of an siRNA-resistant form of PLEKHA4 via lentiviral transduction (Figure 2B). Intriguingly, effects of PLEKHA4 knockdown could also be substantially, but not completely, rescued by overexpression of DVL2 or DVL3, the downstream targets of PLEKHA4 (Figures 2B and S4), suggesting that the established mechanism of action of PLEKHA4 on DVL proteins, via effects on their ubiquitination by CUL3–KLHL12 (36), accounts for a major portion of the effects of PLEKHA4 knockdown in these melanoma cells, though there are likely additional DVL-independent effects, discussed below.

To examine the G1/S phenotype in more detail, including its dynamics, we used the fluorescent ubiquitination-based cell cycle indicator (FUCCI) system, a live cell-compatible, dual color reporter that wherein cells in G1 phase express mRFP (red) and cells in S, G2, or M phase express GFP (green). We generated WM266–4 and SK-MEL-2 cell lines stably expressing the FUCCI reporters and synchronized either control or PLEKHA4 knockdown cells to G1 using serum starvation (44). Upon release from this G1 arrest by addition of serum, we found that, for both cell lines, PLEKHA4 knockdown caused an increase in retention in G1 phase, i.e., a failure to progress to S phase (Figures 2C–D and S4).

To complement this phenotypic characterization of G1/S defects, we examined levels of Cyclin D1 and c-Myc, two well-studied transcriptional targets of Wnt/β-catenin signaling that affect the G1/S cell cycle transition (45,46). In asynchronous populations of WM266–4 or SK-MEL-2 cells, we found that PLEKHA4 knockdown led to decreased levels of both Cyclin D1 and c-Myc in the two human melanoma cell lines (Figure 3A–B) and in YUMM1.7 cells (Figure S2A). Further, PLEKHA4 knockdown on G1-synchronized cells (via serum starvation) led to a similar decrease in the levels of Cyclin D1 and c-Myc, as well as DVL2 and DVL3 in WM266–4 or SK-MEL-2 cells (Figures 3C–D) and YUMM1.7 cells (Figure S2B). The decrease in the levels of these proteins induced by PLEKHA4 knockdown could be substantially rescued by lentiviral transduction with an siRNA-resistant form of PLEKHA4-GFP (Figure 3E). Interestingly, whereas the rescue of c-Myc levels was near-complete, the rescue of Cyclin D1 levels was only partial, suggesting other uncharacterized effects in this instance.

In the same experiment, we found that the decrease in levels of Cyclin D1 and c-Myc induced by PLEKHA4 knockdown could also be substantially, but not completely, rescued — again, partially for Cyclin D1 and completely for c-Myc — by expression of DVL2-GFP, DVL3-GFP, or a combination of DVL2-GFP and DVL3-GFP (Figure 3E). Similar overexpression of DVL proteins could also partially rescue the induction of apoptosis markers, cleaved PARP and activated caspase 3, caused by PLEKHA4 knockdown (Figure S5A–B). Though the extent of reversal of these PLEKHA4 knockdown phenotypes by DVL overexpression in these experiments was substantial, further supporting the proposed mechanism of action, it was not complete, indicating additional DVL-independent effects of PLEKHA4 knockdown in melanoma cells, discussed below. Finally, to complement these findings, we found that DVL2 or DVL3 knockdown led to the same effects on Cyclin D1 and c-Myc levels in both human melanoma cell lines (Figure 3F) and in YUMM1.7 cells (Figure S2C). Overall, these data indicate that decreasing PLEKHA4 levels in melanoma

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leads to a Wnt/β-catenin-mediated G1/S cell cycle transition defect largely via effects on the key proliferation markers Cyclin D1 and c-Myc.

PLEKHA4 is required for tumorigenic and malignant properties in melanoma in vitro

The above molecular and phenotypic data implicate PLEKHA4 as a novel modulator of Wnt signaling in melanoma whose removal causes defects in cell cycle progression and proliferation. We therefore envisioned that loss of PLEKHA4 in melanoma cells might attenuate cancer-causing properties in vitro such as clonogenic capacity, or the ability of a single cell to proliferate into a colony.

We first examined effects of PLEKHA4 knockdown on the anchorage-dependent clonogenic capacity of melanoma cells, using crystal violet staining of colonies derived from single cells grown on traditional 2D cell culture surfaces. PLEKHA4 knockdown in both WM266–4 and SK-MEL-2 cell lines led to substantial losses in clonogenic capacity (Figure 4A). Further, a similar effect was observed upon inhibition of Wnt signaling via other mechanisms, including knockdown of DVL2 or DVL3 (Figure 4B) and IWP-4 treatment (Figure 4C). In these assays, though the effects on anchorage-dependent clonogenic capacity were significant for all perturbations relative to control, we noticed a stronger effect for all siRNA experiments in the BRAF-mutant, WM266–4 cells compared to the NRAS mutant, SK-MEL-2 cells, similar to effects observed in proliferation assays (Figure 1B–C).

To evaluate tumorigenic potential of malignant cells grown in a soft substrate that better mimics the tumor environment, we employed an anchorage-independent colony formation assay (40). Here, colony formation was measured after seeding cells in a 3D soft agar environment, followed by nitrotetrazolium blue staining. We found that PLEKHA4 knockdown in both melanoma cell lines strongly, and roughly equivalently, reduced anchorage-independent growth capacities (Figure 4D). Again, inhibition of Wnt signaling via DVL2 or DVL3 knockdown led to decreases in anchorage-independent growth (Figure 4E). The effect of DVL2 knockdown was stronger than DVL3 knockdown, suggesting a greater dependence on DVL2 in this setting. Collectively, these data indicate that loss of PLEKHA4 causes a drastic decrease in tumorigenic and malignant properties in BRAF and NRAS mutant melanoma in vitro.

PLEKHA4 knockdown attenuates melanoma tumor growth in vivo

Buoyed by the in vitro data implicating PLEKHA4 as a factor required for melanoma cell proliferation, we next tested whether PLEKHA4 played a similar role in vivo. Here, we used two different types of mouse models. First, we established xenografts in immunocompromised NOD *scid* gamma (NSG) mice using WM266–4 and SK-MEL-2 cells, the BRAF- and NRAS-mutant human melanoma cell lines that we had used for the in vitro studies above. Separately, to assess effects of PLEKHA4 knockdown within wild-type mice, we established allografts in C57BL6.J mice using the syngeneic, engineered YUMM1.7 mouse melanoma cell line (42).

For these in vivo experiments, we established PLEKHA4 knockdown by generating cell lines stably expressing a doxycycline-inducible shRNA against human PLEKHA4 and mouse *Plekha4*. To accomplish this, we generated stable cell lines expressing different

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shRNA constructs against human PLEKHA4 in WM266–4 cells (Figure S6A–B) and mouse *Plekha4* in YUMM1.7 cells (Figure S7). Cells were grown in vitro, PLEKHA4/*Plekha4* knockdown was induced by addition of doxycycline, and Western blot analysis was performed. We examined the levels of PLEKHA4, DVL2, DVL3, Cyclin D1, and c-Myc to determine the most effective shRNAs from each collection (Figures S6A and S7). We further validated the effectiveness of the human PLEKHA4 shRNAs at suppressing Wnt3a-stimulated Wnt/β-catenin signaling using the TOPFlash system within the PLEKHA4 stable knockdown lines (Figure S6B). The best-performing shRNAs against human PLEKHA4, as validated in WM266–4 cells, were subsequently stably expressed and validated in SK-MEL-2 cells (Figure S8A–B).

We then generated xenograft/allograft models by subcutaneous injection into the shoulder or hind leg flanks in the absence of doxycycline to allow tumors to form. For the WM266–4 xenograft and YUMM1.7 allografts, after 12 days in the absence of doxycycline to allow tumors to form, doxycycline was administered for 10–12 days to induce PLEKHA4/*Plekha4* knockdown (Figure 5A). As negative controls, stable cell lines expressing luciferase shRNA were employed. Importantly, in the absence of doxycycline, the rate of tumor formation was identical for all cells from the same parental cell line. We monitored tumor progression over this time span and, following the addition of doxycycline to induce shPLEKHA4 expression, observed a major attenuation of tumor growth for both BRAF-mutant models (Figure 5B–C). Further analysis of the tumors at the experimental endpoint revealed that shPLEKHA4-expressing tumors were approximately four-fold smaller in the WM266–4/NSG model (Figure 5B) and three-fold smaller in the YUMM1.7/C57BL6.J model (Figure 5C).

To test the effect of PLEKHA4 knockdown on NRAS-mutant melanoma in vivo, we established an SK-MEL-2 xenograft, and once visible tumors appeared at 1.5 months post-injection, doxycycline administration was carried out for 14 days (Figure 5A). Analysis of tumor progression and endpoint data revealed that tumor growth was attenuated two-fold in the PLEKHA4 knockdown samples compared to control (Figure 5D). These data demonstrate that PLEKHA4 knockdown in an in vivo, tumor xenograft or allograft setting results in a substantial decrease in tumor growth and implicate PLEKHA4 and, by extension, Wnt signaling, as a regulator of BRAF and NRAS-mutant melanoma progression in vivo.

To examine whether the mechanism underlying the effects of PLEKHA4 knockdown in vivo was similar to that determined in vitro, we performed Western blot analysis on tumor samples (Figure 6). We found that, for xeno-/allografts from all three cell lines — WM266–4, (Figure 6A), YUMM1.7 (Figure 6B), and SK-MEL-2 (Figure 6C) — the PLEKHA4 shRNAs were highly effective at reducing PLEKHA4 protein levels, relative to the control tumors expressing control shRNA. Further, this analysis revealed substantial decreases in DVL2, DVL3, Cyclin D1, and c-Myc in PLEKHA4 shRNA-expressing tumors compared to controls expressing control shRNA (Figure 6). This analysis is consistent with the effects of PLEKHA4 siRNA and shRNA observed in vitro and supports the conclusion that PLEKHA4 knockdown attenuates proliferation via effects on Wnt/β-catenin signaling.

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PLEKHA4 knockdown has an additive effect with a BRAF inhibitor on preventing tumor proliferation in vivo

Finally, we wanted to establish the feasibility of targeting PLEKHA4 in a model of a therapeutic setting. Targeted BRAF therapy, i.e., BRAF and/or MEK inhibitors, represents a frontline treatment for melanoma (47,48). Though effective, this treatment has its limitations, including resistance, leading to relapse (6,49). PLEKHA4 and its effect on Wnt signaling could represent a second, parallel druggable pathway to block melanoma progression. Thus, we examined whether the anti-proliferative effects of encorafenib (BRAFi), an FDA-approved BRAF inhibitor used routinely to treat BRAF-mutant melanoma, would be enhanced by simultaneous knockdown of PLEKHA4 in vivo (39). We generated WM266–4 xenografts bearing doxycycline-inducible PLEKHA4 or control shRNA as before. On day 12 post-injection, following the formation of tumors, mice were administered both doxycycline to induce shRNA expression and encorafenib, via daily oral gavage, to inhibit BRAF and downstream MAP kinase signaling (Figure 7A).

This study was divided into two phases. In the first phase, we examined effects of PLEKHA4 knockdown and encorafenib treatment separately or in combination. We found that encorafenib treatment prevented tumor growth compared to control, similar to effects of PLEKHA4 knockdown alone (Figure 7B). Encouragingly, encorafenib treatment in the context of PLEKHA4 knockdown resulted in significantly reduced tumor growth compared to either PLEKHA4 knockdown or encorafenib treatment alone (Figure 7B). These data indicate an additive effect of PLEKHA4 knockdown and BRAF inhibition. Further analysis of tumor size and endpoint data confirmed that encorafenib treatment and PLEKHA4 knockdown exhibited similar effects on tumor size compared to control samples (Figure 7C–D).

Clinically, melanoma tumors can relapse upon development of resistance to BRAF inhibitors such as encorafenib, as well as withdrawal of the inhibitor (6,49). This relapse is problematic, leading to further disease progression and poor patient outcomes. As a model for resistance, we examined the effects of continued PLEKHA4 inhibition on tumor regrowth of residual melanoma cells after removal of encorafenib. In the second phase of the study, we extended the study on both control and PLEKHA4 knockdown groups that had been treated with encorafenib during the first phase of the study. Here, we removed encorafenib but continued doxycycline treatment for an additional 14 days to sustain PLEKHA4 knockdown. We observed that, upon encorafenib withdrawal, both the control and PLEKHA4 knockdown samples started to grow, but to different extents (Figure 7B). Further analysis of the tumor xenografts during the 14-day timecourse and at the endpoint confirmed that upon encorafenib withdrawal, both the encorafenib + PLEKHA4 knockdown and encorafenib alone samples had grown, but to different extents (Figure 7C–D). Notably, the encorafenib + PLEKHA4 knockdown sample exhibited a slower growth during the regrowth phase compared to encorafenib only.

From these data, we conclude that PLEKHA4 knockdown, in combination with BRAF inhibition, prevents melanoma growth in a xenograft model more efficaciously than BRAF inhibition alone. Further, sustained PLEKHA4 knockdown following encorafenib removal, which in this setting serves as a model for melanoma relapse from minimal persister cells

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(50) that had survived the encorafenib treatment, had a partial but substantial effect on proliferation, suggesting that inactivation of PLEKHA4 might be therapeutically beneficial in combination with existing targeted therapies.

DISCUSSION

Wnt/β-catenin signaling is a central pathway in embryonic development. In adults, it controls many aspects of cell and tissue homeostasis, including cell proliferation, differentiation, migration (10). Alterations that perturb Wnt signaling beyond the normal homeostatic range occur in many diseases; in particular, elevated Wnt signaling occurs in many cancers. In certain instances, mutations to core Wnt components are clearly understood to be drivers of oncogenesis, e.g., in colorectal cancer, where more than 80% of cases feature mutations in adenomatous polyposis coli (APC) that lead to hyperactive Wnt signaling and associated pathogenesis (27). In other cancers with elevated levels of Wnt signaling, the causal nature of this pathway in oncogenesis is not as clear.

Several studies have implicated increased Wnt signaling in melanoma, and yet the functional consequences of this dysregulation in melanoma are not entirely understood (11–13). In particular, elevated levels of nuclear β-catenin have been implicated in both increased proliferation but also, unexpectedly, better prognosis, and they are not a marker of the initial transformation event (13,51). Nuclear β-catenin alone may not necessarily correlate with cellular phenotype, suggesting an interplay of additional factors in the regulation of Wnt/β-catenin signaling in melanoma (52). Though the role of Wnt signaling as a sole driver of melanoma progression is controversial, its role in supporting proliferation in certain mutant backgrounds is clearer (14–18). In this context, our study provides important additional evidence implicating Wnt/β-catenin in melanoma proliferation in both BRAF and NRAS mutant backgrounds.

Inhibition of Wnt signaling is a promising route to new anti-cancer therapies, if achievable in a selective or targeted manner that minimizes damage to non-cancerous tissues (12,27,28,53). Because of challenges associated with targeting core Wnt pathway components, efforts have shifted in recent years toward gaining a deeper understanding of proteins that regulate the strength of Wnt signaling. Among this growing list of modulators, or tuners, PLEKHA4 stands out as a protein with a unique mechanism of action and potential relevance to melanoma.

Previously, we established that PLEKHA4 enhances Wnt signaling by sequestering and inactivating the Cullin-3 (CUL3) substrate-specific adaptor KLHL12 and preventing DVL polyubiquitination by the CUL3–KLHL12 E3 ubiquitin ligase (36,37). Here, we establish that this fundamental mechanism of tuning Wnt signaling strength could be highly beneficial in the context of melanoma. Melanoma cells express higher levels of PLEKHA4 than more than other 20 cancers, and even partial removal of PLEKHA4 by siRNA or shRNA dramatically lowers proliferation and increases apoptosis in vitro and in vivo.

PLEKHA4 knockdown exhibited similar effects in melanoma cells, i.e., on DVL levels and Wnt signaling strength, as well as strong effects on clonogenic capacity in vitro. These

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results point to Wnt/β-catenin signaling, and its regulator PLEKHA4, as important players controlling proliferation in both BRAF- and NRAS-mutant melanomas. PLEKHA4 knockdown in melanoma cells strongly affected levels of the canonical Wnt/β-catenin targets Cyclin D1 and c-Myc, which ensure progression through the G1/S cell cycle transition. Disruption of Wnt signaling via other means (DVL knockdown or global pharmacological inhibition of Wnt production) resulted in similar phenotypes to PLEKHA4 knockdown.

The ability of DVL overexpression to partially rescue the effects of PLEKHA4 knockdown both supports this mechanism and also highlights potential undetermined, DVL-independent mechanisms underlying the effect of PLEKHA4 knockdown on melanoma cell proliferation and apoptosis. In particular, effects on other CUL3–KLHL12 ubiquitination substrates, including the COPII coat component SEC31, may be responsible (54). Notably, CUL3 loss of function attenuates proliferation in various settings, including mouse embryonic stem cells and the *Drosophila* pupal wing epithelium, and CUL3–KLHL12 has further been proposed to control proliferation in other contexts (54,55).

In tumor xenograft and allograft models using both BRAF- and NRAS-mutant melanomas, removal of PLEKHA4 by shRNA prevented tumor growth. Further, in a BRAF-mutant melanoma, PLEKHA4 shRNA exhibited an additive effect with a clinically used BRAF inhibitor, leading to much stronger anti-proliferative effects, and its effects help to keep growth slow even after removal of the inhibitor. These results from the combination treatment studies reinforce that, whereas MAP kinase signaling is a predominant player in melanoma, Wnt/β-catenin plays important roles in supporting proliferation. Other modulators of Wnt signaling affect melanoma proliferation. For example, Dkk-1, a negative regulator of Wnt signaling, exhibits reduced expression in melanoma, and its activation inhibits tumorigenicity and induces apoptosis in melanoma (56,57). Another negative regulator of Wnt signaling, WIF-1 (Wnt inhibitory factor-1), is downregulated in melanoma progression (58). Both MAP kinase and Wnt/β-catenin signaling regulate the activity of MITF, a master regulator of melanoma progression in both BRAF and NRAS mutant backgrounds (59,60).

Yet, the interactions between Wnt/β-catenin and MAP kinase signaling in melanoma are complex. Elevated levels of the former, rather than its inhibition, enhanced the efficacy of BRAF inhibition at inducing apoptosis (61). However, chronic BRAF inhibition-induced resistance caused elevated levels of Wnt5a, which were associated with increased cell growth, suggesting that Wnt5a inhibition might counteract these effects (25). In light of this work, our study, performed using different melanoma cell lines and showing that a combination of PLEKHA4 shRNA and BRAF inhibition has stronger anti-proliferative effects compared to BRAF inhibition alone, further highlights the context-dependent effects of Wnt signaling and its relationship to BRAF and MAP kinase signaling in melanoma (11–13).

Our results suggest that PLEKHA4 inhibition might be therapeutically beneficial in both NRAS-mutant melanomas, for which there are no targeted therapies, and for BRAF-mutant melanomas, where PLEKHA4 inhibition could be investigated in combination with existing

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BRAF and/or MEK inhibitors. In principle, PLEKHA4 inhibition in combination with immunotherapies could also represent an interesting future direction.

PLEKHA4 is not a canonical drug target. It is a multidomain adaptor protein, not a receptor, ion channel, or enzyme. Yet, our previous work sheds light on several protein-lipid and protein-protein interactions that could be targeted (36). Its tripartite N-terminal region, which includes a pleckstrin homology (PH) domain, binds to anionic phosphoinositides to localize the protein to the plasma membrane. C-terminal coiled-coil and intrinsically disordered regions mediate oligomerization into membrane-associated clusters that are potentially phase-separated. A central proline-rich domain binds to KLHL12, and all three of these molecular elements (lipid binding, oligomerization, and KLHL12 binding) are featured in its mechanism of action to prevent DVL ubiquitination and enhance Wnt signaling.

In principle, small-molecule ligands could be developed to target the phosphoinositide binding site of the PH domain (62) or disrupt interactions between the proline-rich domain and KLHL12 or homotypic interactions involved in oligomerization and cluster formation. Further, ligands that bind to PLEKHA4 but do not disrupt function could still serve as starting points for development of PROTACs/degraders (63). Finally, a global knockout of the *Drosophila* ortholog of PLEKHA4, *kramer*, is viable (36), raising the possibility that mammalian PLEKHA4 may be dispensable for development and less critical for maintaining homeostatic Wnt signaling. This study, however, implicates it as a vulnerability for melanoma cells. Thus, we believe that PLEKHA4 defines a new type of drug target for melanoma.

Interestingly, our previous work on PLEKHA4 and *kramer* established that these proteins can also mediate non-canonical, β-catenin-independent Wnt signaling (36). In particular, in *Drosophila*, *kramer* knockout resulted in defects in planar cell polarity through effects on *dishevelled*, a pathway that shares key aspects with mammalian non-canonical Wnt signaling, including profound effects on the actin cytoskeleton (64). In melanoma, non-canonical Wnt signaling is implicated in a migratory phenotype, whereas canonical Wnt/β-catenin signaling controls proliferation. Melanoma progression has been described to involve a phenotype switching scenario, wherein alternating cycles of proliferation and migration lead to disease spread and eventually to metastasis (52).

Crucially, DVL is a central signaling molecule in both the canonical and non-canonical pathways (31), and thus it is not surprising that PLEKHA4, which regulates DVL levels, has the potential to affect multiple types of Wnt signaling, depending on the context (36). In the in vitro and xenograft models here, which are geared toward evaluation of the proliferative stages of melanoma, we found a strong effect on removal of PLEKHA4. Examination of effects of PLEKHA4 removal on non-canonical Wnt signaling in the context of a migratory phenotype represents an interesting future direction and could reveal that a single protein, PLEKHA4, might be relevant in suppressing later stages of melanoma, including metastasis, where the cancer cells exhibit an invasive phenotype. Notably, chronic inhibition of mutant BRAF in melanoma causes an elevation in levels of Wnt5a (25). Whereas that study examined effects on Wnt5a-induced cell growth, Wnt5a can also mediate non-canonical Wnt

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signaling, which is implicated in migration and metastasis, suggesting potential interplay in melanoma between BRAF and Wnt signaling pathways in multiple contexts.

In summary, we have identified PLEKHA4 as an important mediator of a proliferative phenotype in BRAF- and NRAS-mutant melanoma. We demonstrate that PLEKHA4 knockdown negatively regulates Wnt/β-catenin signaling in this context, helping to clarify the role of Wnt/β-catenin signaling in melanoma and revealing another layer of regulation in the Wnt/β-catenin signaling axis that controls the G1/S cell cycle transition to maintain melanoma proliferation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Miller AJ, Mihm MC. Melanoma. N Engl J Med. 2006;355:51–65. [PubMed: 16822996]
2. Akbani R, Akdemir KC, Aksoy BA, Albert M, Ally A, Amin SB, et al. Genomic Classification of Cutaneous Melanoma. Cell. 2015;161:1681–96. [PubMed: 26091043]
3. Hayward NK, Wilmott JS, Waddell N, Johansson PA, Field MA, Nones K, et al. Whole-genome landscapes of major melanoma subtypes. Nature. 2017;545:175–80. [PubMed: 28467829]
4. Long G V, Stroyakovskiy D, Gogas H, Levchenko E, de Braud F, Larkin J, et al. Combined BRAF and MEK Inhibition versus BRAF Inhibition Alone in Melanoma. N Engl J Med. 2014;371:1877–88. [PubMed: 25265492]
5. Larkin J, Ascierto PA, Dréno B, Atkinson V, Liszkay G, Maio M, et al. Combined Vemurafenib and Cobimetinib in BRAF-Mutated Melanoma. N Engl J Med. 2014;371:1867–76. [PubMed: 25265494]
6. Lito P, Rosen N, Solit DB. Tumor adaptation and resistance to RAF inhibitors. Nat Med. 2013;19:1401–9. [PubMed: 24202393]
7. Hamid O, Robert C, Daud A, Hodi FS, Hwu W-J, Kefford R, et al. Safety and Tumor Responses with Lambrolizumab (Anti–PD-1) in Melanoma. N Engl J Med. 2013;369:134–44. [PubMed: 23724846]
8. Hu-Lieskovan S, Mok S, Homet Moreno B, Tsoi J, Robert L, Goedert L, et al. Improved antitumor activity of immunotherapy with BRAF and MEK inhibitors in BRAF–V600E melanoma. Sci Transl Med. 2015;7:279ra41.
9. Zhan T, Rindtorff N, Boutros M. Wnt signaling in cancer. Oncogene. 2017;36:1461–73. [PubMed: 27617575]
10. MacDonald BT, Tamai K, He X. Wnt/β-Catenin Signaling: Components, Mechanisms, and Diseases. Dev Cell. 2009;17:9–26. [PubMed: 19619488]
11. Webster MR, Weeraratna AT. A Wnt-er Migration: The Confusing Role of β-Catenin in Melanoma Metastasis. Sci Signal. 2013;6:pe11.
12. Jackstadt R, Hodder MC, Sansom OJ. WNT and β-Catenin in Cancer: Genes and Therapy. Annu Rev Cancer Biol. 2020;4:177–96.
13. Gajos-Michniewicz A, Czyz M. Wnt Signaling in Melanoma. Int J Mol Sci. 2020;21:4852.

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14. Delmas V, Beermann F, Martinozzi S, Carreira S, Ackermann J, Kumasaka M, et al. β-Catenin induces immortalization of melanocytes by suppressing p16INK4a expression and cooperates with N-Ras in melanoma development. Genes Dev. 2007;21:2923–35. [PubMed: 18006687]
15. Pawlikowski JS, McBryan T, van Tuyn J, Drotar ME, Hewitt RN, Maier AB, et al. Wnt signaling potentiates nevogenesis. Proc Natl Acad Sci USA. 2013;110:16009–14. [PubMed: 24043806]
16. Juan J, Muraguchi T, Iezza G, Sears RC, McMahon M. Diminished WNT -> β-catenin -> c-MYC signaling is a barrier for malignant progression of BRAFV600E-induced lung tumors. Genes Dev. 2014/03/03. 2014;28:561–75. [PubMed: 24589553]
17. Damsky WE, Curley DP, Santhanakrishnan M, Rosenbaum LE, Platt JT, Gould Rothberg BE, et al. β-Catenin Signaling Controls Metastasis in Braf-Activated Pten-Deficient Melanomas. Cancer Cell. 2011;20:741–54. [PubMed: 22172720]
18. Sun Q, Lee W, Mohri Y, Takeo M, Lim CH, Xu X, et al. A novel mouse model demonstrates that oncogenic melanocyte stem cells engender melanoma resembling human disease. Nat Commun. 2019;10:1–16. [PubMed: 30602773]
19. Chien AJ, Haydu LE, Biechele TL, Kulikauskas RM, Rizos H, Kefford RF, et al. Targeted BRAF Inhibition Impacts Survival in Melanoma Patients with High Levels of Wnt/β-Catenin Signaling. PLoS One. 2014;9:e94748.
20. Kageshita T, Hamby C V, Ishihara T, Matsumoto K, Saida T, Ono T. Loss of β-catenin expression associated with disease progression in malignant melanoma. Br J Dermatol. 2001;145:210–6. [PubMed: 11531781]
21. Bachmann IM, Straume O, Puntervoll HE, Kalvenes MB, Akslen LA. Importance of P-Cadherin, β-Catenin, and Wnt5a/Frizzled for Progression of Melanocytic Tumors and Prognosis in Cutaneous Melanoma. Clin Cancer Res. 2005;11:8606 LP – 8614. [PubMed: 16361544]
22. Chien AJ, Moore EC, Lonsdorf AS, Kulikauskas RM, Rothberg BG, Berger AJ, et al. Activated Wnt/ß-catenin signaling in melanoma is associated with decreased proliferation in patient tumors and a murine melanoma model. Proc Natl Acad Sci USA. 2009;106:1193–1198. [PubMed: 19144919]
23. Xue G, Romano E, Massi D, Mandalà M. Wnt/β-catenin signaling in melanoma: Preclinical rationale and novel therapeutic insights. Cancer Treat Rev. 2016;49:1–12. [PubMed: 27395773]
24. Webster MR, Xu M, Kinzler KA, Kaur A, Appleton J, O'Connell MP, et al. Wnt5A promotes an adaptive, senescent-like stress response, while continuing to drive invasion in melanoma cells. Pigment Cell Melanoma Res. 2015;28:184–95. [PubMed: 25407936]
25. Anastas JN, Kulikauskas RM, Tamir T, Rizos H, Long G V, von Euw EM, et al. WNT5A enhances resistance of melanoma cells to targeted BRAF inhibitors. J Clin Invest. 2014;124:2877–90. [PubMed: 24865425]
26. Hoek KS, Eichhoff OM, Schlegel NC, Döbbeling U, Kobert N, Schaerer L, et al. In vivo Switching of Human Melanoma Cells between Proliferative and Invasive States. Cancer Res. 2008;68:650 LP – 656. [PubMed: 18245463]
27. Nusse R, Clevers H. Wnt/β-Catenin Signaling, Disease, and Emerging Therapeutic Modalities. Cell. 2017;169:985–99. [PubMed: 28575679]
28. Tran FH, Zheng JJ. Modulating the Wnt signaling pathway with small molecules. Protein Sci. 2017;26:650–61. [PubMed: 28120389]
29. Kahn M Can we safely target the WNT pathway? Nat Rev Drug Discov. 2014;13:513–32. [PubMed: 24981364]
30. Clevers H, Nusse R. Wnt/β-catenin signaling and disease. Cell. 2012;149:1192–205. [PubMed: 22682243]
31. Mlodzik M The Dishevelled Protein Family: Still Rather a Mystery After Over 20 Years of Molecular Studies [Internet]. 1st ed. Curr. Top. Dev. Biol. 2016.
32. Nielsen CP, Jernigan KK, Diggins NL, Webb DJ, MacGurn JA. USP9X Deubiquitylates DVL2 to Regulate WNT Pathway Specification. Cell Rep. 2019;28:1074–1089.e5. [PubMed: 31340145]
33. Tauriello DVF, Haegebarth A, Kuper I, Edelmann MJ, Henraat M, Canninga-van Dijk MR, et al. Loss of the Tumor Suppressor CYLD Enhances Wnt/β-Catenin Signaling through K63-Linked Ubiquitination of Dvl. Mol Cell. 2010;37:607–19. [PubMed: 20227366]

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34. Ding Y, Zhang Y, Xu C, Tao QH, Chen YG. HECT domain-containing E3 ubiquitin ligase NEDD4L negatively regulates Wnt signaling by targeting dishevelled for proteasomal degradation. J Biol Chem. 2013;288:8289–98. [PubMed: 23396981]
35. Wei W, Li M, Wang J, Nie F, Li L. The E3 Ubiquitin Ligase ITCH Negatively Regulates Canonical Wnt Signaling by Targeting Dishevelled Protein. Mol Cell Biol. 2012;32:3903–12. [PubMed: 22826439]
36. Shami Shah A, Batrouni AG, Kim D, Punyala A, Cao W, Han C, et al. PLEKHA4/kramer Attenuates Dishevelled Ubiquitination to Modulate Wnt and Planar Cell Polarity Signaling. Cell Rep. 2019;27:2157–2170.e8. [PubMed: 31091453]
37. Angers S, Thorpe CJ, Biechele TL, Goldenberg SJ, Zheng N, MacCoss MJ, et al. The KLHL12–Cullin-3 ubiquitin ligase negatively regulates the Wnt–β-catenin pathway by targeting Dishevelled for degradation. Nat Cell Biol. 2006;8:348–57. [PubMed: 16547521]
38. Hruz T, Laule O, Szabo G, Wessendorp F, Bleuler S, Oertle L, et al. Genevestigator V3: A Reference Expression Database for the Meta-Analysis of Transcriptomes. Adv Bioinformatics. 2008;2008:1–5.
39. Koelblinger P, Thuerigen O, Dummer R. Development of encorafenib for BRAF-mutated advanced melanoma. Curr Opin Oncol. 2018;30:125–33. [PubMed: 29356698]
40. Rafehi H, Orlowski C, Georgiadis GT, Ververis K, El-Osta A, Karagiannis TC. Clonogenic assay: Adherent cells. J Vis Exp. 2011;15–7.
41. Rodgers L Measurement of DNA Content Using Propidium Iodide (PI) Staining of Fixed Whole Cells. Cold Spring Harb Protoc. 2006;2006:pdb.prot4436.
42. Meeth K, Wang JX, Micevic G, Damsky W, Bosenberg MW. The YUMM lines: a series of congenic mouse melanoma cell lines with defined genetic alterations. Pigment Cell Melanoma Res. 2016;29:590–7. [PubMed: 27287723]
43. Chen B, Dodge ME, Tang W, Lu J, Ma Z, Fan C-W, et al. Small molecule-mediated disruption of Wnt-dependent signaling in tissue regeneration and cancer. Nat Chem Biol. 2009;5:100–7. [PubMed: 19125156]
44. Sakaue-Sawano A, Kurokawa H, Morimura T, Hanyu A, Hama H, Osawa H, et al. Visualizing Spatiotemporal Dynamics of Multicellular Cell-Cycle Progression. Cell. 2008;132:487–98. [PubMed: 18267078]
45. Shtutman M, Zhurinsky J, Simcha I, Albanese C, D'Amico M, Pestell R, et al. The cyclin D1 gene is a target of the β-catenin/LEF-1 pathway. Proc Natl Acad Sci USA. 1999;96:5522–5527. [PubMed: 10318916]
46. He T-C, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, et al. Identification of c-MYC as a Target of the APC Pathway. Science. 1998;281:1509–1512. [PubMed: 9727977]
47. Flaherty KT, Puzanov I, Kim KB, Ribas A, McArthur GA, Sosman JA, et al. Inhibition of Mutated, Activated BRAF in Metastatic Melanoma. N Engl J Med. 2010;363:809–19. [PubMed: 20818844]
48. Bollag G, Hirth P, Tsai J, Zhang J, Ibrahim PN, Cho H, et al. Clinical efficacy of a RAF inhibitor needs broad target blockade in BRAF-mutant melanoma. Nature. 2010;467:596–9. [PubMed: 20823850]
49. Rizos H, Menzies AM, Pupo GM, Carlino MS, Fung C, Hyman J, et al. BRAF Inhibitor Resistance Mechanisms in Metastatic Melanoma: Spectrum and Clinical Impact. Clin Cancer Res. 2014;20:1965–1977. [PubMed: 24463458]
50. Shen S, Vagner S, Robert C. Persistent Cancer Cells: The Deadly Survivors. Cell. 2020;183:860–74. [PubMed: 33186528]
51. Lucero OM, Dawson DW, Moon RT, Chien AJ. A Re-evaluation of the "Oncogenic" Nature of Wnt/β-catenin Signaling in Melanoma and Other Cancers. Curr Oncol Rep. 2010;12:314–8. [PubMed: 20603725]
52. Webster MR, Kugel CH, Weeraratna AT. The Wnts of change: How Wnts regulate phenotype switching in melanoma. Biochim Biophys Acta - Rev Cancer. 2015;1856:244–51.
53. Krishnamurthy N, Kurzrock R. Targeting the Wnt/beta-catenin pathway in cancer: Update on effectors and inhibitors. Cancer Treat Rev. 2018;62:50–60. [PubMed: 29169144]
54. Jin L, Pahuja KB, Wickliffe KE, Gorur A, Baumgärtel C, Schekman R, et al. Ubiquitin-dependent regulation of COPII coat size and function. Nature. 2012;482:495–500. [PubMed: 22358839]

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55. Strutt H, Searle E, Thomas-MacArthur V, Brookfield R, Strutt D. A Cul-3-BTB ubiquitylation pathway regulates junctional levels and asymmetry of core planar polarity proteins. Development. 2013;140:1693–702. [PubMed: 23487316]
56. Kuphal S, Lodermeyer S, Bataille F, Schuierer M, Hoang BH, Bosserhoff AK. Expression of Dickkopf genes is strongly reduced in malignant melanoma. Oncogene. 2006;25:5027–36. [PubMed: 16568085]
57. Mikheev AM, Mikheeva SA, Rostomily R, Zarbl H. Dickkopf-1 activates cell death in MDA-MB435 melanoma cells. Biochem Biophys Res Commun. 2007;352:675–80. [PubMed: 17141200]
58. Haqq C, Nosrati M, Sudilovsky D, Crothers J, Khodabakhsh D, Pulliam BL, et al. The gene expression signatures of melanoma progression. Proc Natl Acad Sci USA. 2005;102:6092–7. [PubMed: 15833814]
59. Widlund HR, Horstmann MA, Roydon Price E, Cui J, Lessnick SL, Wu M, et al. β-Catenin-induced melanoma growth requires the downstream target Microphthalmia-associated transcription factor. J Cell Biol. 2002;158:1079–87. [PubMed: 12235125]
60. Hartman ML, Czyz M. MITF in melanoma: mechanisms behind its expression and activity. Cell Mol Life Sci. 2014/11/30. 2015;72:1249–60. [PubMed: 25433395]
61. Biechele TL, Kulikauskas RM, Toroni RA, Lucero OM, Swift RD, James RG, et al. Wnt/β-catenin signaling and AXIN1 regulate apoptosis triggered by inhibition of the mutant kinase BRAF V600E in human melanoma. Sci Signal. 2012;5.
62. Sierecki E, Sinko W, McCammon JA, Newton AC. Discovery of Small Molecule Inhibitors of the PH Domain Leucine-Rich Repeat Protein Phosphatase (PHLPP) by Chemical and Virtual Screening. J Med Chem. 2010;53:6899–911. [PubMed: 20836557]
63. Lai AC, Crews CM. Induced protein degradation: An emerging drug discovery paradigm. Nat Rev Drug Discov. 2017;16:101–14. [PubMed: 27885283]
64. Simons M, Mlodzik M. Planar Cell Polarity Signaling: From Fly Development to Human Disease. Annu Rev Genet. 2008;42:517–40. [PubMed: 18710302]

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STATEMENT OF SIGNIFICANCE

This study establishes that melanoma cell proliferation requires the protein PLEKHA4 to promote pathological Wnt signaling for proliferation, highlighting PLEKHA4 inhibition as a new avenue for the development of targeted therapies.

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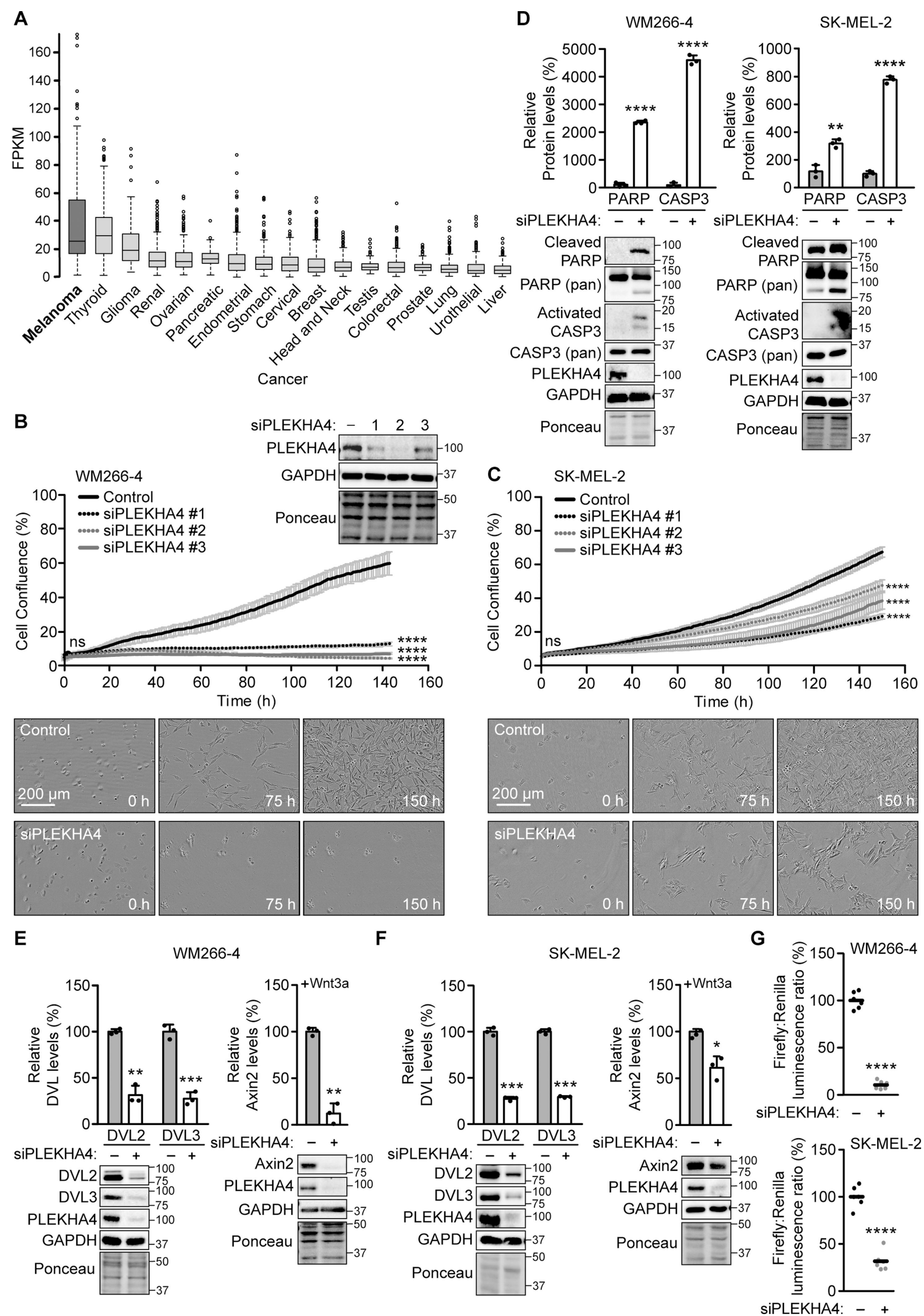

Figure 1. PLEKHA4 loss from melanoma cells reduces proliferation and increases apoptosis via attenuation of Wnt/β-catenin signaling.

(A) Analysis of PLEKHA4 mRNA levels in various cancers, based on data generated by the TCGA Research Network. FPKM, fragments per kilobase of transcript per million mapped reads. (B and C) PLEKHA4 knockdown by siRNA inhibits melanoma cell proliferation in vitro. Automated brightfield imaging of cell proliferation via IncuCyte of (B) WM266–4 and (C) SK-MEL-2 melanoma cells treated with siRNA duplexes targeting different regions of PLEKHA4 (siPLEKHA4 #1, #2 and #3) or a negative control siRNA (n=3). Western blot validation of siRNA duplexes is shown in WM266–4 cells (B). (D–F) PLEKHA4

knockdown (using siPLEKHA4 #2) causes increased levels of apoptotic markers (cleaved PARP and activated Caspase 3 (CASP3)) and reduction in Wnt signaling (DVL2, DVL3, and Axin2) in mutant melanoma cells. Shown is Western blot analysis of WM266–4 and SK-MEL-2 cells subjected to siPLEKHA4 or a negative control siRNA (–) (n=3). For Axin2 analysis, cells were stimulated with Wnt3a-containing conditioned media concurrently with siRNA. (G) PLEKHA4 modulates Wnt/β-catenin signaling in WM266–4 and SK-MEL-2 cells. Shown is TOPFlash assay signal, i.e., ratio of β-catenin-dependent firefly luciferase activity to constitutive Renilla luciferase activity in WM266–4 or SK-MEL2 cells treated with siPLEKHA4 (#2) or negative control siRNA (–) and stimulated with Wnt3a-containing conditioned media (n=6). For Western blot analysis, GAPDH and Ponceau are shown as loading controls. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; ns, not significant. Scale bars: 200 μm.

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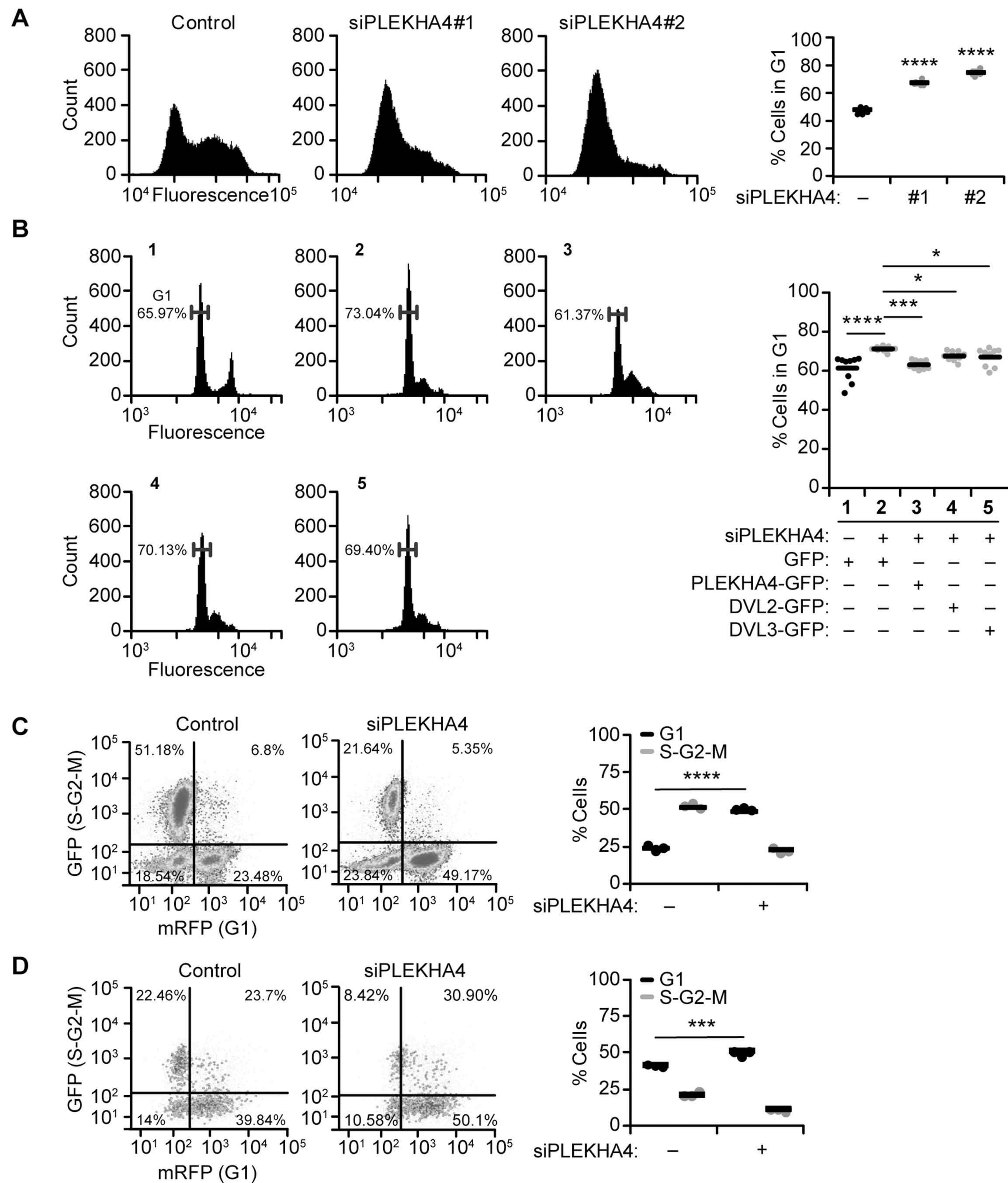

Figure 2. PLEKHA4 knockdown inhibits Wnt/β-catenin mediated G1/S cell cycle transition.
(A) PLEKHA4 knockdown leads to accumulation of WM266–4 cells in G1 phase. An asynchronous population of WM266–4 cells was treated with one of two different siRNA duplexes against PLEKHA4 (siPLEKHA4 #1 and #2) or a negative control siRNA (–), followed by fixation, propidium iodide staining, and flow cytometry analysis. (n=6) (B) PLEKHA4-GFP, DVL2-GFP, and DVL3-GFP can rescue the attenuation of the G1/S transition defect induced by PLEKHA4 knockdown (using siPLEKHA4 #2). WM266–4 cells were synchronized to G1 phase, subjected to siPLEKHA4 or negative control siRNA

(–), and stimulated with media containing FBS and simultaneously transduced with conditioned media containing lentivirus encoding GFP, siRNA-resistant PLEKHA4-GFP, DVL2-GFP, or DVL3-GFP, followed by fixation, propidium iodide staining, and flow cytometry analysis (n=9). (C–D) PLEKHA4 knockdown (using siPLEKHA4 #2) impairs G1/S transition in synchronized melanoma cells stably expressing the FUCCI cell cycle indicator. WM266–4-FUCCI (C) and SK-MEL-2-FUCCI (D) stable cells were synchronized to G1 phase via serum starvation and concurrent treatment with the indicated siRNA duplex for 48 h. Cells were then released into fresh medium containing FBS, followed by the quantification of mRFP (G1) and GFP (S-G2-M) fluorescence via flow cytometry (n=3). * $p < 0.05$; *** $p<0.001$; **** $p < 0.0001$.

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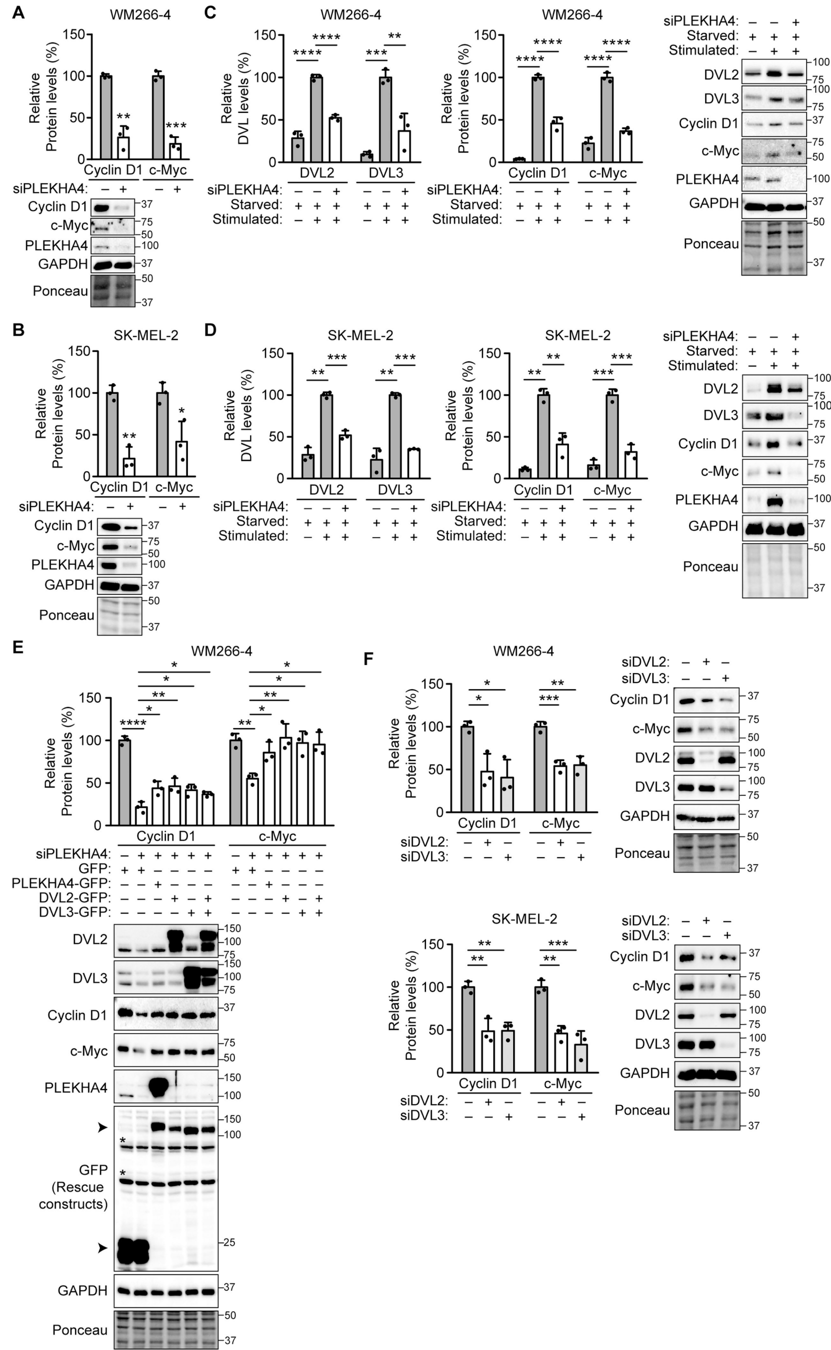

Figure 3. PLEKHA4 knockdown reduces levels of Wnt/β-catenin-controlled markers of proliferation.

(A and B) PLEKHA4 knockdown decreases Cyclin D1 and c-Myc levels in asynchronous WM266–4 (A) and SK-MEL-2 (B) cells. Shown is quantification and representative blot images of Western blot analysis of lysates from the indicated cells treated with an siRNA duplex against PLEKHA4 (siPLEKHA4, #2) or a negative control siRNA (–) (n=3). (C and D) PLEKHA4 modulates the levels of DVL2, DVL3, Cyclin D1, and c-Myc in G1-synchronized WM266–4 (C) and SK-MEL-2 (D) cells. Shown is Western blot analysis and quantification of lysates from melanoma cells synchronized to G1 phase via serum

starvation that were treated with siPLEKHA4 (#2) or a negative control siRNA (–) and then stimulated with FBS-containing medium (n=3). (E) PLEKHA4-GFP, DVL2-GFP, and DVL3-GFP can partially rescue the changes in DVL2, DVL3, Cyclin D1, and c-Myc levels induced by PLEKHA4 knockdown in WM266–4 cells. Shown is quantification and representative blot images of Western blot analysis of lysates from WM266–4 cells subjected to siPLEKHA4 (#2) or negative control siRNA (–) and transduced with conditioned media containing lentivirus encoding GFP, siRNA-resistant PLEKHA4-GFP, DVL2-GFP, DVL3-GFP, or a combination of DVL2-GFP and DVL3-GFP (n=3). (F) Knockdown of DVL2 or DVL3 leads to a decrease in levels of Cyclin D1 and c-Myc. Shown is quantification and representative blot images of Western blot analysis of lysates from WM266–4 and SK-MEL-2 cells treated with the indicated siRNA duplex or negative control siRNA (n=3). GAPDH and Ponceau are shown as loading controls. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

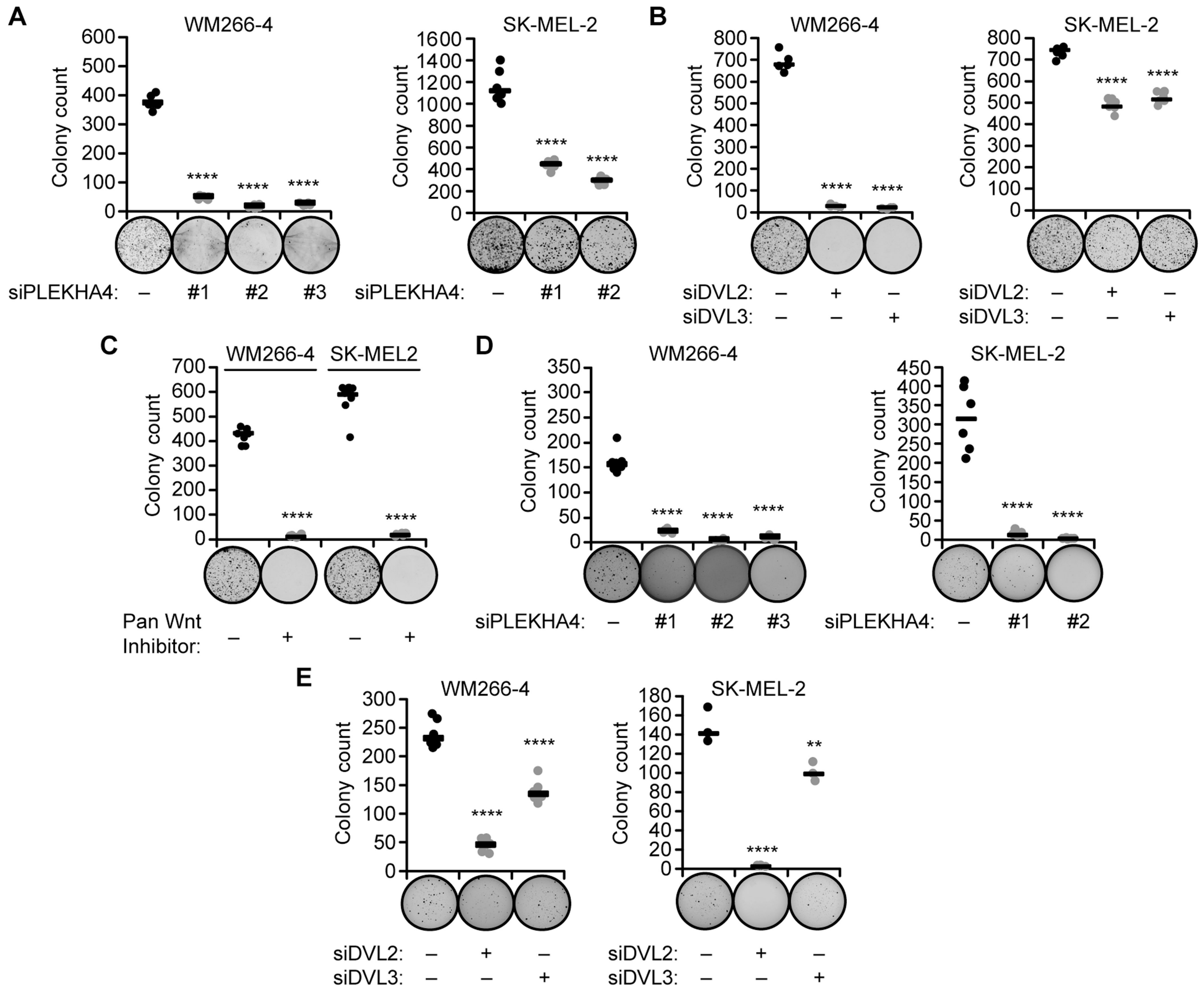

Figure 4. PLEKHA4 knockdown and Wnt inhibition causes loss of tumorigenic and malignant properties in melanoma cells in vitro.

Cells treated as described below were analyzed via anchorage-dependent colony formation assay with crystal violet staining (A–C) or anchorage-independent soft agar assay (D–E). Representative brightfield images are shown for each treatment, and graphs indicate colony count. (A and D) Cells were treated with the indicated siRNA duplex against PLEKHA4 or negative control siRNA (–) (n=6). (B and E) Cells were treated with siRNA duplexes against DVL2, DVL3, or negative control siRNA (n=6 for all, except for n=3 for SK-MEL-2 in (E)). (C) Cells were treated with the pan Wnt inhibitor IWP-4 or DMSO control (–) (n=6). ** $p < 0.01$; **** $p < 0.0001$.

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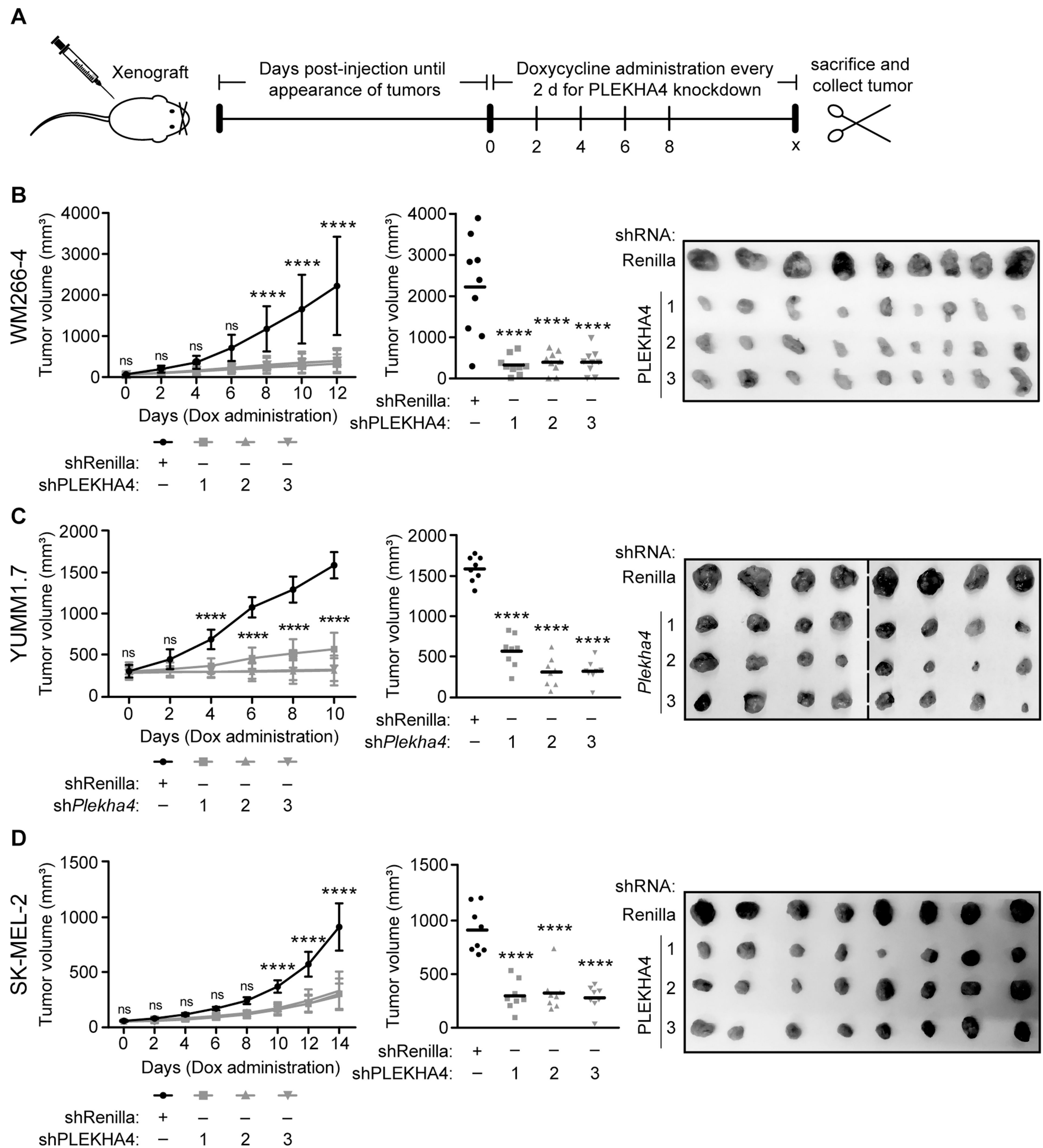

Figure 5. Inducible PLEKHA4 knockdown inhibits melanoma tumor xenograft/allograft growth in vivo.

(A) Schematic representation of experimental setup and timeline for xenograft/allograft analyses. Cell lines stably expressing doxycycline-inducible shRNA against human PLEKHA4 (shPLEKHA4; WM266–4 and SK-MEL-2) or mouse *Plekha4* (sh*Plekha4*; YUMM1.7) or a negative control shRNA (Renilla) were xenografted into NSG (for WM266–4 and SK-MEL-2) and C57BL/6J (for YUMM1.7) mice. Mice were monitored, and after small tumor bumps appeared (12 d for WM266–4 and YUMM1.7; 45 d for SK-MEL-2), doxycycline was administered through the drinking water for a total of 10–16 d to

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induce PLEKHA4 knockdown. Tumor progression over this time period was monitored by measurement of tumor dimensions using a digital caliper and calculation of tumor volume using the formula $v = 0.5233*l*w^2$. Mice were then sacrificed, and tumors were collected (n=12 for WM266–4-xenografted NSG mice, n=10 for YUMM1.7-allografted C57BL/6J mice, and n=14 for SK-MEL-2-xenografted NSG mice). (B–D) Data from studies using WM266–4 xenografts (B), YUMM1.7 allografts (C), and SK-MEL-2 xenografts (D). The plots at left show changes in tumor volume over time, and the plot in the middle show final tumor volumes measured with a caliper post-harvesting, with images of tumors harvested at the endpoint shown at right. n=9 for WM266–4 and n=8 for YUMM1.7 and SK-MEL-2. **** $p < 0.0001$; ns, not significant.

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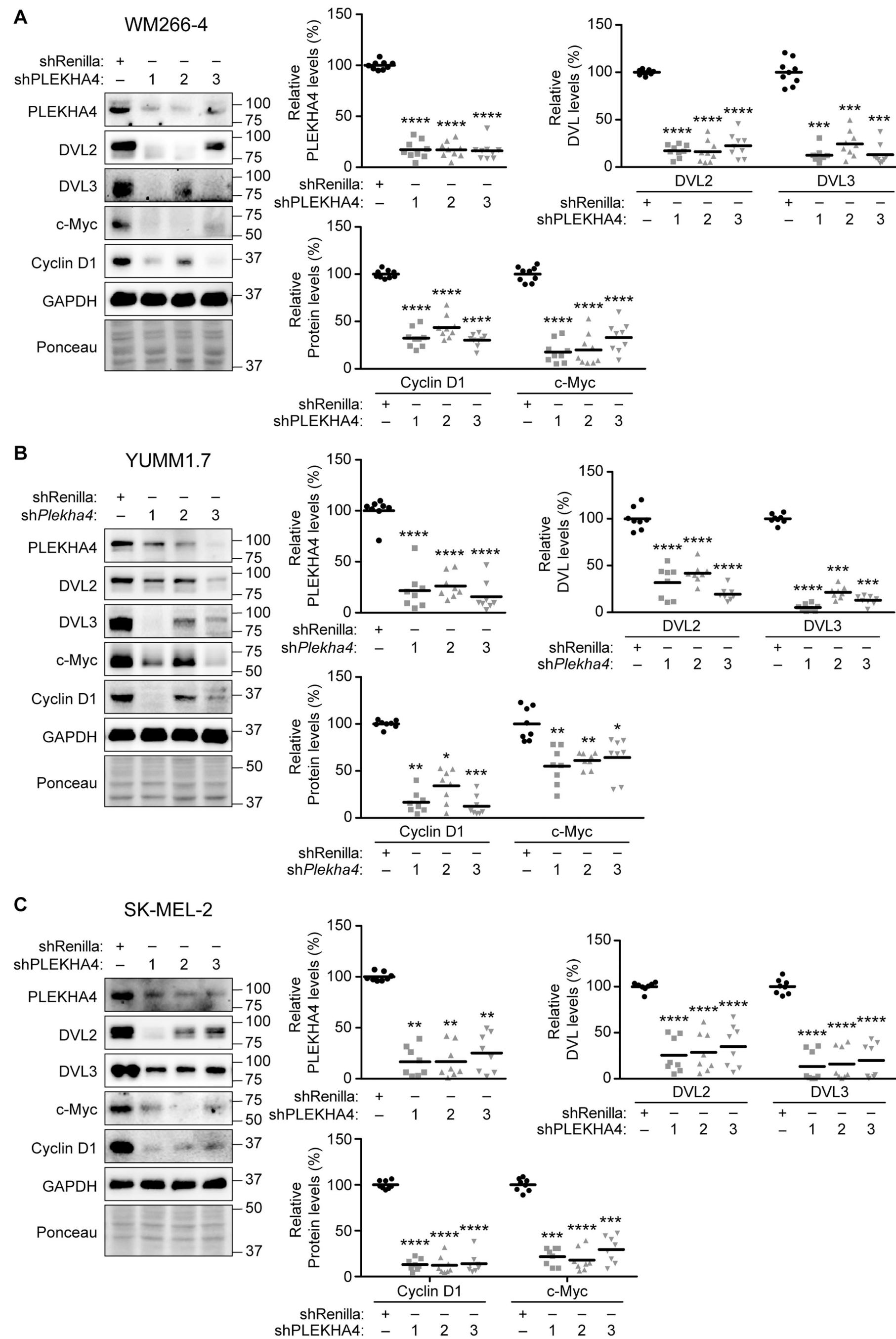

Figure 6. Inducible PLEKHA4 knockdown in tumor xenograft/allografts reduces levels of Wnt/β-catenin signaling and proliferation markers.

Tumor samples were harvested at the endpoints of the xenograft experiments described in Figure 6. Lysates were generated and analyzed by Western blot for PLEKHA4, to verify shRNA efficacy, and for DVL2, DVL3, Cyclin D1, and c-Myc, to assess effects on Wnt/β-catenin signaling and proliferation. GAPDH and Ponceau are shown as loading controls. Representative Western blots are shown at top, with quantification in scatter plots below. Results are shown for all xenograft studies, from WM266–4 (A), YUMM1.7 (B), and SK-

MEL-2 (C). n=9 for WM266–4 and n=8 for YUMM1.7 and SK-MEL-2. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

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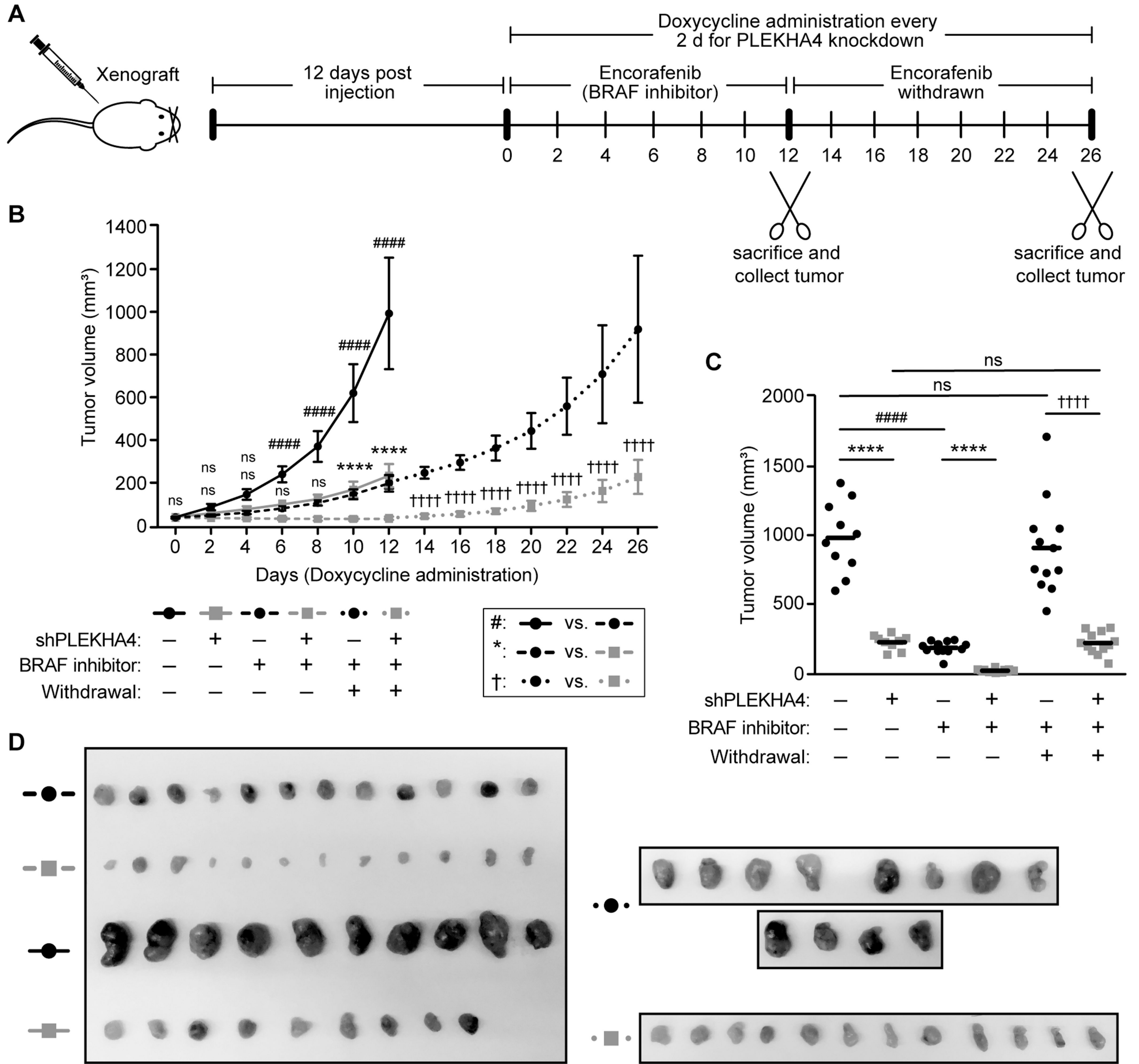

Figure 7. PLEKHA4 knockdown exhibits an additive effect with the BRAF inhibitor encorafenib to attenuate melanoma tumor xenograft growth in vivo.

(A) Schematic representation of experimental setup and timeline. WM266–4 cells stably expressing a doxycycline-inducible shRNA hairpin against PLEKHA4 (shPLEKHA4 #1) or a control shRNA (–) were xenografted into NSG mice. Mice were monitored, and after tumors became visible 12 d post-injection (labeled as day 0), doxycycline was administered through the drinking water and the BRAF inhibitor encorafenib or vehicle control was administered via oral gavage every day for 12 d. On day 12, all vehicle-treated mice and half of the encorafenib-treated mice bearing control and PLEKHA4 knockdown tumors were sacrificed for tumor collection. For the remaining mice, doxycycline treatment was

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continued but encorafenib was withdrawn to assess effect of PLEKHA4 knockdown on relapse for another 14 d. On day 26, mice were sacrificed for tumor collection. (B–D) Data from these studies. (B) Plot showing changes in tumor volume over time, with dimensions determined as described in the Figure 6 legend. (C) Final tumor volumes measured with a caliper post-harvesting. (D) Images of tumors harvested at endpoints: day 12 (left) and day 26 (right) (n=10–12 for each group). ****, ####, and ††††: $p < 0.0001$; ns, not significant.