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About half of all melanomas harbor a mutation that results in a constitutively active BRAF kinase mutant (BRAF^{V600E/K}) that can be selectively inhibited by targeted BRAF inhibitors (BRAFi). While patients treated with BRAFi initially exhibit measurable clinical improvement, the majority of patients eventually develop drug resistance and relapse. Here, we observed marked elevation of *WNT5A* in a subset of tumors from patients exhibiting disease progression on BRAFi therapy. *WNT5A* transcript and protein were also elevated in BRAFi-resistant melanoma cell lines generated by long-term in vitro treatment with BRAFi. RNAi-mediated reduction of endogenous *WNT5A* in melanoma decreased cell growth, increased apoptosis in response to BRAFi challenge, and decreased the activity of prosurvival AKT signaling. Conversely, overexpression of *WNT5A* promoted melanoma growth, tumorigenesis, and activation of AKT signaling. Similarly to *WNT5A* knockdown, knockdown of the WNT receptors FZD7 and RYK inhibited growth, sensitized melanoma cells to BRAFi, and reduced AKT activation. Together, these findings suggest that chronic BRAF inhibition elevates *WNT5A* expression, which promotes AKT signaling through FZD7 and RYK, leading to increased growth and therapeutic resistance. Furthermore, increased *WNT5A* expression in BRAFi-resistant melanomas correlates with a specific transcriptional signature, which identifies potential therapeutic targets to reduce clinical BRAFi resistance.

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WNT5A enhances resistance of melanoma cells to targeted BRAF inhibitors

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About half of all melanomas harbor a mutation that results in a constitutively active BRAF kinase mutant (BRAF^{V600E/K}) that can be selectively inhibited by targeted BRAF inhibitors (BRAFi). While patients treated with BRAFi initially exhibit measurable clinical improvement, the majority of patients eventually develop drug resistance and relapse. Here, we observed marked elevation of WNT5A in a subset of tumors from patients exhibiting disease progression on BRAFi therapy. WNT5A transcript and protein were also elevated in BRAFi-resistant melanoma cell lines generated by long-term in vitro treatment with BRAFi. RNAi-mediated reduction of endogenous WNT5A in melanoma decreased cell growth, increased apoptosis in response to BRAFi challenge, and decreased the activity of pro-survival AKT signaling. Conversely, overexpression of WNT5A promoted melanoma growth, tumorigenesis, and activation of AKT signaling. Similarly to WNT5A knockdown, knockdown of the WNT receptors FZD7 and RYK inhibited growth, sensitized melanoma cells to BRAFi, and reduced AKT activation. Together, these findings suggest that chronic BRAF inhibition elevates WNT5A expression, which promotes AKT signaling through FZD7 and RYK, leading to increased growth and therapeutic resistance. Furthermore, increased WNT5A expression in BRAFi-resistant melanomas correlates with a specific transcriptional signature, which identifies potential therapeutic targets to reduce clinical BRAFi resistance.

Introduction

Over two-thirds of melanomas exhibit activating mutations in the MAPK pathway—activating enzymes NRAS and BRAF, which include BRAF^{V600E} and to a lesser extent BRAF^{V600K} (1, 2). Small molecules such as vemurafenib and dabrafenib have been developed to specifically target mutant BRAF^{V600E}, although they also exhibit activity against BRAF^{V600K} (3, 4). Use of these BRAF inhibitors (BRAFi) in metastatic melanoma patients with activating BRAF mutations has resulted in unprecedented decreases in tumor burden and improvement in overall survival (5–7). Unfortunately, the majority of patients develop resistance to BRAFi and subsequently relapse (5–8). Studies to date on BRAFi-resistant melanoma cells have focused largely on reactivation of the MAPK signaling pathway downstream of BRAF through somatic mutations in MAPK pathway members including MEK and NRAS (9, 10), and by splice variants in BRAF (11). While patients on combination therapies involving a BRAFi plus a MEK inhibitor (MEKi) exhibit significantly higher response rates than patients on a BRAFi alone, the observation of resistance in patients on combined BRAFi/MEKi therapy suggests that factors independent of MAPK reactivation are also involved in the development of therapeutic resistance (12, 13). For instance, BRAFi-resistant melanoma cells exhibit aberrations in the expression or activity of receptor tyrosine kinases such as PDGFR β , VEGFR, EGFR, and IGFR (9, 14, 15), as well as changes in the expression of pro- and antiapoptotic proteins (16–18).

The WNT gene family encodes secreted proteins that act as ligands to stimulate β -catenin-dependent and β -catenin-independent sig-

naling pathways. While most cancer research has focused on the WNT/ β -catenin signaling pathway, numerous studies now identify aberrations in β -catenin-independent WNT pathways in melanoma, most notably those activated by WNT5A (19, 20).

Recent studies have reported that altered WNT/ β -catenin signaling can change the sensitivity of tumor cells to therapeutic drugs (21–24), yet potential roles for β -catenin-independent WNT signaling in drug resistance are not well understood. In the present study, we found that WNT5A protein and transcript levels were dramatically increased in BRAFi-resistant cell lines and in patient tumors. Functional studies revealed that endogenous WNT5A was required for the growth and survival of naive melanoma cells that had not been exposed to BRAFi. WNT5A was also required for the proliferation and survival of melanoma cells that have acquired resistance to BRAFi due to chronic drug treatment. In pursuing the underlying mechanisms that might account for these effects of WNT5A on melanoma cells, we found that WNT5A activated PI3K/AKT signaling, another critical pathway in melanoma proliferation and resistance to apoptosis (25, 26). Further mechanistic studies suggested that WNT5A promotes melanoma growth and survival via its receptors RYK and FZD7. Collectively, these studies revealed an unsuspected role for WNT5A-dependent signaling in promoting the resistance of melanoma cells to BRAFi.

Results

Chronic inhibition of BRAF^{V600E} with PLX4720 elevates WNT5A expression. Previous expression microarray profiling revealed that melanoma cell lines inherently insensitive to low doses of a BRAFi (PLX4032) express elevated levels of WNT5A (27). In the present study, we first asked whether WNT5A expression is similarly increased in melanoma cells exhibiting de novo resistance to chronic inhibition of

Conflict of interest: The authors have declared that no conflict of interest exists.

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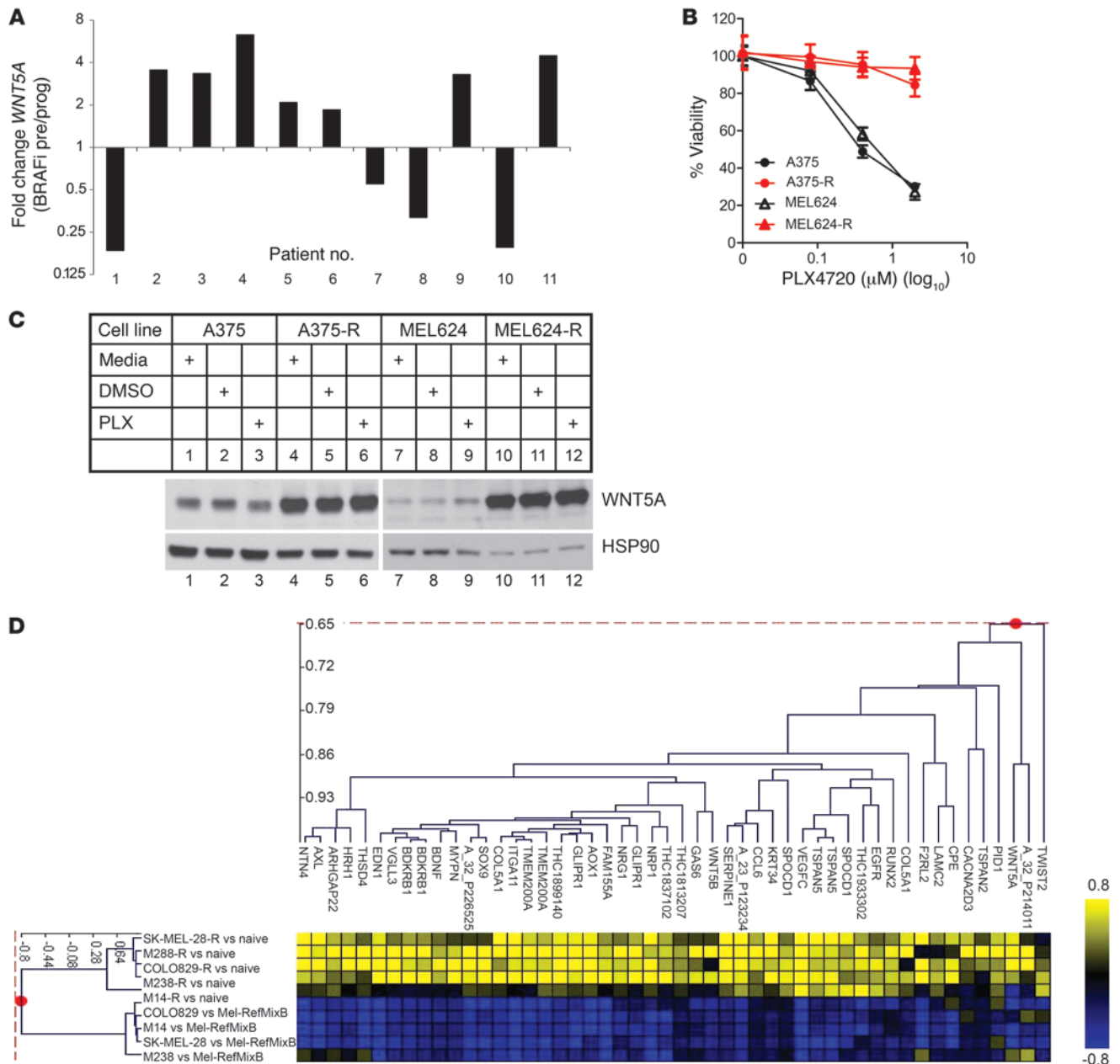


Figure 1 WNT5A expression is increased in response to chronic inhibition of BRAF^{V600E} with PLX4720. (A) Fold change in WNT5A mRNA expression normalized to GAPDH in BRAFi-resistant patient tumors (as marked by disease progression [prog]) compared with paired tumor biopsies collected before the start of treatment (pre). (B) Normalized viability of A375 and MEL624 parental and PLX-resistant cells (-R) after treatment with either medium, DMSO, or increasing doses of PLX4720 for 48 hours. Resistant cell lines (A375-R and MEL624-R) were derived by treatment of melanoma cells with 2 μM PLX4720 for more than 10 weeks. (C) Western blots of lysates from A375, A375-R, MEL624, and MEL624-R cells after either culturing in standard growth medium or treatment with vehicle control (DMSO) or 2 μM PLX4720 for 2 days. (D) Hierarchical clustering of gene expression in PLX-resistant (-R) and PLX-naive cells displaying the subset of genes that significantly correlate with WNT5A expression. The top 5 rows represent relative expression changes in naive cell lines compared with BRAFi-resistant (-R) cell lines generated by chronic treatment with PLX4032. The lower 5 rows display the relative expression of these genes in naive melanoma cells in comparison with a reference data set determined as an average of the expression profiles of 47 different melanoma cell lines (Mel-RefMixB).

BRAF^{V600E}. Specifically, we used real-time quantitative RT-PCR (qRT-PCR) to monitor the relative levels of WNT5A transcripts in melanoma tumors from patients who developed resistance to BRAF^{V600E}-targeted therapies (as measured by disease progression) and compared expression with samples taken before the start of

treatment. We found that WNT5A expression was increased in a subset of BRAFi-resistant patient tumors (7 of 11) compared with pretreated samples (Figure 1A). In this small patient cohort, we did not observe a significant association between WNT5A expression and either melanoma-specific survival ($P = 0.059$) or progression-

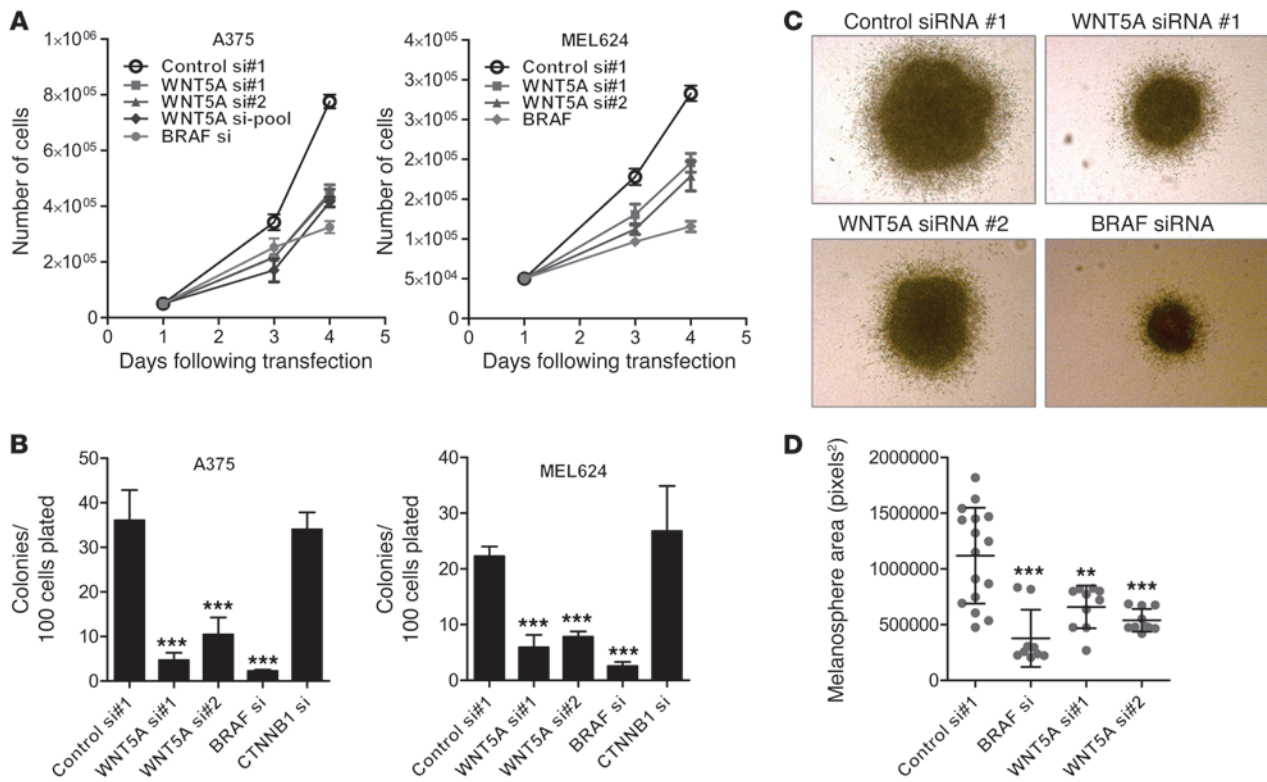


Figure 2

WNT5A loss of function reduces melanoma cell number. **(A)** Growth curves of A375 (left) and MEL624 (right) melanoma cells transfected with control siRNA #1, WNT5A siRNAs #1 and #2, and BRAF siRNA as a positive control. Error bars indicate SD calculated from 3 independent experiments with triplicate wells counted for each time point. **(B)** Average number of colonies per 100 A375 (left) or MEL624 (right) melanoma cells plated in soft agar, scored 1 week after siRNA transfection and embedding in soft agar. Error bars indicate SD calculated from 3 independent experiments with duplicate wells for each siRNA. **(C)** Representative photographs of A375 cells grown in 3D Collagen I culture after siRNA transfection. **(D)** Quantification of A375 spheroid area at day 4 following siRNA transfection. Each point represents the area of a single melanosphere determined by image analysis. Center bars indicate the mean melanosphere area, and error bars show the SD. See Methods for further details on measurement procedure. Statistically significant differences in colony formation in soft agar and in melanosphere size were calculated using 1-way ANOVA. (** $P < 0.01$, *** $P < 0.001$.)

free survival ($P = 0.068$). Another independent study has similarly observed increased WNT5A expression in patient melanomas exhibiting resistance to BRAFi, suggesting that upregulation of WNT5A is a common molecular aberration in drug-resistant melanoma (28).

To investigate the potential consequences of increased WNT5A in BRAFi-resistant melanomas, we next established BRAFi-resistant melanoma cell lines in culture. A375 and MEL624 human melanoma cells were treated with 2 μ M of the BRAFi PLX4720 for more than 10 weeks to generate “resistant” cell lines (subsequently referred to as A375-R and MEL624-R). We validated the resistance of these cells to BRAFi by treating equal numbers of parental and resistant cells with increasing doses of BRAFi for 3 days and then determining cell viability. BRAFi treatment decreased the viability of parental cells in a dose-dependent manner as previously reported, yet did not affect the growth of either A375-R or MEL624-R cells (Figure 1B), confirming the insensitivity of these cells to BRAFi treatment.

We then analyzed the expression of WNT5A protein in both parental and resistant cell lines. Immunoblots revealed a dramatic increase in levels of WNT5A in the resistant cells (Figure 1C). Returning the resistant cells to either normal medium or medium containing vehicle (DMSO) did not diminish the high levels of

WNT5A observed in BRAFi-resistant samples (Figure 1C), suggesting that the upregulation of WNT5A in resistant cells does not require the continual presence of BRAFi. WNT5A protein was similarly increased following knockdown of endogenous BRAF with siRNA (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI70156DS1), indicating that increased WNT5A expression is a consequence of loss of BRAF activity rather than an off-target effect of PLX4720 treatment.

We next asked whether WNT5A levels are increased in additional melanoma cell lines as a consequence of long-term BRAF^{V600E} inhibition. We used microarray profiling to analyze changes in gene expression in COLO829, SK-MEL-28, M288, M238, and M14 melanoma cell lines chronically treated with BRAFi (PLX4032). Although baseline expression of WNT5A varied between different melanoma cell lines (Supplemental Figure 1B), chronic treatment with BRAFi correlated with elevated WNT5A in 4 of 5 additional cell lines tested (Figure 1D). We further analyzed the expression profiles of these BRAFi-resistant cell lines using hierarchical clustering in order to identify additional transcripts with expression profiles similar to that of WNT5A. The top 50 genes significantly correlating with WNT5A expression included transcription factors

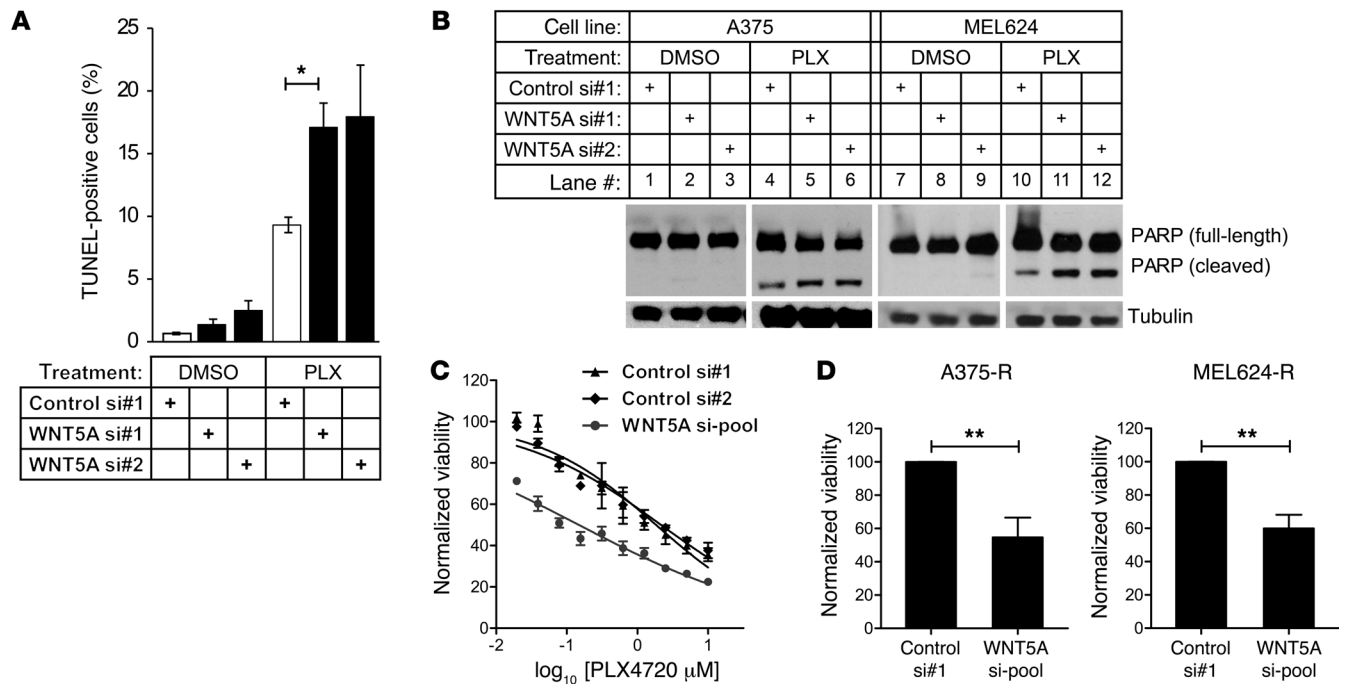


Figure 3

WNT5A loss of function decreases the viability of both PLX-naive and PLX-resistant melanoma cells. (A) Quantification of the percentage of A375 cells that were apoptotic (TUNEL⁺) after siRNA transfection and treatment with either DMSO or 2 μM PLX4720 for 2 days. Error bars indicate the SD. (**P* < 0.05, 2-tailed *t* test.) (B) Western blot analysis of lysates from A375 (lanes 1–6) or MEL624 cells (lanes 7–12) transfected with the indicated siRNAs and then treated 2 days later with either DMSO (lanes 1–3, 7–9) or 2 μM PLX4720 (lanes 4–6, 10–12) for 48 hours. Blots were probed with either PARP antibody or β-tubulin antibody as a loading control. (C) Normalized viability of A375 cells after transfection with siRNAs followed by treatment with increasing doses of PLX4720 for 2 days. Normalized viability was determined with the mock + DMSO condition set at 100%. Error bars show SD calculated from 3 independent experiments. Nonlinear best fit curves were obtained using GraphPad software (control siRNA #1, *R*² = 0.9472; control siRNA #2, *R*² = 0.9144; and WNT5A siRNA pool, *R*² = 0.9016). (D) Normalized viability of A375-R (left) and MEL624-R (right) cells that were plated in medium containing 2 μM PLX4720 48 hours after siRNA transfection and allowed to grow for 3 days. Data were normalized to the control siRNA #1 condition set to 100%, and error bars indicate SD of 3 independent experiments. (***P* < 0.01, 2-tailed *t* test.)

such as *SOX9*, *RUNX2*, and *TWIST2*, extracellular matrix components such as *COL5A1* and *LAMC*, and proteins involved in signal transduction such as *VEGFC* and *EGFR* (Figure 1D). These results suggest that increased *WNT5A* expression is induced in BRAFi-resistant cell populations as part of a unique transcriptional program associated with drug resistance.

We observed increased *WNT5A* expression in both patient tumors that developed resistance to BRAF-targeted therapy and multiple cultured melanoma cell lines chronically treated with BRAFi *in vitro*. Our finding that *WNT5A* levels were increased in melanoma cells grown *in vitro* suggests that increased *WNT5A* observed in patient tumors is derived from the tumor tissue rather than other cells in the tumor microenvironment. This hypothesis is further supported by a recent study reporting increased expression of *WNT5A* by immunohistochemistry in the tumor cells in melanoma biopsies from patients showing a poor clinical response to BRAFi compared with patients who initially responded well to BRAFi (28).

WNT5A loss of function reduces melanoma cell growth. The observation that *WNT5A* expression was elevated in BRAFi-resistant melanomas raises the question of whether *WNT5A* functionally contributes to the growth and survival of melanoma cells. To address this question we determined whether knockdown of endogenous *WNT5A* regulates melanoma growth. For these studies we used 2 independent *WNT5A* siRNAs that consistently reduce levels

of endogenous *WNT5A* protein and transcripts (Supplemental Figure 1, A and C). *WNT5A* knockdown inhibited the growth of both A375 and MEL624 cells to a similar degree to BRAF knockdown (Figure 2A). To test for the specificity of these siRNAs, we performed a rescue experiment by treating siRNA-transfected cells with *WNT5A*-conditioned medium. *WNT5A*-conditioned medium partially rescued growth inhibition induced by *WNT5A* knockdown (Supplemental Figure 1D). We then asked whether reducing endogenous *WNT5A* with siRNA also reduces melanoma growth in anchorage-independent growth assays (29). *WNT5A* siRNAs reduced colony formation in soft agar by more than 80% in both A375 and MEL624 cells and similarly reduce melanosphere growth (Figure 2, B–D).

WNT5A loss of function decreases the viability of BRAFi-resistant melanoma cells. We then determined whether *WNT5A*-dependent signaling also contributes to melanoma cell viability in the presence of BRAFi. siRNA knockdown of *WNT5A* enhanced the apoptotic response induced by short-term cotreatment with BRAFi, as indicated by a significantly increased proportion of cells labeled by TUNEL and by an increase in the abundance of cleaved PARP, an additional marker of apoptosis (Figure 3, A and B). Since a relatively small percentage (~10%–25% TUNEL⁺) of melanoma cells underwent apoptosis under these conditions, we also tested whether combining *WNT5A* siRNAs with BRAFi can reduce cell



viability as measured using resazurin, a metabolic indicator (see Methods). We found that pooled *WNT5A* siRNA reduced A375 cell viability at all doses of BRAFi (PLX4720) to 40%–50% of levels observed following mock transfection or transfection with control siRNAs (#1 and #2) (Figure 3C). As *WNT5A* expression was elevated in BRAFi-resistant melanoma cells (Figure 1), we then asked whether *WNT5A* is also necessary for the viability of the BRAFi-resistant A375-R and MEL624-R cells. Similarly to the naive cell lines, transfection with pooled *WNT5A* siRNA decreased viability of both MEL624-R and A375-R cells when grown in PLX4720-containing medium (Figure 3D). As 2 independent *WNT5A* siRNAs similarly inhibited the growth of A375-R cells (see below), reduced A375-R and MEL624-R cell numbers observed following transfection with pooled *WNT5A* siRNAs are therefore not likely to be due to an off-target effect of a single siRNA. Collectively, these data suggest that *WNT5A* is required for optimal growth and survival of both naive and BRAFi-resistant melanoma cells.

Overexpression of *WNT5A* enhances melanoma cell growth. Since loss of endogenous *WNT5A* reduces the growth and viability of both naive and BRAFi-resistant melanoma cells, we next asked whether *WNT5A* overexpression is sufficient to promote melanoma growth. We infected melanoma cells expressing low levels of endogenous *WNT5A* (Supplemental Figure 1B) with lentiviruses to overexpress either GFP as a control or *WNT5A* and then monitored the expression of exogenous *WNT5A* by immunoblotting (Figure 4A). As we predicted, *WNT5A* overexpression promoted the growth of both A2058 and A375 cells in soft agar, as *WNT5A*-expressing cells produced both an increased number of colonies compared with GFP control cells and larger colonies than control cells (Figure 4, B–D). Since the growth of cancer cells in soft agar involves the clonogenic expansion of individual cells, we hypothesized that *WNT5A* might promote the survival of single melanoma cells. To test this hypothesis, we plated single SK-MEL-28 melanoma cells in 96-well low-attachment plates and allowed these cells to grow undisturbed for 1 week. Approximately 30% of the single GFP control cells survived and proliferated to form colonies (Figure 4E), while a significantly higher percentage of the *WNT5A*-expressing cells (48%) formed colonies in this assay ($P < 0.001$).

Given that *WNT5A* overexpression enhanced the growth of melanoma cells in vitro, we then asked whether *WNT5A* can also promote tumorigenesis in vivo using a xenograft model. We transplanted 50,000 GFP- or *WNT5A*-transduced A2058 cells s.c. in the flanks of immunocompromised nude mice and then monitored the formation of tumors. Overexpression of *WNT5A* decreased the time to palpable tumor formation following cell transplantation (Figure 4F). Together, these results reveal that overexpression of *WNT5A* is sufficient to promote the growth of melanoma cells in vitro and enhances the tumorigenic potential of melanoma cells in vivo.

***WNT5A* negatively regulates the WNT/ β -catenin pathway in melanoma.** In order to gain further insight into the observed role for *WNT5A* in promoting the viability of both naive and BRAFi-resistant melanomas, we then focused on identifying signaling pathways acting downstream of *WNT5A* in melanoma. We and others have recently shown that activation of WNT/ β -catenin-dependent signaling with WNT3A can inhibit the proliferation of melanoma cells in vitro and in vivo (24, 30, 31). Since several studies indicate that *WNT5A* can act as a negative regulator of WNT/ β -catenin-dependent transcription (32, 33), these data suggest that *WNT5A* might promote melanoma growth by inhibiting WNT/ β -catenin signaling. We

monitored WNT/ β -catenin signaling using a luciferase reporter of β -catenin-dependent transcription (“pBAR”) (34) and found that WNT3A-conditioned medium but not *WNT5A*-conditioned medium enhanced β -catenin-dependent transcription in both A375 and A2058 melanoma cells (Supplemental Figure 2, A and B). As a control, we confirmed that activation of this luciferase reporter activity was due to increased activity of the β -catenin pathway by blocking reporter activation with a siRNA targeting *CTNNB1* (encodes for β -catenin). Knocking down β -catenin effectively reduced pBAR reporter activity induced by WNT3A (Supplemental Figure 2C).

Since A375 cells endogenously expressed *WNT5A*, we then asked whether reducing *WNT5A* by siRNA transfection can either enhance or inhibit the activation of β -catenin-dependent transcription induced by WNT3A. In contrast to the *CTNNB1* siRNA, knockdown of endogenous *WNT5A* with multiple individual siRNAs increased the sensitivity of A375 cells to stimulation with WNT3A-conditioned medium as indicated by a leftward curve shift (Supplemental Figure 2C). These data revealed that endogenous *WNT5A* acts as a negative regulator of the WNT/ β -catenin pathway in A375 cells. We then asked whether exogenous *WNT5A* can also negatively regulate β -catenin-dependent transcription. As we predicted, adding *WNT5A*-conditioned medium inhibited β -catenin-dependent transcription induced by WNT3A in multiple human melanoma cell lines (Supplemental Figure 2D). Consistent with previous studies involving other cell and tissue types (32, 35), these data suggest that *WNT5A* negatively regulates the WNT/ β -catenin pathway in melanoma.

Since *WNT5A* promoted melanoma growth and survival (Figures 2–4), whereas WNT/ β -catenin signaling inhibits melanoma growth and survival (24, 30, 31), we then determined the consequences of simultaneously activating WNT/ β -catenin-dependent signaling with WNT3A-conditioned medium and inactivating *WNT5A*-dependent signaling by siRNA-mediated knockdown of *WNT5A*. Either knockdown of *WNT5A* with siRNAs or treatment with WNT3A-conditioned medium resulted in reduced A375 cell number (Supplemental Figure 3A). Cotreatment with WNT3A-conditioned medium and *WNT5A* siRNAs further reduced A375 cell proliferation compared with either treatment alone (Supplemental Figure 3A). These data revealed that a shift from elevated WNT/ β -catenin signaling to elevated *WNT5A*-dependent signaling can result in increased melanoma growth and survival.

Cross-talk between WNT/ β -catenin-dependent signaling and *WNT5A*-dependent signaling does not account for decreased melanoma proliferation induced by *WNT5A* siRNAs. Based on previous reports suggesting that *WNT5A* can act as a negative regulator of WNT/ β -catenin signaling, we next tested our hypothesis that *WNT5A* loss of function might inhibit melanoma cell growth by promoting WNT/ β -catenin-dependent signaling. We determined the consequences of blocking the activation of WNT/ β -catenin-dependent transcription induced by *WNT5A* siRNA by treating cells with XAV939, a small-molecule inhibitor of WNT/ β -catenin signaling (36). XAV939 treatment efficiently blocked β -catenin-dependent transcription induced by *WNT5A* siRNAs (Supplemental Figure 3B). However, cotreatment with XAV939 and *WNT5A* siRNAs did not prevent the reduction in melanoma cell number induced by *WNT5A* loss of function (Supplemental Figure 3C). These results suggest that *WNT5A* acts as a negative regulator of the WNT/ β -catenin signaling pathway, yet increased activity of the WNT/ β -catenin signaling pathway does not cause the growth defects observed following *WNT5A* knockdown.

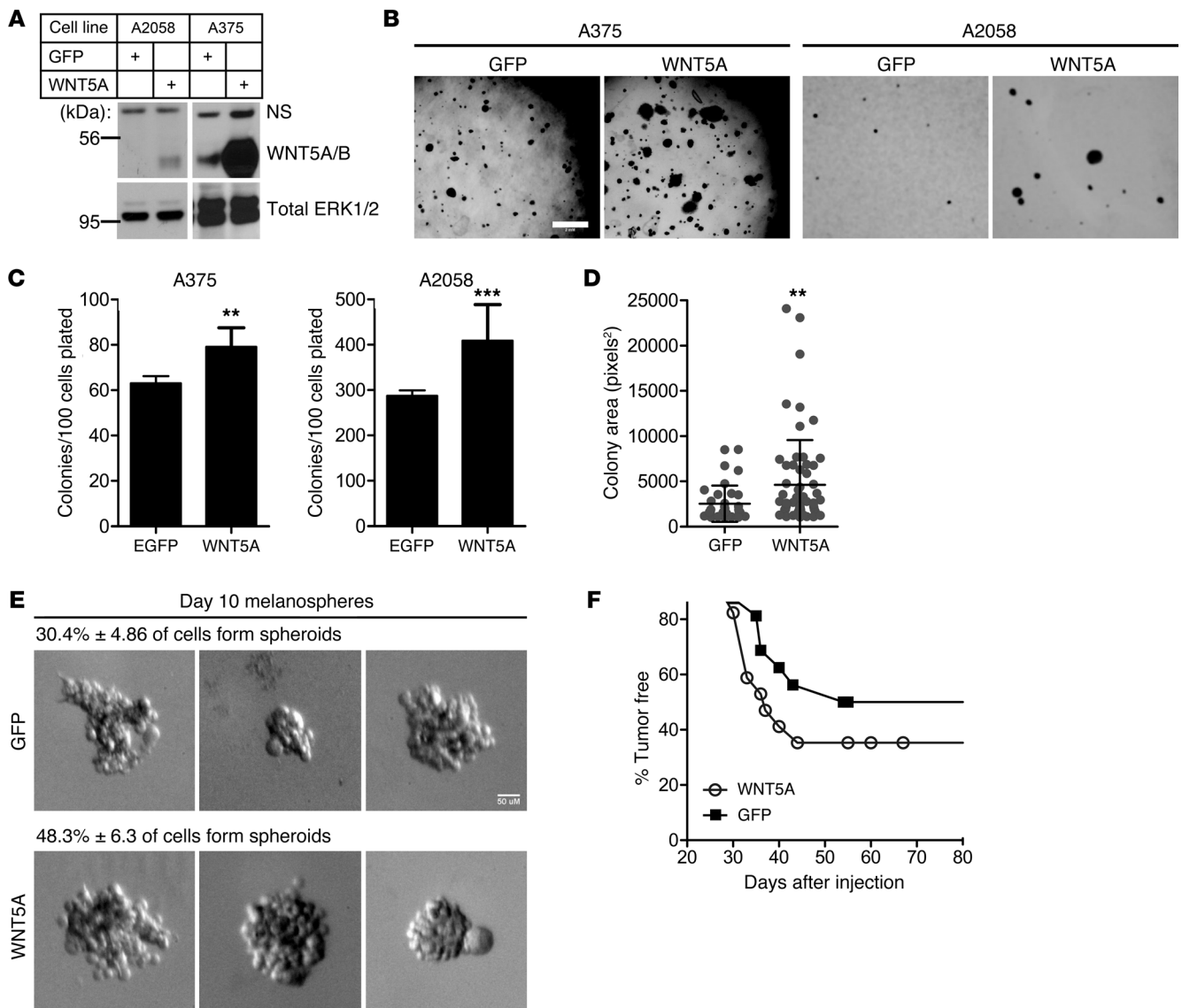


Figure 4
 WNT5A overexpression enhances melanoma cell proliferation. (A) Representative Western blots of lysates collected from A2058 and A375 stable cell lines generated by infection with either WNT5A-IRES-GFP or IRES-GFP control lentiviruses. Blots were probed with antibodies to detect the expression of WNT5A and total ERK1/2. (B) Representative photographs of colonies formed 3 weeks after embedding of 100 A375 cells or 1,000 A2058 cells in soft agar. (C) Average number of colonies formed in anchorage-independent growth conditions per 100 A375 cells (left) or 1,000 A2058 cells (right) plated in soft agar. (D) Quantification of A2058 soft agar colony area. Each point represents the area of a single colony determined by image analysis. Center bars indicate the mean colony area, and error bars show the SD. Statistical significance of differences in colony number and melanosphere size was calculated using a 2-tailed Student's *t* test. (***P* < 0.01, ****P* < 0.001.) (E) Summary of the spheroid-forming potential of SK-MEL-28 cells infected with either WNT5A or GFP control lentiviral particles. Representative photographs of melanospheres taken 10 days after plating of single cells are included below the table summarizing the proportion of sphere-forming cells compiled from 3 independent experiments. (F) Kaplan-Meier survival curve indicating the tumor-free survival of mice injected with 50,000 A2058 melanoma cells expressing either WNT5A or GFP controls.

WNT5A enhances AKT pathway activity in melanoma. Since WNT5A did not appear to promote melanoma growth and survival by acting as an inhibitor of WNT/β-catenin signaling, we then focused on identifying other signaling pathways that might be modulated by WNT5A in melanoma. We next determined whether WNT5A enhances melanoma cell growth via kinase-dependent signaling pathways. For these experiments, we monitored the phosphorylation of different kinases in A375 cells by

transfecting the cells with either control or WNT5A siRNAs and then subjecting them to a short time course of serum stimulation (Figure 5A). As a positive control, we examined the phosphorylation of PKC, which is normally induced by WNT5A in melanoma (37, 38). As expected, transfection of A375 cells with both pooled WNT5A siRNAs and individual WNT5A siRNAs #1 and #2 reduced the phosphorylation of PKC in comparison with control siRNA (Figure 5A).

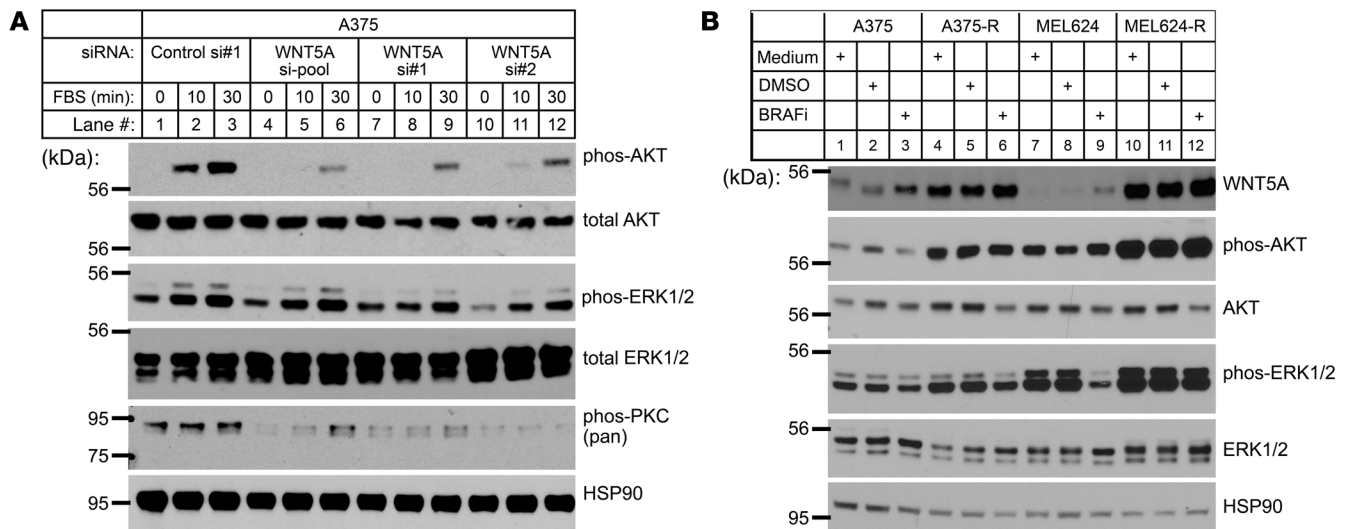


Figure 5 WNT5A enhances AKT pathway activity in melanoma. (**A** and **B**) Cell lysates analyzed by Western blotting. Blots were probed with antibodies to detect total ERK, AKT, and HSP90 proteins as well as the phosphorylated forms of ERK1/2 (Thr202, Tyr204), AKT (Ser473), and PKC (pan-phospho- β II Ser660). In **A**, melanoma cell lysates were collected after transfection of A375 cells with control siRNA #1 (lanes 1–3) or WNT5A siRNAs (lanes 4–12) followed by stimulation with 5% FBS for 0, 10, or 30 minutes. In **B**, protein lysates were collected from either naive A375 cells (lanes 1–3), A375-R cells (lanes 4–6), naive MEL624 cells (lanes 7–9), or MEL624-R cells (lanes 10–12) that were treated with either medium, DMSO, or 2 μ M PLX4720 for 24 hours before sample collection.

Since the cell lines used in this study have activating BRAF mutations that lead to constitutively high levels of ERK phosphorylation, we were not surprised to find that WNT5A siRNAs neither enhanced nor reduced the phosphorylation of ERK1 and ERK2 (Figure 5A). In contrast, WNT5A knockdown reduced AKT phosphorylation at Ser473 induced by serum stimulation (Figure 5A). Loss of WNT5A resulted in a similar delay in AKT phosphorylation induced by serum in MEL624 cells (Supplemental Figure 4A), which suggests that these observed changes in protein kinase activity are not specific to 1 cell line. We also noted that WNT5A siRNAs reduced the phosphorylation of kinases that are downstream targets of AKT, including S9 and GSK3 β (Supplemental Figure 4B). As reducing the levels of WNT5A led to reduced phosphorylation of AKT, we then tested whether overexpression of WNT5A conversely promotes the phosphorylation of AKT. In support of this prediction, we found that exogenous WNT5A enhanced AKT phosphorylation at Ser473 in both UACC1273 and A2058 melanoma cells (Supplemental Figure 4C). As a positive control we monitored the phosphorylation of PKC by immunoblotting and found that WNT5A overexpression enhanced the phosphorylation of PKC in UACC1273 cells, as previously reported (38, 39), but did not regulate PKC phosphorylation in A2058 cells (Supplemental Figure 4C).

One important caveat of these experiments using cell lines constitutively overexpressing WNT5A is that it is difficult to determine whether activation of downstream signaling events is due to either a direct effect of increased WNT5A or an indirect effect of artificially overexpressing WNT5A, such as a gradual rewiring of kinase network activity. We then asked whether treatment with recombinant WNT5A is sufficient to promote AKT phosphorylation in melanoma. For these experiments we serum-starved A2058 cells, which lacked endogenous WNT5A expression (Supplemental Figure 1B), and then treated them with 100 ng/ μ l recombinant human WNT5A (r-hWNT5A) for 10, 30, or 60 minutes and treated

control wells with CHAPS (a vehicle control). r-hWNT5A induced both increased phospho-PKC (a positive control) and increased phospho-AKT after 10 or 30 minutes of stimulation, and this response tapered off after 60 minutes (Supplemental Figure 5). We did not observe strong differences in levels of phosphorylated JNK using this assay (Supplemental Figure 5, bottom), which suggests that r-hWNT5A does not simply induce a global increase in protein phosphorylation, but regulates specific signaling pathways. These results showing that exogenous WNT5A can promote the phosphorylation of AKT are consistent with our data showing that knockdown of WNT5A decreased AKT phosphorylation in response to serum stimulation and that exogenous WNT5A enhanced AKT phosphorylation (Figure 5A, Supplemental Figure 4, A–C, and Supplemental Figure 5).

To address whether WNT5A-dependent phosphorylation of AKT requires PI3K, we pretreated UACC1273 cells infected with lentiviruses to express either control protein (GFP) or WNT5A-IRES-GFP with LY294002, a potent PI3K inhibitor (40), and then stimulated these cells with a short time course of serum to activate PI3K/AKT signaling. Consistent with our above results (Supplemental Figure 4C), we found that overexpression of WNT5A increased AKT phosphorylation (Supplemental Figure 6). Treatment with LY294002 completely blocked AKT phosphorylation in both GFP control and WNT5A-IRES-GFP-expressing cells (Supplemental Figure 6), which suggests that the increased phosphorylation of AKT induced by WNT5A requires PI3K activity.

Our results showing that both exogenous and endogenous WNT5A enhanced the phosphorylation of AKT predict that the elevated levels of WNT5A observed in BRAFi-resistant melanomas might similarly promote the activity of PI3K/AKT signaling. We therefore analyzed the phosphorylation status of various kinases in our BRAFi-resistant cell lines. As expected, the phosphorylation of ERK1/2 was decreased by PLX4720 treatment in naive A375 and

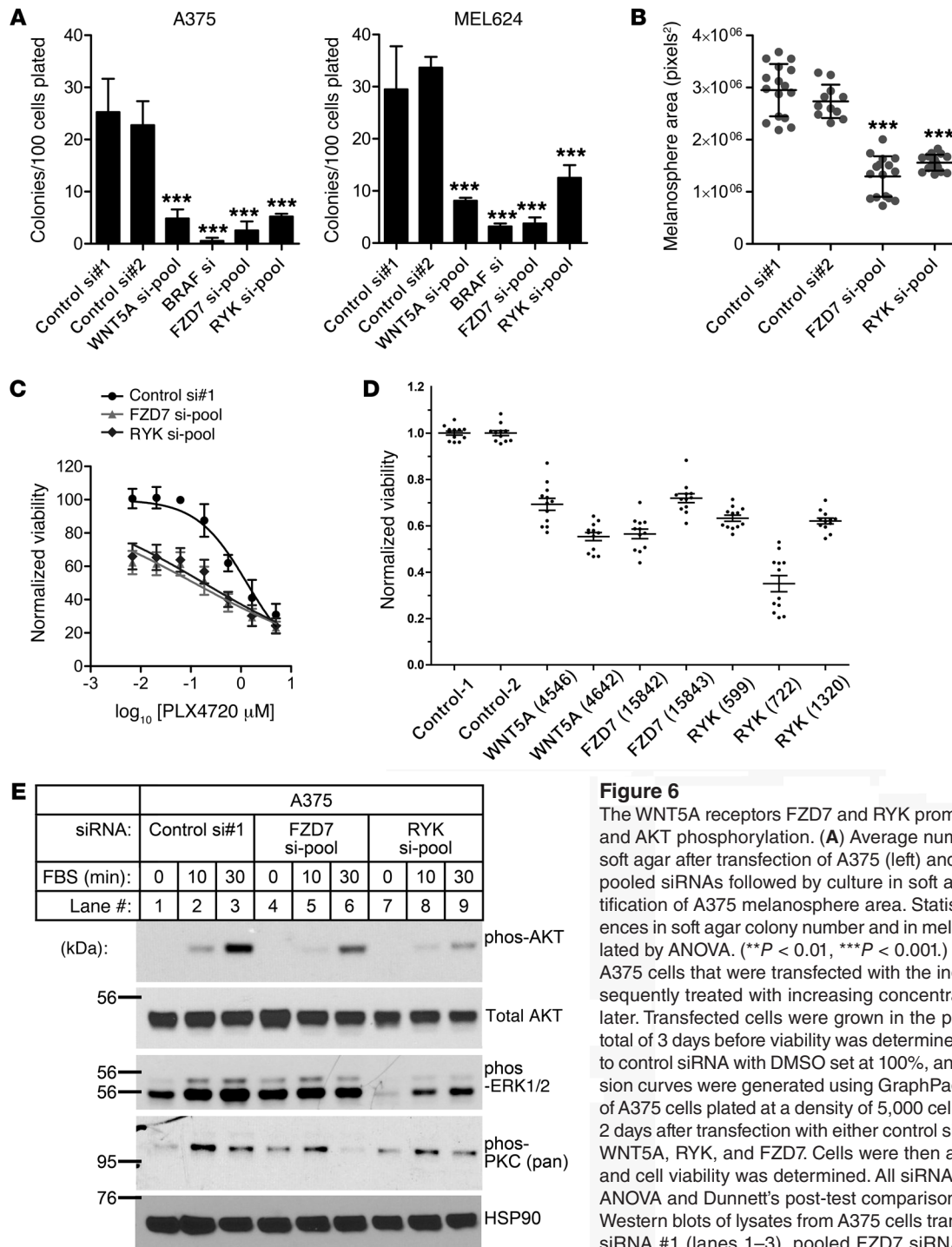


Figure 6

The WNT5A receptors FZD7 and RYK promote melanoma cell growth and AKT phosphorylation. (A) Average number of colonies formed in soft agar after transfection of A375 (left) and MEL624 (right) cells with pooled siRNAs followed by culture in soft agar for 1 week. (B) Quantification of A375 melanosphere area. Statistical significance of differences in soft agar colony number and in melanosphere size was calculated by ANOVA. (***P* < 0.01, ****P* < 0.001). (C) Normalized viability of A375 cells that were transfected with the indicated siRNAs, then subsequently treated with increasing concentrations of PLX4720 2 days later. Transfected cells were grown in the presence of PLX4720 for a total of 3 days before viability was determined. Values were normalized to control siRNA with DMSO set at 100%, and nonlinear, best fit regression curves were generated using GraphPad. (D) Average cell viability of A375 cells plated at a density of 5,000 cells per well in 96-well plates 2 days after transfection with either control siRNAs or siRNAs targeting WNT5A, RYK, and FZD7. Cells were then allowed to grow for 3 days, and cell viability was determined. All siRNAs are *P* < 0.0001 by 1-way ANOVA and Dunnett's post-test comparison with control columns. (E) Western blots of lysates from A375 cells transfected with either control siRNA #1 (lanes 1–3), pooled FZD7 siRNAs (lanes 4–6), or pooled RYK siRNAs (lanes 7–9). Blots were probed with antibodies to detect the phosphorylated forms of ERK1/2 (Thr202, Tyr204), AKT (Ser473), and PKC (pan-phospho-βII Ser660) and HSP90 as a loading control.

MEL624 cells (Figure 5B). In line with previous studies (14, 41–43), we noted an overall increase in the ratio of phosphorylated ERK1/2 to total ERK1/2 in the BRAFi-resistant cell lines compared with naive control cells. We also observed an increase in the ratio of AKT phosphorylated at Ser473 compared with total AKT in both A375-R and MEL624-R cells compared with naive cells (Figure 3B).

These data were consistent with previous reports of hyperactivated PI3K/AKT signaling in BRAFi-resistant melanomas (16, 43, 44).

Several studies have reported that cotreating melanoma cells with BRAF/MAPK and PI3K/AKT inhibitors synergistically reduces cell viability (41, 42, 44–47), suggesting that the BRAF/MAPK and PI3K/AKT pathways cooperate to promote cell growth. We



similarly found that cotreating naive melanoma cells with an IC₅₀ dose of PLX4720 and with increasing doses of either GDC-0941 (a PI3K inhibitor) or BEZ235 (a dual PI3K/mTOR inhibitor) further reduced cell viability compared with treatment with BRAFi alone (Supplemental Figure 7, A and B). As a control, we then asked whether our BRAFi-resistant cell lines also require PI3K/AKT pathway activity for their growth and found that GDC-0941 and BEZ235 also reduced the viability of A375-R and MEL624-R cells (Supplemental Figure 7, C and D). In summary, these results revealed that WNT5A can activate PI3K/AKT signaling that is required for the optimal viability of both naive and BRAFi-resistant cells. Our findings are consistent with the hypothesis that WNT5A enhances the growth and survival of melanoma cells by activating PI3K/AKT signaling.

The WNT5A receptors FZD7 and RYK are highly expressed in melanoma. Previous work has identified multiple putative WNT5A receptors that act in a cell- and tissue type-dependent manner (48, 49). Since previous studies indicate that WNT5A is highly expressed in metastatic melanomas that display poor clinical outcomes (38, 50–52), we reasoned that functional WNT5A receptors would also be highly expressed in melanomas. We analyzed a large microarray data set profiling transcript expression in 950 human tissue samples including multiple normal and cancerous tissues (ArrayExpress, E-MTAB-37). We found that *FZD6* ($P = 4.93 \times 10^{-5}$), *FZD7* (probe 1, $P = 3.84 \times 10^{-9}$; probe 2, $P = 4.94 \times 10^{-10}$), and *RYK* (probe 1, $P = 4.76 \times 10^{-4}$; probe 2, $P = 2.80 \times 10^{-6}$) were significantly elevated in melanoma while other WNT receptors were not (Supplemental Figure 8A). We analyzed additional microarray data sets and found that *FZD7* was highly expressed in both normal skin and melanoma (Supplemental Figure 4B), while *RYK* was overexpressed in melanoma (Supplemental Figure 8B).

siRNAs targeting the WNT receptors FZD4, FZD5, FZD7, and RYK reduce melanoma cell viability and growth. In parallel to our analysis of gene expression profiles, we also carried out functional tests to determine whether any of these WNT receptors contributed to melanoma cell viability in a manner similar to that of WNT5A. As expected from previous results, we found that *BRAF* and *WNT5A* siRNAs reduced A375 cell viability, whereas transfection with either control siRNAs or siRNAs targeting *LRP6* (Supplemental Figure 9H) and β -catenin (24) to block WNT/ β -catenin signaling had no effect on cell viability (Supplemental Figure 10A). Previous reports have suggested that the receptor tyrosine kinases *ROR1* and *ROR2* can act as WNT5A receptors and that they enhance the proliferation of cancer cells (28, 53, 54). We observed efficient knockdown of *ROR1* and *ROR2* transcripts with all 3 siRNAs (Supplemental Figure 9, F and G), yet cell viability decreased with only 1 of 3 *ROR1* and 1 of 3 *ROR2* siRNAs (Supplemental Figure 10A). These results suggest that an off-target effect of a single siRNA rather than variable knockdown of *ROR1* and *ROR2* by different siRNAs probably accounts for the lack of consistent changes in cell number observed following *ROR1* or *ROR2* depletion. In contrast to *ROR1* and *ROR2*, we found that multiple validated siRNAs targeting the WNT5A receptor *RYK* (55) inhibited the growth of A375 melanoma cells (Supplemental Figure 10A).

We also tested whether members of the Frizzled family of receptors found to be highly expressed in melanoma (Supplemental Figure 8A) also regulate the growth of melanoma cells in vitro, by transfecting A375 cells with additional siRNAs targeting *FZD1*, *FZD4*, *FZD5*, *FZD6*, and *FZD7*. We first confirmed that

these siRNAs effectively reduce their target transcripts by qRT-PCR, and found that all siRNAs effectively reduced their target mRNAs (Supplemental Figure 9, A–E). We did not consistently observe a statistically significant change in cell viability following transfection with siRNAs targeting *FZD1* or *FZD6* (Supplemental Figure 10B), as only 1 of 2 *FZD1* siRNAs and 1 of 2 *FZD6* siRNAs significantly reduced A375 cell growth in this assay. We did observe a reduction in cell viability after transfection with multiple, independent *FZD7*-, *FZD4*-, and *FZD5*-targeted siRNAs (Supplemental Figure 10B).

Since *FZD4* and *FZD5* were expressed at very low levels in the melanoma cells used in this study (Supplemental Figure 9, A and B), we decided to focus our further studies on *RYK* and *FZD7*. We further validated that multiple siRNAs targeting both *RYK* and *FZD7* reduced melanoma cell number by manually counting A375 cells following transfection with these siRNAs and observed that multiple, independent siRNAs targeting these receptors decreased cell number (Supplemental Figure 10C). Since loss of WNT5A inhibited melanoma proliferation in 3D culture assays (Figure 2, B–D), we asked whether loss of *FZD7* and *RYK* receptors similarly regulates melanoma cell growth in these assays. Like depletion of WNT5A, depletion of either *FZD7* or *RYK* by transfection with pooled siRNAs inhibited the growth of both A375 and MEL624 melanoma cells when grown in soft agar assays and when grown as melanospheres embedded in Collagen I (Figure 6, A and B).

We then determined whether *RYK* and *FZD7* also regulate melanoma cell growth in the presence of BRAFi. We again treated melanoma cells with increasing concentrations of PLX4720 following transfection with pooled *RYK* and pooled *FZD7* siRNAs. Similar to our findings with reduced WNT5A levels (Figure 3), siRNA-mediated reduction of either *RYK* or *FZD7* further reduced melanoma cell growth at all doses of PLX4720 (Figure 6C). Finally, we asked whether *FZD7* and *RYK* are also required for the growth and survival of BRAFi-resistant melanoma cells. Similarly to the naive cell lines, siRNA knockdown of either *RYK* or *FZD7* using pooled siRNAs decreased the growth of both A375-R and MEL624-R cells, as did knockdown of these receptors using multiple independent siRNAs (Figure 6D and Supplemental Figure 11). Taken together, these results support the hypothesis that WNT5A enhances the growth and viability of both naive and BRAFi-resistant melanoma cells via the receptors *RYK* and *FZD7*.

FZD7 and RYK enhance AKT signaling in melanoma. As our data demonstrated that elevating levels of WNT5A enhanced AKT signaling in melanoma, we predicted that increased expression of receptors for WNT5A, such as *FZD7* and *RYK*, would also regulate AKT signaling in melanoma. Transfection of A375 cells with ssFLAG-*FZD7* enhanced AKT phosphorylation, but not ERK phosphorylation, in comparison with FLAG-GFP (Supplemental Figure 12). Overexpression of *RYK* had little effect. We also investigated whether endogenous *FZD7* and *RYK* can regulate the phosphorylation of AKT in melanoma cells. As with WNT5A siRNAs, transfecting A375 cells with either pooled *RYK* or pooled *FZD7* siRNAs reduced AKT phosphorylation induced by serum stimulation, but did not significantly affect either ERK1/2 or PKC phosphorylation (Figure 6E).

WNT5A is upregulated as part of a gene signature associated with clinical phenotypes in melanoma. Our microarray analysis suggested that WNT5A expression is elevated in concert with a group of other transcripts in BRAFi-resistant versus naive melanoma cell lines (Figure 1D). Since WNT5A loss and gain of function regulated



concurrently with *WNT5A* in BRAFi-resistant melanomas (Figure 1D) also correlate with high *WNT5A* expression in other melanoma cell data sets independently of BRAFi treatment. Together, these data suggest that cells with higher levels of *WNT5A* expression may represent a distinct cellular phenotype that can be either induced or enriched by long-term BRAFi treatment.

Discussion

A more complete understanding of melanoma progression and responses to targeted therapeutics such as BRAFis will be crucial for developing more durable therapies. In this study, we found that *WNT5A* was dramatically upregulated in a subset of BRAFi-resistant melanoma tumors and in BRAFi-resistant melanoma cells derived in vitro. We further showed that *WNT5A* promoted the growth and survival of both naive parental cells and BRAFi-resistant melanoma cells. Previous reports found that high expression of *WNT5A* similarly results in reduced responsiveness of colon cancer cells to histone deacetylase inhibitors (21) and reduced responsiveness of pancreatic cancer cells to gemcitabine (58). Along with these previous studies, our current work highlights an emerging role for *WNT5A*-dependent signaling in mediating drug resistance in cancer.

Resistance to the targeted inhibition of the BRAF/MAPK pathway in melanoma involves perturbations in multiple different signaling axes. BRAFi-resistant melanoma cells often exhibit cross-resistance to other BRAF/MAPK pathway inhibitors such as drugs targeting MEK (44, 59, 60), suggesting that aberrant activity of other signaling pathways and cellular processes can compensate for the loss of BRAF/MAPK signaling. Indeed, many studies have observed increased activity of key mitogenic signaling proteins such as RTKs and AKT in BRAFi-resistant melanomas (9, 14, 15, 47), while other studies report that BRAFi-resistant melanoma cells inactivate apoptotic pathways through downregulation of *BIM* (16) and upregulation of *MDM4*, a negative regulator of TP53 (18). In addition to these cell-autonomous mechanisms of resistance to RAF inhibitors, recent studies suggest an important role for secreted proteins such as HGF in the development of BRAFi resistance (61, 62). The results of the present study add *WNT5A* to this growing list of secreted signaling proteins that may promote both intrinsic and acquired resistance to BRAF/MEK inhibition.

Specific interest in the role of *WNT5A* in melanoma dates back to transcriptional profiling studies that observed elevated levels of *WNT5A* in metastatic melanoma (50). Subsequent immunohistochemistry studies showed that high expression of cytoplasmic *WNT5A* correlates with disease progression, decreased progression-free survival, and decreased overall survival (51). Further mechanistic studies focused largely on the role of *WNT5A* in regulating cell motility through the ROR2/PKC pathway as well as through regulating the polarization of cell adhesion molecules (38, 39, 63). Our results suggested that *WNT5A* can activate multiple signaling pathways in melanoma cells, since we found that *WNT5A* and its receptors FZD7 and RYK all promoted AKT activation. During the course of our studies, another group similarly observed that FZD7 can activate AKT in muscle progenitor cells (64), suggesting that WNT/FZD7/AKT signaling is active in multiple cell types. Although we cannot rule out the possibility that other WNT receptors also contribute to *WNT5A*-dependent cell proliferation and survival in melanoma, these studies represent an initial characterization of a *WNT5A*/AKT pathway that may be therapeutically relevant in metastatic and drug-resistant melanomas.

Our detailed analysis of gene expression in BRAFi-resistant melanoma cells revealed that *WNT5A* was not upregulated in isolation, but rather in concert with numerous other genes as part of a unique gene expression signature. This gene expression signature was also coordinately upregulated in metastatic subsets of melanomas even in the absence of BRAF or MAPK inhibitors (56, 57), which suggests that this gene signature identifies a phenotypic subtype of melanomas that can arise independently of therapeutic intervention. Future studies will address whether the genes upregulated in concert with *WNT5A* in both BRAFi-resistant and metastatic melanomas might collaborate with *WNT5A* in a unified signaling network to promote disease progression and drug resistance. Importantly, we found that *WNT5A* was upregulated not only in BRAFi-resistant cells derived in cell culture, but also in patients who have relapsed on BRAF/MAPK inhibitors. While we did not observe a significant correlation between *WNT5A* expression and survival in the small cohort of 11 patients analyzed in this study, further analysis of larger numbers of patient-derived BRAFi-resistant tumors may reveal whether *WNT5A* expression can predict clinical outcomes with better statistical power. Future studies will also determine whether the gene signature upregulated in concert with *WNT5A* in metastatic and BRAFi-resistant melanomas can more accurately predict clinical responses to BRAF/MAPK inhibition.

On the basis of our analysis of *WNT5A*-dependent signal transduction in cultured melanoma cells, we speculate that high *WNT5A* expression in patient tumors might similarly lead to increased PI3K/AKT activity. It will be interesting to determine whether *WNT5A* expression can serve as a biomarker to predict patient response to the PI3K and AKT inhibitors that are the subject of investigation as therapeutics for metastatic melanoma in ongoing clinical trials. Although it is still challenging to target *WNT5A* signaling pharmacologically, our results suggest that inhibiting this *WNT5A*/RYK/FZD7 pathway might be therapeutically beneficial in melanoma patients who have developed resistance to BRAF/MAPK-targeted therapies. FZD7 has recently garnered attention for its potential connection to the development and progression of multiple cancer subtypes (65). Blocking FZD7 activity with siRNAs or peptides can induce apoptosis and inhibit proliferation in vitro and in vivo in colon, breast, pancreatic, lung, and hepatocellular cancer cells (65–69). Whether these methods of blocking FZD7 activity could be adapted for the treatment of melanomas or other cancers exhibiting chemotherapeutic resistance due to aberrant hyperactivation of *WNT5A*-dependent signaling remains to be explored.

Methods

Analysis of WNT5A expression in melanoma patient samples. Access to patients undergoing early-phase clinical trials with vemurafenib and dabrafenib enabled us to acquire a unique set of triplicate melanoma biopsies before treatment and upon disease progression to study biomarkers of response and resistance to these drugs. Patients with BRAF-mutant metastatic melanoma with at least 2 lesions amenable to excision biopsy before and during systemic therapy with a BRAFi were included in this study. Informed consent was obtained for each patient under approved Human Ethics Review Committee protocols. Excision biopsies were taken within 7 days prior to commencement of the BRAFi (pretreatment samples). Biopsies of new or growing melanoma metastases (progressed or resistant samples) were performed in a subset of patients. The BRAFi was ceased no more than 24 hours before surgery for those requiring a general anesthetic for therapeutic surgical resection of progressed lesions. Patient samples were used to isolate mRNA, which was subsequently converted to cDNA and then



analyzed for levels of *WNT5A* using SYBR Green real-time qRT-PCR. The team performing measurements of *WNT5A* transcript was blinded to the clinical outcome of patients. Statistical analysis for patient outcomes data was performed using IBM SPSS version 21 (SPSS).

Cell culture. Melanoma cell lines used in this study were cultured in various growth media purchased from Life Technologies supplemented with penicillin/streptomycin and FBS. Briefly, A375 cells were cultured in DMEM + 5% FBS, A2058 cells were cultured in DMEM + 10% FBS, SK-MEL-28 cells were grown in MEM + 10% FBS, and COLO829, MEL624, M288, M238, and M14 cells were all cultured in RPMI + 10% FBS. A375 and MEL624 BRAFi-resistant cell lines were generated by growing of these cells in 2 μ M PLX4720 (a BRAFi) for at least 10 weeks. During this process, cells were split once they reached confluence, and PLX4720-containing medium was replenished every 3–4 days. In order to generate additional resistant cell lines, COLO829, SK-MEL-28, M288, M238, and M14 were treated at their growth-adjusted inhibitory concentration of 50% (IC_{50}) of PLX4032 (another BRAFi), or with DMSO as a control. Concentrations of PLX4032 were increased as resistance developed.

siRNA transfection. Melanoma cells were reverse-transfected with 10–20 nM siRNA (final concentration) in 6-well plates with RNAiMAX reagent per the manufacturer's suggestions (Life Technologies). The catalog numbers corresponding to the siRNAs used in this study can be found in Supplemental Table 1.

Generation of stable cell lines. A2058, A375, and UACC1273 melanoma cell lines expressing either IRES-GFP or *WNT5A*-IRES-GFP were generated by lentiviral transfection followed by FACS to isolate GFP⁺ cells (30). SK-MEL-28 cells expressing either GFP or *WNT5A* were generated by infection of these cells with additional lentivirus constructs to express either GFP or *WNT5A* followed by selection with hygromycin.

Analysis of WNT/ β -catenin signaling. Production of *WNT3A*- and *WNT5A*-conditioned medium as well as assays of WNT/ β -catenin-dependent transcriptional activation were conducted using a β -catenin-activated luciferase reporter as previously described (34).

Cell proliferation and viability assays. Melanoma cells were trypsinized and counted 24–48 hours after transfection of the indicated siRNAs and then replated for various growth assays. To assess 2-dimensional growth, 50,000 A375 or MEL624 cells were plated in multiple wells of a 24-well plate 24 hours after siRNA transfection. At various time points following initial plating, cells were trypsinized and counted using a hemocytometer. All growth curves summarize 3 independent experiments performed with triplicate technical replicates for each time point and condition. For rescue experiments, cells were plated in either medium containing 25% control L cells, or 25% *WNT5A*-conditioned medium prepared as previously described. For resazurin-based viability assays, 2,500 to 5,000 cells were plated in 96-well plates 48 hours after siRNA transfection in normal growth medium and allowed to grow for 48–72 hours. At this time, the normal cell culture medium was exchanged with medium containing resazurin (Promega), and plates were incubated for 2.5–3 hours in the tissue culture incubator before assessment of the fluorescent end product (resorufin) using an EnVision plate reader (PerkinElmer).

Three-dimensional cell culture. Soft agar assays were performed by embedding of 100–1,000 siRNA-transfected cells at single-cell density in 0.3% agarose overlaid on a 0.6% base agarose layer in 6-well plates. Macroscopic colonies were scored and colony-forming efficiencies were calculated 6–7 days after single cells were embedded in soft agar. Melanosphere assays were performed as previously described with minor modifications (29). Briefly, 24 hours after transfection of melanoma cells with various siRNAs, 5,000 cells were plated in multiple wells of ultralow-attachment round-bottom 96-well plates (Corning), where they grew into melanospheres. Melanospheres were embedded in Collagen I (BD Biosciences) 48 hours

later and imaged 24 hours after embedding. Melanosphere areas were determined using ImageJ (NIH) to measure the diameter of each sphere from micrographs. All measurements were done blinded to avoid potential experimenter bias.

In vivo tumorigenesis. Fifty thousand A2058 cells expressing either GFP or *WNT5A*-IRES-GFP were injected s.c. into the flanks of nude *FoxNI*^{-/-} mice (Harlan Laboratories). Mice were checked 3 times a week and the presence of palpable tumors was determined. Tumor volumes were calculated using caliper measurements as previously described (70).

Western blotting. Cells were washed with ice-cold PBS (pH 7.5), and then lysed before quantification of protein by bicinchoninic acid assay to equalize samples for Western blotting. Combinations of the following antibodies were then used to detect proteins of interest: anti-FLAG (clone M2, Sigma-Aldrich), anti-*WNT5A*/B (9562, Cell Signaling), anti-phospho-AKT (Ser473) (Cell Signaling), anti-total AKT (Cell Signaling), anti-phospho-ERK1/2 (Cell Signaling), anti-total ERK1/2 (Cell Signaling), anti-phospho-JNK (Cell Signaling), anti-pan-phospho-PKC (Cell Signaling), anti-phospho-PKC α/β (Cell Signaling), anti-PARP1 (Cell Signaling), anti-total GSK3 β (Cell Signaling), anti-phospho-GSK3 β (Millipore), anti-HSP90 (Santa Cruz Biotechnology), and anti- β -tubulin (Millipore). See complete unedited blots in the supplemental material.

Analysis of gene expression by microarray. For microarray analysis of gene expression, the RNA was isolated using the QIAGEN RNeasy protocol and quantitated using a NanoDrop Spectrophotometer (Agilent Technologies). Seven hundred fifty nanograms of high-quality total RNA with RIN (RNA integrity number) greater than 8.0 was labeled with cyanine 5-CTP or cyanine 3-CTP using the Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies) and purified on RNeasy Mini columns (QIAGEN). Labeled RNA was then hybridized to Agilent Human 44K expression arrays that include 44,000 probes and compared with a labeled mixed reference sample consisting of a pool of equal amounts of RNA from each of 47 melanoma cell lines. The analysis of the microarray data was done using Rosetta Biosoftware's Resolver system. ROAST analysis was used to perform similarity searches to determine the correlation between *WNT5A* expression and other probes detecting transcripts with similar expression patterns. Raw and normalized array data have been uploaded to Gene Expression Omnibus at NCBI (GSE55583). All RNA isolation and expression microarray experiments were performed in triplicate.

Analysis of gene expression by RT-PCR. RNA was purified with the RNeasy kit according to the manufacturer's protocol (QIAGEN). cDNA was synthesized with RevertAid M-MuLV Reverse Transcriptase (Fermentas). LightCycler FastStart DNA Master SYBR Green 1 (Roche) was used for real-time PCR using various primers (Supplemental Table 2) to detect human mRNA transcripts.

Statistics. Statistically significant differences between data were determined using GraphPad software. One-way ANOVA or 2-tailed *t* tests as indicated in the figure legends were used to compare most data sets. Microarray data sets were compared using 1-way ANOVA test for statistical significance. Unless otherwise indicated, all error bars indicate SD. *P* values less than 0.05 were considered significant.

Study approval. All experiments involving patients and analysis of patient samples were carried out using protocols approved by the Royal Prince Alfred Hospital Ethics Review Committee under Protocol X10-0305 (HREC/10/RPAH/539). All experiments involving animals were approved by the IACUC at the University of Washington.

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