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Characterization of novel, context-dependent modulators of WNT signaling in cancer

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Abstract

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WNTs comprise a family of secreted proteins that play critical roles in both embryogenesis and in the etiology of human diseases such as cancer. Even after more than three decades of research, our understanding of the complex roles of various WNT signaling pathways in cancer is rudimentary at best. Just as either activating or inactivating different WNT signaling pathways results in unique consequences at different stages of animal development and in different tissues types during embryogenesis, so too do WNTs contribute to cancer progression in a context-dependent manner. The work presented in this dissertation identifies and characterizes novel modulators of WNT signaling in cancer and explores possible mechanisms of action for these signaling effectors including crosstalk with other signal transduction pathways. First, I describe a novel role for AGGF1 in colorectal cancer as a chromatin-associated enhancer of β-catenin-dependent transcription. Second, I find that the planar cell polarity protein SCRIB does

not universally act as a tumor suppressor as presumed by many researchers, but rather that SCRIB can either promote or inhibit tumorigenesis in a context-dependent manner. My working hypothesis is that this switch from pro- to anti-tumorigenic function likely involves changes in the proteins associated with SCRIB such as NOS1AP, ARHGEF7 and VANGL. Finally, I characterize a novel role for a WNT5A/FZD7/RYK/AKT pathway in promoting the growth and viability of both naïve melanoma cells and melanoma cells that have acquired resistance to BRAF/MAPK pathway inhibition. With the exception of the work on AGGF1, these studies highlight context-dependent functional roles for β-catenin-independent WNT signaling in modulating numerous cancer cell behaviors ranging from the regulation of cell motility to the acquirement of drug resistance.

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Dedication

For Andrew and the girls.

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Chapter 1: WNT signaling pathways as therapeutic targets in cancer

Jamie N. Anastas and Randall T. Moon

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WNT signaling pathways as a therapeutic targets in cancer.

J.N. Anastas and R. T. Moon (2012) Nature Reviews Cancer. 13(1):11-26.

Abstract

Since the initial discovery of the oncogenic activity of WNT1 in mouse mammary glands, our appreciation for the complex roles for WNT signaling pathways in cancer has increased dramatically. WNTs and their downstream effectors regulate various processes that are important for cancer progression, including tumor initiation, tumor growth, cell senescence, cell death, differentiation and metastasis. Although WNT signaling pathways have been difficult to target, improved drug-discovery platforms and new technologies have facilitated the discovery of agents that can alter WNT signaling in preclinical models, thus setting the stage for clinical trials in humans.

At a glance

- WNTs are secreted glycoproteins that regulate multiple signaling pathways through both CTNNB1/β-catenin-dependent and –independent mechanisms.

- The activation of WNT signaling pathways can be both positively and negatively correlated with patient outcomes in different types of cancer.

- WNT/CTNNB1 signaling can either promote or inhibit tumor initiation, growth, metastases and drug resistance in a cancer stage- and a cancer type-specific manner.

- CTNNB1-independent WNT signaling pathways also contribute to tumorigenesis and cancer progression in a context-dependent manner.

- Aberrations in WNT signaling pathways and alterations in other oncogene and tumor suppressor pathways cooperate to drive cancer initiation and progression.

- Multiple strategies for targeting WNT signaling ranging from small molecules, to blocking antibodies, to peptide agonists and antagonists are now in development paving the way for initial clinical trials using WNT modulators in cancer patients.

WNTs are a family of 19 secreted glycoproteins[1] that play crucial roles in the regulation of diverse processes including cell proliferation, survival, migration, polarity, specification of cell fate, and self-renewal in stem cells. While perturbation of the levels of WNT ligands or altered activities of the proteins necessary for WNT signal transduction can result in defects in embryonic development, abnormal WNT signaling in adults may contribute to disease etiology. A role for WNTs in cancer was first described three decades ago in mouse models of mammary cancer and in colon cancer. Aberrant overexpression of WNT1 induced by pro-viral insertion at the int-1 locus induces spontaneous mammary hyperplasia and tumors in mice[2,3], while Wnt1 transgenic mice similarly develop mammary tumors, suggesting a causative role for WNT1 in mammary tumorigenesis[4]. Further studies found that WNT1 and other WNTs promoted the stabilization of free pools of CTNNB1[5] (also known as β-catenin) and the activation of CTNNB1-dependent transcription.

Shortly after the characterization of WNT1 in mouse models of mammary cancer, other studies pointed to a critical role for hyper-activated WNT/CTNNB1 signaling in colorectal cancer[6,7]. Inherited inactivating mutations in adenomatous polyposis coli (APC), a negative regulator of CTNNB1 stability (Figure 1), are found in patients to Familial Adenomatous Polyposis (FAP), which can subsequently progress to colorectal carcinomas with concomitant activating mutations in KRAS and inactivating mutations in TP53 (reviewed in 6,7). Both APC and CTNNB1 are also frequently mutated in colorectal cancers of non-FAP patients as well[8,9], and overexpression of constitutively active CTNNB1 or loss of APC function leading to the hyper-activation of WNT/CTNNB1 signaling can result in colorectal tumorigenesis in

mouse models[6,7]. These observations demonstrate that mutations leading to the unregulated activation of WNT/CTNNB1 signaling contribute to tumorigenesis in the colon.

It is now clear that WNTs modulate both CTNNB1-dependent (Figure 1), often referred to as "canonical" WNT signaling) and CTNNB1-independent (Figure 2), often referred to as "non-canonical" WNT signaling) pathways. The precise mechanisms by which a WNT stimulates CTNNB1-dependent versus –independent cellular responses are not fully elucidated, but likely involves stimulation of multiple WNT receptors. Reported transmembrane WNT receptors include the ten members of the FZD (Frizzled) family of G-protein coupled receptors (GPCRs), as well as the receptor tyrosine kinases (RTKs) ROR1 and ROR2 and the RTK-like protein RYK[10,11]. Though it is beyond the scope of the present dissertation, it is worth noting that the while labeling WNT signaling pathways as either "canonical" or "non-canonical" has some utility for enabling discussion, the reality is that WNT ligands likely stimulate complex, non-linear, networks that share many downstream effectors.

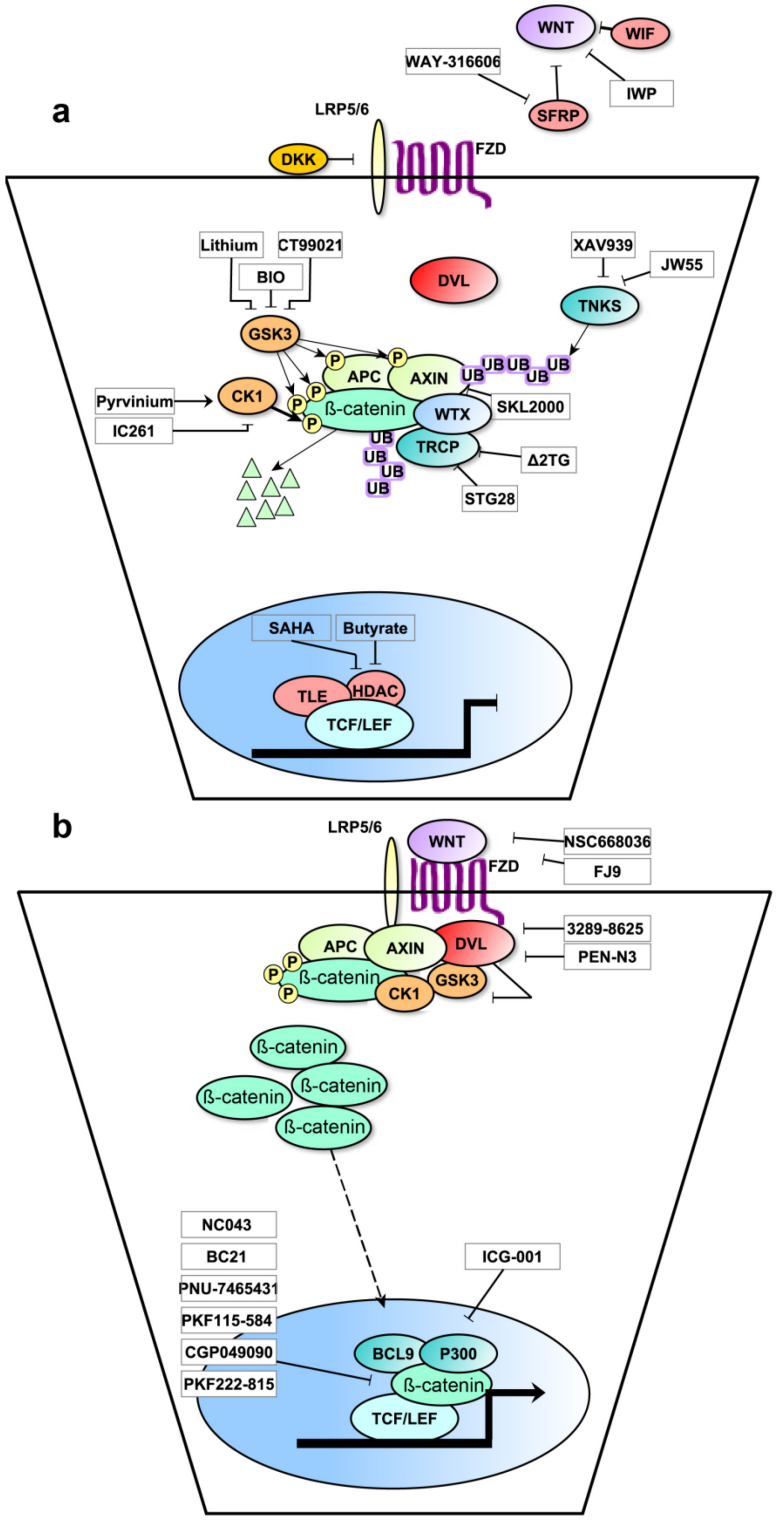

Figure 1: β-catenin (CTNNB1)-dependent WNT signaling

β-catenin (CTNNB1)-dependent WNT signaling pathways have crucial roles in the regulation of diverse cell behaviors, including cell fate, proliferation, survival, differentiation, migration and polarity. Recently, numerous studies have identified small-molecule inhibitors and activators of various pathway components; these are indicated in this figure in blue boxes. For a more detailed description of these inhibitors refer to Table 3. **a** In the absence of WNT stimulation, a destruction complex — containing the proteins adenomatous polyposis coli (APC), glycogen synthase kinase 3β (GSK3β) and AXIN — phosphorylates (P) and targets CTNNB1 for ubiquitylation (Ub) and proteasomal degradation. In the absence of WNTs, members of the TCF/LEF family of high-mobility-group transcription factors associate in a repressive complex with transducin-like enhancer protein (TLE; also known as Groucho) co-repressor proteins, which promote the recruitment of histone deacetylases (HDACs) to repress CTNNB1 target genes. **b** The binding of WNTs, such as WNT3A and WNT1, to frizzled (FZD) and LRP5 or LRP6 co-receptors transduces a signal across the plasma membrane that results in the activation of the Dishevelled (DVL) protein. Activated DVL inhibits the destruction complex, resulting in the accumulation of CTNNB1, which then enters the nucleus where it can act as a co-activator for TCF/LEF-mediated transcription. CTNNB1 acts a transcriptional switch, as the presence of CTNNB1 reduces the association of TLE with TCF/LEF, while recruiting various transcriptional cofactors including BCL9, Pygopus and histone acetyltransferases. WNT–CTNNB1-dependent transcription ultimately modulates changes in cell behaviours such as proliferation, survival and differentiation. CK1, casein kinase 1; DKK, dickkopf homologue; SFRP, secreted frizzled-related protein; TNKS, tankyrase; βTRCP, β-transducin repeat-containing E3 ubiquitin protein ligase; WIF, WNT inhibitory factor; WTX, Wilms tumor gene on the X chromosome.

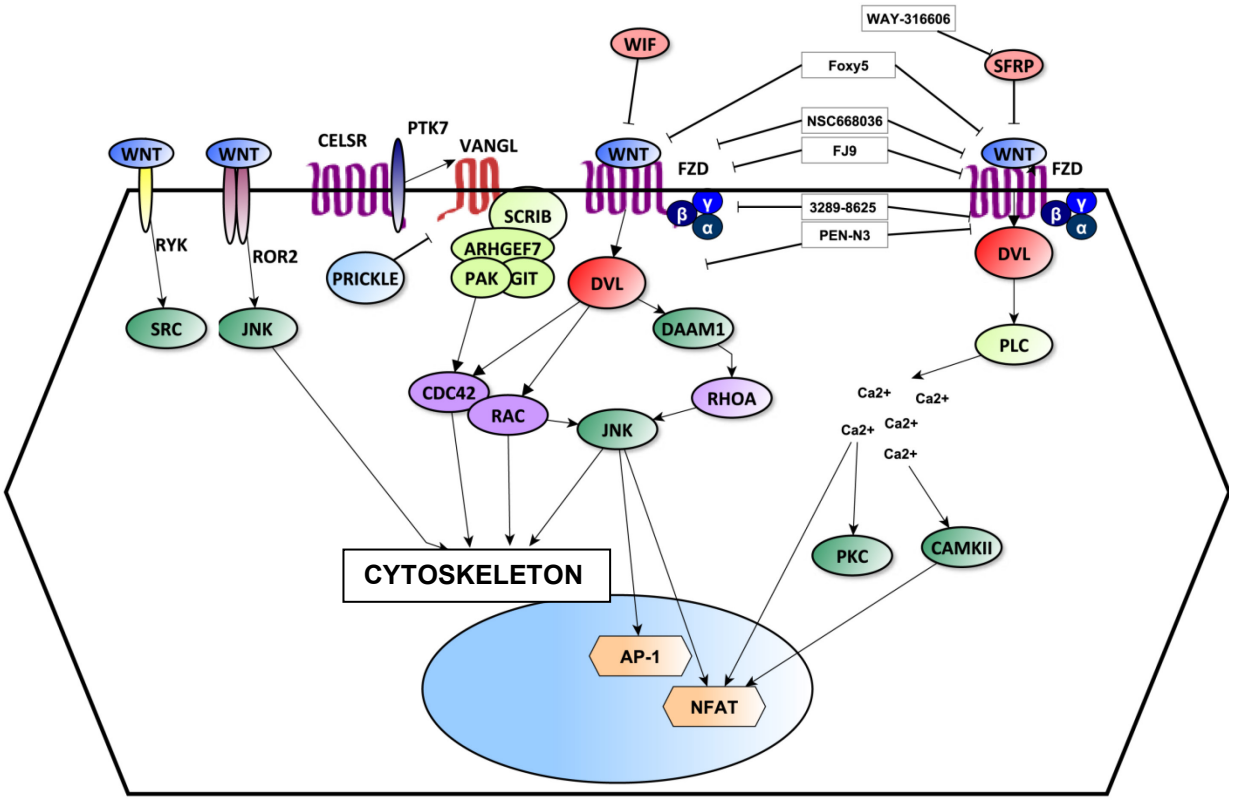

Figure 2: β-catenin-independent WNT signaling pathways.

Some WNTs, such as WNT5A and WNT11, fail to stabilize β-catenin (CTNNB1), but, instead, regulate signaling pathways associated with cell polarity and migration. CTNNB-1 independent WNT pathways are also initiated by the binding of certain WNTs, such as WNT11 and WNT5A to frizzled (FZD) receptors in order to activate dishevelled (DVL), which can then activate a variety of downstream effectors. In addition to regulating calcium- and small GTPase-dependent signaling networks, CTNNB1-independent WNTs also regulate the planar cell polarity (PCP) signaling pathway. Disruption of FZD receptors also results in PCP defects[12–14], suggesting that a WNT/FZD signaling pathway may regulate PCP. In addition to FZD receptors, other transmembrane proteins such as VANGL, PTK7, and CELSR, genetically or biochemically interact with WNTs and FZDs to regulate PCP signaling in vertebrates[15]. RYK and ROR receptor tyrosine kinases can also act as WNT receptors and activate CTNNB1-independent signaling[10]. Like other WNT pathways, PCP also requires the intact function of DVL, as well as a number of other cytosolic factors including, SCRIB, and PRICKLE[15]. In some contexts, CTNNB1-independent WNTs regulate small GTPases such as RHOA, RAC, and CDC42 in a DVL-dependent manner[16]. WNT5A and WNT11 can also induce a calcium flux, which results in the activation of a variety of signaling pathways such as protein kinase C (PKC), calcium/calmodulin-dependent protein kinase II (CAMKII), and JUN N-terminal kinase (JNK)[11].

In this chapter we present evidence that the activity of WNT signaling networks can unexpectedly correlate in either a positive or negative manner with patient outcomes in different types of cancer. We further review evidence that WNT signaling can either promote or inhibit tumor initiation, growth, metastases and drug resistance in a cancer stage- and a cancer type-specific manner. Our first goal is to paint a more complex picture than the widely-held presumption that elevated WNT/CTNNB1 signaling consistently leads to negative clinical outcomes in cancer. Our second goal is to emphasize potential roles for CTNNB1-independent WNT signaling in cancer. It is our hope that appreciating the complexity of WNT signaling in different contexts will facilitate the development of improved therapies.

Context-dependent aberrations in WNT signaling in cancer

The high frequency of WNT pathway mutations in many different cancers underscores the significance of WNT/CTNNB1 signaling to carcinogenesis. In addition to APC mutations, sequencing of patient colorectal tumors by the Cancer Genome Atlas Network and others has identified mutations in other WNT pathway genes such as TCF7L2 (previously known as TCF4), CTNNB1, and FAM123B that are predicted to activate WNT/CTNNB1signaling[17,18]. Both missense mutations and mutations predicted to disrupt the phosphorylation and degradation of CTNNB1 are also frequent in hepatocellular carcinoma (HCC)[19,20], medulloblastoma[21], and

ovarian cancer[22], while deletions and truncation mutations in AXIN1 are common in HCC and colorectal tumors[23,24](Table 1). We note that most WNT pathway mutations observed in cancer result in hyper-activation of WNT/CTNNB1 signaling. Although mutations in genes like FZD4 and LRP5/6 that are thought to inhibit the WNT signaling have been identified in other disorders, such as coronary artery disease and neurodegenerative disorders[25–27], similar WNT-pathway inactivating mutations have not been characterized in cancer.

WNTs and WNT pathway components are also frequently over- or under-expressed in different human cancers, and these changes in expression profiles often correlate with epigenetic activation or inactivation of gene promoters. The expression patterns of WNT signaling components can serve as a prognostic indicator of patient outcomes (Table 2). As predicted by the seminal studies linking WNT/CTNNB1 signaling to carcinogenesis of the breast and colon, high levels of nuclear CTNNB1, which are normally interpreted as a sign of increased WNT/CTNNB1 signaling activity, correlate with poor prognosis in these cancers[28–36].

Table 1: Mutations in WNT pathway genes observed in cancers

Curated from the Catalogue of Somatic Mutations in Cancer (COSMIC) database. Genes that are mutated in at least 10% of the analysed samples for each cancer type are included in the table. APC, adenomatous polyposis coli; CTNNB1, β-catenin; WTX, Wilms tumor gene on the X chromosome.

Gene	Type of mutation	Primary tissues	Num. Mutated Samples	% Mutated	Total Samples
APC	Primarily frameshift and deletion mutations leading to compromised ability to degrade CTNNB1	large intestine	2152	39%	5517
		stomach	129	61%	214
		soft tissue	50	12%	430
		small intestine	34	16%	214
		pancreas	26	14%	184
		liver	11	12%	94
CTNNB1	Mutations in CTNNB1 cluster around the N-terminus and prevent the phosphorylation of amino acids, S33, S37, T41 and S45, which promote protein degradation	liver	907	23%	3933
		soft tissue	673	42%	1601
		endometrium	218	20%	1098
		kidney	168	14%	1225
		pancreas	125	26%	476
		ovary	104	11%	913
		adrenal gland	100	19%	534
		pituitary	86	24%	360
		billary tract	43	10%	433
AXIN1	Many mutations prevent AXIN1 from acting as a scaffold to degrade CTNNB1	billary tract	10	38%	26
		liver	49	11%	448
FAM123B (WTX)	Predicted to be loss of function mutations	kidney	125	13%	949
		large intestine	19	13%	151
TCF7L2	Unknown	large intestine	13	28%	47

However, recent studies suggest that high levels of WNT and nuclear CTNNB1 do not always predict poor prognosis. In medulloblastoma, patients with activating mutations in CTNNB1 had greater disease free survival than patients with mutations in the SHH pathway, which likely reflects the unique cell-type origins of medulloblastomas driven by these different mutations[37,38]. Elevated nuclear CTNNB1 also correlates with improved patient outcomes in malignant melanoma, ovarian, and prostate cancers[39–41]. Although information regarding CTNNB1-independent WNT signaling pathways in cancer is still limited, several studies suggest that increased expression of WNT5A leading to CTNNB1-independent signaling correlates with poor clinical outcomes in melanoma[42–44], and gastric cancer[45], yet correlates with improved patient survival in breast[46] and colon cancers[47]. Importantly, some of these studies have not yet been independently confirmed. Furthermore, it is not always clear which of these aberrations in WNT signaling are causative of poor clinical outcomes in cancer patients, and which of these aberrations are merely correlative. Despite these important caveats, these findings indicating that changes in the expression of WNT signaling components can be predictive of either improved or worsened patient outcomes highlight the need to better understand the context-dependent roles for WNT signaling in cancer, which is further discussed below.

Table 2: WNT signaling proteins are associated with distinct patient outcomes in a cancer-subtype-specific manner

Gene	Cancer type	Clinical significance
LEF1	**colorectal carcinoma**	High LEF1, but low TCF4 were correlated with a better prognosis in colorectal carcinoma [48]. However, in another study high levels of LEF1 were associated with reduced survival in colorectal cancer [49]. In this study expression was low in primary colorectal tumors, but high in metastatic tumors obtained from the livers of colorectal cancer patients.
TCF4	**colorectal carcinoma**	Reduced TCF4 correlates with better prognosis.
CTNNB1	**colorectal carcinoma**	High nuclear CTNNB1 expression and high P27 expression were significantly correlated with patient deaths in colorectal carcinoma[30] High nuclear CTNNB1 staining at the invasion front of colorectal carcinomas was significantly associated with poor survival prognosis in colorectal carcinoma [28]
CTNNB1	**prostate cancer**	WNT1 and CTNNB1 protein levels were increased in advanced prostate cancer and in metastatic lesions [50].CTNNB1 was present in both the cytosol and the nucleus of these tissues. In contrast to other cancer types, decreased nuclear CTNNB1 correlates with decreased relapse-free survival prostate cancer [39] and decreases in nuclear catenin were observed in prostate cancer metastases [51].
WNT5A	**prostate cancer**	Loss of WNT5A especially in combination with high Ki67+ or high AR staining was predictive of reduced relapse-free survival in prostate cancer [52]
SFRP4	**prostate cancer**	Although all tumors expressed sFRP4 in the cytosol, decreased membranous sFRP4 was associated with decreased patient survival in prostate cancer [53]
DKK3	**breast cancer**	DKK3 promoter methylation was significantly associated with decreased overall survival and relapse free survival in a study of 150 primary invasive breast cancer samples, while promoter methylation of WIF1 gene was not [54]
DKK3	**gastric cancer**	DKK3 promoter methylation was also significantly correlated with decreased overall survival in gastric cancer patients (n=104), but not in colorectal patients (n=84) [55]. However DKK3 is divergent from other DKKs and has not been shown to significantly affect WNT signaling [56]
WNT5A	**gastric cancer**	Increased levels of WNT5A protein were associated with high grade tumors and with decreased survival time in gastric cancer [45]
CTNNB1	**gastric cancer**	Decreased nuclear CTNNB1 was observed in high grade gastric cancers [45]
CTNNB1	**non small-cell lung carcinomas**	CTNNB1+ (cytoplasmic and/or nuclear staining) was associated with increased survival in NSCLCs and nonsquamous cell carcinomas in comparison to CTNNB1- samples (solely at the plasma membrane)[57]
CTNNB1	**glioblastoma**	High nuclear and cytoplasmic CTNNB1 was associated with poor survival in glioblastoma [58]
WNT1	**glioblastoma**	Increased WNT1 staining was associated with poor survival in glioblastoma [58].

Gene	Cancer type	Clinical significance
WNT2	**esophageal carcinoma**	Overexpression of WNT2 was correlated with poor survival in esophageal squamous cell carcinoma. Interestingly, WNT2 was highly overexpressed in tumor associated fibroblasts indicating that it might function non-cell autonomously to regulate WNT signaling in these sarcomas [59]
CTNNB1	**AML**	CTNNB1 is expressed in freshly prepared CD34+ blood progenitors [60] cells and also expressed in a subset of primary AML samples[60,61]. High CTNNB1 in AML patient samples correlates with decreased relapse-free survival and decreased overall survival [61]
CTNNB1	**esophageal carcinoma**	Nuclear CTNNB1 was increased in esophageal adenocarcinoma in comparison to premalignant dysplasias [62,63], and nuclear CTNNB1 was significantly correlated with poor one year survival in this cancer, but not with lymph node metastases[63]
WNT/CTNNB1	**neuroblastoma**	One study noted increased expression of a subset of WNT ligands (WNT1, WNT6, WNT7A, WNT10B), DVL1 and TCF7 expression in high-risk NBs without MYCN amplification [64]
WNT7A	**ovarian carcinoma**	WNT7a was overexpressed in ovarian carcinoma and significantly associated with patient deaths [65]
WNT1	**breast cancer**	WNT1 protein expression was increased in tumor tissue in comparison to non-cancerous adjacent tissue in Hong Kong breast cancer patient samples in low grade breast carcinomas. However, there was no significant difference in WNT1 expression in high-grade breast tumors versus adjacent non-tumor tissue, indicating that WNT1-dependent signaling events may be particularly important during early stages of breast tumorigenesis [66].
CTNNB1	**breast cancer**	Nuclear CTNNB1 accumulation was correlated with a variety of prognostic indicators in breast cancer including grade and presence of lymph node metastases. Nuclear CTNNB1 is also associated with reduced metastases-free and overall survival in breast cancer [29,67]. However, cancer subtype differences have also been observed in breast cancer as invasive ductal carcinomas exhibited membranous but not nuclear CTNNB1 staining, whereas invasive lobular carcinomas lacked any CTNNB1 expression [67]. Nuclear accumulation of CTNNB1 was also strongly associated with the Basal subtype of triple negative subtype of breast cancer (ER,- PR-, HER2-negative) [67]
APC	**breast cancer**	APC was decreased in grade 1 breast tumors in comparison to adjacent normal breast tissue, yet was increased in grade 3 breast tumors in comparison to adjacent normal tissue in a study of Hong Kong breast cancer patient samples [66]
WNT/CTNNB1	**breast cancer**	A high WNT/CTNNB1 gene signature (NFATc3, CDH23, WISP2, FZD9, HDAC1, LRP6, EN1, HOXA7, CDH23, MYCL1, HOXD4) was significantly correlated with basal-like breast cancers, but not luminal A, luminal B, or normal breast expression profiles [68]
CTNNB1	**adrenocortical carcinoma**	High nuclear CTNNB1 was significantly associated with reduced overall and disease free survival in adrenocortical carcinoma a rare, but aggressive disease with a 40% 5 year survival rate [69]
WNT5A	**ovarian carcinoma**	High WNT5A IHC is correlated with poor survival in ovarian carcinoma [70]

Gene	Cancer type	Clinical significance
FZD7, DVL1, AXIN1	**ovarian carcinoma**	DNA methylation of the promoters of several WNT pathway genes including FZD7, DVL1, and AXIN1 was associated with reduced progression-free survival in ovarian carcinoma [71]
LEF1	**ALL**	High LEF1 transcript expression was associated with poor relapse-free survival in B cell ALL [72]
WNT5A	**colorectal carcinoma**	High levels of WNT5A protein expression were correlated with increased patient survival in Dukes B colon cancers, which are locally invasive [47]
CTNNB1	**hepatocellular carcinoma**	High levels of CTNNB1 were predictive of decreased overall survival and increased risk of recurrence in HCC, and tumors staining positively with both CTNNB1 and HIF1alpha boded particularly poor prognosis [73]
WIF1	**hepatocellular carcinoma**	Loss of WIF1 mRNA expression was associated with decreased overall survival in hepatocellular cancer [74]
WNT5A	**ovarian cancer**	Low expression of WNT5A protein expression is observed during progression of epithelial ovarian cancer and loss of WNT5A correlates with decreased overall survival in epithelial ovarian cancer patients [75]

WNT signaling and cancer cell proliferation

WNTs. While different cancer cell types vary dramatically in their growth responses to stimulation by different WNT ligands, it is clear that autocrine WNT signaling plays a critical role in the growth and survival of many cancer cells. For example, early studies of mammary epithelial cells reveal that WNTs 7A, 3A, and 1 efficiently transform these cells, while WNTs 6,4, and 5A do not[76,77]**.** Similarly, overexpression of WNT1, but not WNT7B or WNT5A induced hyperplasia in mouse mammary cells grown in vivo in mammary fat pads[78].

Other cancer subtypes also exhibit unique sensitivities and responses to different WNT ligands. WNT3A promotes the stabilization of CTNNB1 and the activation of TCF/LEF-dependent transcription in both myeloma and prostate cancer cell lines[79–82], and the expression of constitutively active CTNNB1 is sufficient to enhance the growth of myeloma cells[80] and the growth of prostate tumors in mouse models[83,84]. This suggests that WNT3A/CTNNB1 signaling promotes the growth of these cancers. Consistent with the finding that activation of WNT/CTNNB1 signaling can be either positively or negatively correlated with disease progression in cancer, WNT3A/CTNNB1 signaling can also inhibit the growth of some cancers. For example, elevated levels of WNT3A significantly decrease the growth of both human and mouse melanoma cells in xenografts studies, while activating CTNNB1-dependent transcription[41,85].Why might WNTs such as WNT3A and WNT7A promote growth in some cancers but not in others? One possible explanation is that WNTs activate different signaling

pathways depending on the cellular context. WNT7A regulates both proliferation and CTNNB1-dependent transcription in ovarian cancer cells[86], but in leukemic cells[87] Wnt7A inhibits proliferation yet has little effect on CTNNB1-dependent transcription. It is possible that WNT7A regulates different signaling pathways in leukemic cells, which are important for growth inhibition. In endometrial carcinoma cells, co-transfection of WNT7A and FZD5 promotes CTNNB1-dependent transcription, whereas co-transfection of WNT7A and FZD10 activates JUN-N terminal kinases (JNKs) [88]. These data suggest that WNT7A can regulate different signaling cascades depending on the combination of receptors expressed by a particular cancer type. Sometimes the divergent responses of cancer cells to ligand stimulation do not seem to involve the activation of unique signaling pathways, but reflect intrinsic differences in cellular interpretations of WNT/CTNNB1 signaling. WNT3A promotes the expression of genes associated with melanocyte differentiation and decreases the expression of genes associated with proliferation in melanoma cells[41], yet induces the expression of genes important for growth and survival in prostate cancer cells[84,89]. WNT-dependent stabilization of CTNNB1 alters the transcription profiles of many different target genes in a tissue type-specific manner and these expression changes involve crosstalk with other transcription factors and cofactors, which are differentially expressed in cancers.

Increasing or decreasing CTNNB1-independent WNT signaling mediated by the altered expression of WNT5A and WNT11 can also result in profound effects on cancer cell proliferation. WNT5A can act as a growth suppressor in many cancers, including ovarian and

thyroid carcinomas, potentially by acting as a negative regulator of CTNNB1-dependent transcription in these cancers[75,90]. Importantly, ablation of endogenous WNT5A in murine B-cells induces the development of spontaneous B-cell lymphomas and chronic myeloid leukemias (CML)[91], suggesting a tumor suppressor role for WNT5A in some cell types. However, WNT5A does not always act as a growth and tumor suppressor. Reducing WNT5A expression in pancreatic cancer cells attenuates xenograft tumor growth[92], while WNT5A-transduced feeder cell**s** enhance the growth of patient-derived chronic lymphoid leukemia (CLL) cells[93].

WNT Receptors. Targeting WNT receptors that maintain malignant phenotypes, but which are dispensable for normal tissue homeostasis, may provide an attractive strategy for therapeutic intervention in cancer. It is first necessary to identify specific WNT receptor proteins that are expressed in tumors and which are functionally relevant to disease progression. Several WNT receptors including FZD6, are overexpressed in spontaneous B-cell leukemias and lymphomas derived from the T-cell lymphoma breakpoint 1 ($Tcl1^{+/-}$) transgenic mouse model FZD6 has a unique role in promoting leukemia development in these animals as $Tcl1^{+/-}$ $Fzd6^{-/-}$ double transgenic mice are at significantly less risk of leukemia than $Tcl1^{+/-}$ $Fzd6^{+/+}$ littermates, while leukemogenesis is neither enhanced nor inhibited when Fzd9 is deleted[94]. FZD7 expression is increased in many different tumors compared to normal tissues[95] and also acts to promote cancer proliferation and progression. Specifically, FZD7 shRNA reduces the TCF-dependent transcription and xenograft tumor growth of triple negative breast cancer[96] and

knockdown of FZD7 similarly inhibits the growth of HCC cells[97], which also rely on active WNT/CTNNB1 signaling for their proliferation[98].

In addition to FZD receptors, the WNT receptors ROR1 and ROR2 also contribute to cancer proliferation and tumorigenesis. Reducing levels of ROR1 with siRNAs reduces the growth of gastric, lung, and breast cancer cells both in cell culture and in xenografts[99–101], while ROR2 shRNAs significantly reduce the growth of leiomyosarcoma and renal cell carcinoma xenografts tumors[102,103]. Deciphering the signaling pathways acting downstream of ROR1 and ROR2 remains an active area of research. In some cancers, ROR1 supports tumorigenesis through crosstalk with other RTKs such as MET and epidermal growth factor receptor (EGFR)[99,101]. In CLL cells, treatment with anti-ROR1 antisera blocks the enhanced proliferation induced by WNT5A, suggesting that ROR1 might be mediating the mitogenic signal from WNT5A[93]. Whether specific WNTs regulate ROR1 and ROR2 driven tumorigenesis and whether these WNTs also regulate crosstalk between ROR receptors and other RTKs remain as open questions.

SFRPs and WIFs. WNT pathways are also regulated by a variety of secreted proteins, such as WNT inhibitory factors (WIFs) and secreted frizzled-related proteins (SFRPs) that can competitively displace certain WNT ligands from their receptors. In select cancer models, increasing SFRP levels attenuates cancer growth, particularly in cells requiring autocrine WNT stimulation such as myeloma cells[104] and subsets of breast cancer cells[105,106]. Other cell types appear to be insensitive to altered SFRP levels, and, in certain cancer contexts, SFRP can

enhance cell growth. In contrast to other breast cancer cell lines[105,106], SFRP1 is insufficient to inhibit TCF-dependent transcription and has no effect on the xenograft growth of SUM1315 breast cancer cells[68], suggesting cell line-specific differences in SFRP sensitivity. SFRPs also regulate prostate cancer cell proliferation in a context-dependent manner as the overexpression of SFRP4 or SFRP3 decreases the proliferation of human PC3 cells in vitro[53,107], while overexpression of SFRP1 promotes growth of BPH1 prostate cancer cells[108].

WIF1 also regulates CTNNB1-dependent transcription and the proliferation of a variety of cancer cell lines including those derived from cervical and prostate cancers, as well as glioblastoma[109–111]. Of note, overexpression of WIF1 also inhibits osteosarcoma cell growth in soft agar assays and in xenograft assays, yet has little effect on CTNNB1-dependent transcription[112]. It is conceivable that WIFs and other secreted WNT antagonists result in pleiotropic signaling outputs and regulate not only CTNNB1 signaling, but potentially other signaling pathways as well. SFRPs and WIFs associate with multiple WNTs, so altering SFRP and WIF levels could have pleiotropic effects on cancer cell proliferation.

WNTs and tumor initiating cells

WNT signaling pathways contribute to both the maintenance and differentiation of a variety of multi-potent progenitor cells in developing embryos, as well as in adults. Numerous studies indicate that WNT/CTNNB1 signaling analogously contributes to cancer progression via

the maintenance of highly tumorigenic sub-populations of cancer cells called tumor initiating cells[113]. Perhaps the most extensive evidence for the significance of WNT/CTNNB1 signaling in tumor initiating cells comes from studies of mouse and patient-derived leukemias. Sub-populations of leukemia stem cells capable of forming tumors in mice at short latency have increased nuclear CTNNB1 and increased WNT/CTNNB1 reporter activity[90,94,95,114]. as do myeloid progenitors isolated from CML patients[115], suggesting that WNT/CTNNB1 signaling is up-regulated in leukemia-initiating cells. CTNNB1 deletion via a floxed allele inhibits the initiation of mixed lineage leukemia (MLL) driven by MLL-Enl, CML driven by BCR-Abl oncogenes[114,116] and prevents disease progression of acute myeloid leukemia (AML) driven by the Hoxa9 and Meis1a oncogenes in mice[117]. Loss of CTNNB1 also significantly reduces tumor forming ability of serially transplanted CLL cells but not acute lymphoblastic leukemia (ALL) cells, suggesting different requirements for CTNNB1 in leukemia subtypes[116]. In contrast to CLL, CTNNB1-dependent transcription was very low in the ALL cells[116]. The authors of this study suggest that activation of CTNNB1-dependent transcription is required for the initiation of forms of leukemia characterized by a progenitor cell hierarchy.

CTNNB1-dependent transcription in colon progenitor cells similarly drives the initiation of colorectal tumors. Deletion of APC in colon crypt progenitor cells induces rapid and sustained adenoma growth, while APC deletion in non-progenitor cells does not induce sustained tumors in mouse models[118]. Increased CTNNB1 levels in colon and intestinal tissue drives tumorigenesis through the activation of a specific subset of TCF/LEF family transcription factors in order to

promote a progenitor-like gene expression signature[119]. Specifically, TCF7L2 and CTNNB1 regulate the expression of many target genes normally associated with proliferative progenitor cells of colon crypts[119]. Transcription cofactors such as BCL9 and BCL9L enhance the expression of a subset of CTNNB1 target genes associated with progenitor cell phenotypes in the colon[120]. These gene expression changes are likely functionally relevant since ablation of both BCL9 and BCL9L prevents of chemically-induced colorectal adenomas in mice[120]. BCL9L protein levels were also recently found to be up-regulated in patients with ovarian carcinoma persisting after surgery[121], suggesting that these CTNNB1 transcription cofactors might promote tumorigenic properties of multiple carcinomas.

WNT/CTNNB1 signaling also supports the self-renewal of both normal and malignant mammary stem cells. Cells expressing mammary stem cell markers are enriched in both pre-malignant mammary glands and spontaneous tumors in mouse mammary tumor virus (MMTV)-Wnt1 transgenic mice, but not in MMTV-Hras, MMTV-Neu, or MMTV-PyMT transgenic mice[122,123], which indicates that WNT/CTNNB1 tumors contain progenitor-like cells. WNT pathway genes such as Fzd6 and Wnt7b are also highly expressed in undifferentiated mouse mammary tumor cells grown in spheroid conditions in order to enrich for progenitor-like cells, in comparison to differentiated mouse mammary cells[124]. Importantly, WNT/CTNNB1 is not only active, but is functionally relevant in mammary stem cells since Wnt3a is sufficient to promote the self-renewal of mouse mammary stem cells grown as spheres in vitro and

enhances the ability of these mammary progenitor cells to reconstitute mammary glands in mice[124,125].

Human mammary cells expressing progenitor cell surface markers similarly express altered levels of many WNTs and WNT modulators. For instance, a subpopulation of mesenchymal human mammary cells shown to induce xenograft tumors at high efficiency have reduced expression of secreted inhibitors of WNT/CTNNB1 such as, SFRP1 and dickkopf 1 homolog (DKK1), but expressed higher levels of WNT5A than non-tumorigenic mammary cells[126]. Restoration of SFRP1 but not DKK1 in human mesenchymal mammary cells reduces secondary sphere formation in vitro and tumorigenesis in xenograft models[126]. Further research is necessary to determine why SFRP1 inhibits mammary tumorigenesis by mammary progenitor-like cells, while DKK1 has no effect[126]. Together, these studies indicate that WNT signaling pathways can affect progenitor populations in a manner that affects tumorigenesis.

WNT signaling, apoptosis and senescence in cancer

WNT signaling pathways not only drive the initiation of tumorigenesis, but WNT signaling must also be maintained as tumors continue to grow. In some cell types, WNT stimulation prevents cellular senescence. For example, loss of endogenous WNT2 in fibroblasts leads to increased expression of senescence markers, whereas stimulation with WNT3A or

inhibition of GSK3β, a negative regulator of WNT/CTNNB1 (Figure 1), delays both replicative and oncogene-induced senescence[127]. Ectopic expression of WNT1 in mouse mammary cells similarly prevents contact-inhibition induced senescence, and supports the development of three-dimensional outgrowths from cell monolayers[125]. Other WNTs promote rather than prevent senescence. WNT5A levels increase as late passage primary ovarian epithelial cells begin to senesce, and the overexpression of WNT5A in ovarian epithelial carcinoma cells leads to senescence both in vitro and in xenografted tumors[75]**.** Many different cancer cell types require endogenous WNT stimulation for survival when challenged with chemotherapeutic agents or other toxins[128–136]. Finally, identifying WNT receptors that are necessary for cancer cell evasion of senescence and apoptosis could inform clinical strategies. Many studies indicate an important role for ROR1 in cancer cell survival, since ROR1 siRNAs induced apoptosis in CLL, breast, and cervical carcinoma cells[100,137,138]. Blocking FZD7 activity with siRNAs or peptides can similarly induce apoptosis in colon and breast cancer cells[95,96,139,140].

WNT signaling in metastatic disease

CTNNB1-dependent signaling and metastasis. WNT/CTNNB1 signaling also contributes to metastastic progression of cancer, and can either enhance or inhibit migratory behavior in a cancer cell type-specific manner. Both WNT1 and WNT3A promote the migration of myeloma cells isolated from patients[141], while a dominant negative form of LRP5 can negatively regulate

WNT/CTNNB1 signaling and abrogates the invasion and migration of prostate cancer cells[107]. Consistent with these observations indicating that WNT/CTNNB1 signaling enhances cell migration in some cancers, many secreted antagonists of WNT/CTNNB1 pathway such as DKK1 and SFRPs slow cancer cell motility and block invasive behavior[142,126,45,107,132]. Other studies suggest that WNT/FZD signaling can also inhibit cancer cell migration and invasion. For example, ectopic FZD1 decreases the invasion of both primary thyroid cells and thyroid carcinoma cells through Matrigel[143], and the addition of media from cells expressing WNT2 slows the migration and invasion of esophageal carcinoma cells[59].

What are the mechanisms by which WNT/CTNNB1 signaling regulates malignant cell migration and invasion? In some cases hyper- or hypo-activation of WNT/CTNNB1 pathway can regulate cell migration via the differential expression of CTNNB1 target genes. WNT1 overexpression induces invasiveness of MCF7 breast cancer cells, and this effect can be attenuated by blocking CTNNB1-dependent transcription[144]. Activation of WNT/CTNNB1 signaling in cancer often drives a transcriptional program reminiscent of an epithelial-mesenchymal transition (EMT)[145], which can promote cell migration and invasiveness . In addition to promoting EMT-like changes in a subset of cancers, CTNNB1 also regulates the expression of other factors relevant to metastatic progression, notably, matrix metalloproteinases (MMPs) and other factors necessary for the regulation of the extracellular matrix[10].

The development of metastases is thought to involve the delamination of cells from primary tumors through an epithelial-mesenchymal transition (EMT). The process of EMT involves alterations in protein expression resulting in complex changes in cell cellular behaviors such as, reduced cell-cell adhesion and enhanced motility[145,146]. Numerous studies indicate that activation of WNT/CTNNB1 signaling can promote transcriptional changes in order to drive EMT in cancer. Expression of WNT1, stabilized CTNNB1, or AXIN2, WNT/CTNNB1 target, is sufficient to induce EMT like changes in MCF7 breast cancer cells including up-regulation of Snail1 and down-regulation of E-cadherin expression[144]. Other WNT signaling molecules appear to suppress cell migration and invasiveness by inhibiting or reversing EMT. For instance, overexpression of WNT5A or addition of recombinant WNT5A increases the expression of CTNNB1 and E-cadherin at the membrane in breast cancer cells[147], while DKK1 expression inhibits EMT in colorectal cancer[148]. Overexpression of either SFRP3 or a dominant negative LRP5 lacking the cytosolic and extracellular domains similarly induced the expression of E-cadherin and reduced the expression of N-cadherin, SLUG, and TWIST in prostate cancer[107]. Connections between WNT signaling and EMT are further complicated by studies indicating that members of the WNT pathway are also up-regulated in response to EMT. For example, WNT signaling proteins such as, ROR2, and WNT5A, were up-regulated following SNAIL-induced EMT in epidermal carcinoma cells[149]. In renal cell carcinoma ROR2 expression seemed to promote EMT-like changes[102], raising the possibility that positive feedback loops between EMT programs and ROR2-dependent signaling could be important in select cancers.

Components of the WNT/CTNNB1 pathway also directly participate in coordinating changes in cell morphology, polarity, and signaling necessary for migration and invasion. Core WNT signaling pathway components localize to specific subcellular regions in migrating cells and contribute to the establishment of cell polarity as these cells migrate. APC is recruited to the leading edge of astrocytes migrating in wound healing assays where it promotes the localization of discs large homolog 1 (DLG1)[150], another important polarity protein. Dishevelled (DVL) and AXIN are also required for the establishment of organelle polarity of rat embryonic fibroblasts migrating in scratch assays, whereas GSK3β inhibitors, which activate CTNNB1-dependent transcription similarly prevent organelle polarity in migrating fibroblasts[151], suggesting that tight control of pathway activation is necessary for cell migration.

Relatively few studies have addressed potential roles for WNT signaling in animal models of metastasis. Expression of dominant negative TCF1 and TCF4 inhibits the metastatic capabilities of lung adenocarcinoma cells in a mouse model[152], while blocking CTNNB1 function with shRNAs reduces the incidence of both pulmonary and abdominal metastatic lesions in xenograft models of HCC[73]. Although CTNNB1 inhibits melanoma cell migration in vitro[153,154], expression of stabilized, constitutively active, CTNNB1 cooperates with PTEN loss and NRAS overexpression to promote metastatic progression in mouse models of melanoma[155,154]. One possible explanation for this discrepancy is that WNT/CTNNB1 signaling mediates complex cell-cell interactions necessary for metastatic progression in animal models. For example, one recent study finds that metastatic breast cancer cells induce the expression of periostin

(POSTN) in the lung microenvironment, which can, in turn, enhance WNT/CTNNB1 signaling and the growth of metastatic lesions[156].

CTNNB1-independent WNT pathways and metastasis. During development CTNNB1-independent WNT signaling pathways play crucial roles in embryo morphogenesis, in part, through the regulation of directed cell migration. These pathways may analogously contribute to the migration and invasion of cancer cells in a context-dependent manner. In melanoma, WNT5A acts through CTNNB1-independent signaling mechanisms to enhance melanoma cell invasiveness in vitro[43]. However, in other cancer cell lines, WNT5A inhibits cell migration and invasiveness. For example, recombinant WNT5A reduces the invasion of 22Rv1 and DU145 prostate cancer cells[52], while overexpression of WNT5A inhibits the migration of T47D breast cancer cells[157,158].

Studies investigating the mechanisms by which WNTs can regulate cell migration and metastasis independently of CTNNB1 are still in their infancy. Several studies have suggested that altering WNT5A signaling may induce changes in cell polarity. Supportively, expression of WNT5A enhances the polarization of melanoma cell adhesion molecule (MCAM) in melanoma cells that are responding to a chemokine gradient[159] . Moreover, knockdown of endogenous WNT5A with siRNAs similarly disrupts organelle polarity in rat embryonic fibroblasts migrating in scratch assays[151]. CTNNB1-independent WNTs may also regulate cell polarity in migrating cancer cells by cooperating with other PCP proteins, such as SCRIB, VANGL and PTK7[160–164]. In

developing embryos, WNT5A regulates the phosphorylation and the localization of VANGL[165], which suggests that WNTs can either directly or indirectly modulate the activity of PCP proteins. Further research is necessary to fully understand crosstalk between WNT/FZD signaling and PCP signaling networks in metastatic cancer cells, especially in vivo.

In addition to either directly or indirectly regulating cell polarity, CTNNB1-independent WNTs can also regulate cell motility and invasiveness by activating kinase-dependent signaling cascades, as is evident from the observations that WNT5A-induced invasion of MCF-7 cells can be inhibited by blocking JNK signaling[142] and that WNT5A-induced invasiveness of melanoma requires the phosphorylation of PKC[43]. Although many studies find that WNT3A can activate CTNNB1-dependent signaling, WNT3A-induced migration of myeloma cells can be blocked by PKC inhibition, but not by DKK1[141]. In a previous study DKK-1 expression was sufficient to block WNT3A induced CTNNB1-dependent transcription in myeloma cells[79], suggesting that WNT3A can regulate myeloma cell migration independently of CTNNB1. Finally, recent studies highlight an important role for the WNT5A receptor ROR2, in mediating metastatic cell behavior. siRNA-mediated reduction in ROR2 in B16 mouse melanoma cells reduces the frequency and severity of lung metastases in mice[166]. ROR2 siRNA also inhibits both baseline and WNT5A-induced invasion of sarcoma cells in vitro[103,167], while ROR2 overexpression enhances leiomyosarcoma and osteosarcoma cell migration[103,167]. Although the mechanisms are not entirely clear, these studies indicate that ROR2 may enhance migration by promoting the expression of MMPs[103,167].

Mechanisms of context-dependent WNT signaling in cancer

Unique outcomes of specific WNT pathway aberrations. WNT pathway genes are mutated at different frequencies in different cancer subtypes suggesting that these mutations do not equivalently activate WNT signaling and may have unique functional outcomes. For instance, APC mutations are common in colorectal tumors, but are rare in HCC[20] and in melanoma[168]. Mutations in APC can present unique phenotypes partially due to the multiplicity of APC functions as a component of the destruction complex in the WNT pathway, as a microtubule-binding protein, and as a guardian of genome integrity[169].

It is also likely that activation of WNT signaling pathways by different means results in differences in signaling amplitude or duration, which might lead to unique physiological or pathological consequences. Expression of a stabilized, constitutively active, form of CTNNB1 driven by MMTV is sufficient to induce extensive lobuloalveolar development and adenocarcinomas in mouse mammary glands but does not induce ductal branching[170], whereas expression of WNT1 induces extensive ductal branching, and mammary tumors at long latency[4,170,171]. These results suggest that WNT1 and constitutively activate CTNNB1 promote different phenotypes in mouse mammary tissue. Interestingly, MMTV-Wnt1 induces CTNNB1-dependent transcription in a basally located mammary cells, whereas and MMTV-CTNNB1 drives CTNNB1-dependent transcription in mammary cells expressing luminal markers[171]. These data suggest that different subsets of cells in mouse mammary tissue vary in their

responsiveness to different means of activating WNT/CTNNB1 signaling. Surprisingly, overexpression of a constitutively active form of CTNNB1 in other secretory epithelia also induces tumors in a tissue-specific manner. Specifically, CTNNB1 induces neoplastic growth of the prostate, but promotes cell hyperplasia and keratinization of the salivary glands and skin[172].

Crosstalk with other oncogenes and tumor suppressors. Aberrations in WNT signaling pathways drive tumorigenesis in cooperation with other signaling pathways, oncogenes and tumor suppressors. Although a comprehensive review of crosstalk between WNT signaling and other signaling pathways is beyond the scope of this review, below we discuss a few instances of crosstalk between WNT/CTNNB1 signaling and key tumor suppressors and oncogenes.

Altered activity of WNT signaling pathways is not sufficient to drive tumorigenesis. This is evident from studies revealing that the expression of a constitutively active form of CTNNB1 simultaneously enhances proliferation and increases apoptosis in mouse intestinal tissue[173]. Overexpression of constitutively active CTNNB1 in mouse embryonic fibroblasts (MEFs), similarly induces a senescence-like phenotype rather than enhanced proliferation[174]. However, CTNNB1-induced senescence is not observed when constitutively active CTNNB1 is expressed in Trp53 null, or in Arf null MEFs (encoded by the Cdkn2a gene)[174]. Inactivation of p53 also plays an important role in mouse models of WNT/CTNNB1-induced tumorigenesis. $Apc^{min/min}$ mice in $Trp53^{-/-}$ background developed more intestinal adenomas than $Apc^{min/min}$ mice in a $Trp53^{+/+}$

background[175]. These results suggest that apoptosis or senescence observed in response to hyper-activation of WNT/CTNNB1 signaling arises at least in part by p53 signaling.

Research in mouse models of other cancers has similarly reported nodes of crosstalk between WNT signaling networks and various oncogenes. In one model of lung cancer, over-expression of a form of CTNNB1 that has been mutated in a manner enhancing its stability and thus rendering it constitutively active produces no lung tumors, while co-expression of a constitutively active Kras with the stabilized CTNNB1 induces lung tumors[176]. Expression of stabilized CTNNB1 also synergizes with active forms of NRAS to induce melanoma tumors at short latency[177]. Along the same lines, expression of either the human papillomavirus 16 (HPV16) E7 viral oncoprotein and/or a constitutively active form of CTNNB1 promotes the development of cervical tumors at long latency, whereas double-transgenic mice developed cervical pathologies at a much higher rate[178]. Finally, co-transfection of hepatitis C virus (HCV) core protein with CTNNB1 can enhance growth of xenografted HCC cells, and co-expression if these proteins, again, has a synergistic effect[98].

Other studies indicate that simultaneous activation of WNT/β-catenin signaling and of the PI3K/AKT pathway can support tumorigenesis in variety of epithelial tissues. Co-expression of MMTV-PTEN to inhibit AKT signaling decreased β-catenin levels and delayed tumorigenesis driven by MMTV-Wnt1 in mouse mammary tissue [179]. Co-transduction of liver tissue with stabilized form of CTNNB1 and with either MET or activated AKT to activate PI3K/AKT pathway with results in hepatocellular carcinoma in mice, while none of these single genes results in

tumors [180]. Interestingly, combined expression of stabilized CTNNB1 with either the MET oncogene or with AKT induced liver tumors with unique histopathological features. Specifically, AKT/ CTNNB1 tumors, but not the MET/ AKT/ S37A-CTNNB1 were observed to display an unusual accumulation of intracellular lipid deposits[180]. These results suggest that PI3K/AKT signaling may cooperate with β-catenin dependent signaling to induce tumorigenesis, yet different means of activating these pathways are not equivalent and could lead to unique pathological consequences that must be considered when developing targeted therapies.

Context-dependent transcriptional outputs of TCF/LEF and CTNNB1. Activating or inhibiting the function of different WNT pathways proteins can induce unique transcriptional responses in a cell type-dependent manner. For example, activating mutations in CTNNB1 and loss of function mutations in AXIN1 induce different patterns of gene expression in hepatocellular carcinoma (HCC)[181]. These different transcriptional responses could arise from different levels of WNT/CTNNB1 pathway activation given that AXIN1 mutations induced much lower levels of CTNNB1-dependent reporter activity CTNNB1 mutations[181], but may also involve CTNNB1-independent functions of AXIN1. Furthermore, disruption of specific TCF/LEF family members (mammals express four different TCF/LEF family transcription factors with unique roles in regulating WNT target gene transcription[182]) is not equivalent to the ablation of CTNNB1 as revealed by expression profiling studies indicating very little overlap between CTNNB1 and TCF7L2 siRNA-induced changes[183]. Furthermore, while a dominant negative

TCF7L2 reduced CTNNB1-dependent transcription and induced cell cycle arrest in colorectal cells[119,183], increasing or decreasing TCF7L2 expression could both activate and repress CTNNB1 dependent reporters in other cell lines[183]. These data suggest that CTNNB1, AXIN1 and TCF family transcription factors regulate gene expression in a cell-type specific manner. Cell type-specific WNT/CTNNB1 transcriptional programs may arise, in part, from direct interactions between CTNNB1 and other transcription factors. In addition to TCF proteins, CTNNB1 also associates with additional transcription factors such as PITX1, SOX17, and FOXO1[184–187]. Other studies indicate that CTNNB1 can interact with nuclear hormone receptors such as the androgen receptor [188], the vitamin D receptor[189], and the retinoic acid receptor[190]. Importantly, CTNNB1 can regulate the transcriptional activity of these receptors and vice versa. Overexpression of either CTNNB1, or treatment with WNT3A can regulate androgen receptor-dependent transcription[188,191], whereas activation of vitamin D receptor inhibits CTNNB1-dependent transcription in colorectal cancer cells[189,192].

Collectively, these studies indicate that aberrant WNT signaling can cooperate with alterations in the activity of variety of other oncogenes and signaling proteins to promote the development of aggressive carcinomas. Other studies described above find that β-catenin cooperates with other transcription factors in order to drive specific patterns of gene expression in different cancer cell types. These data indicating synergy or cooperation between WNT/β-catenin signaling and other signaling pathways and gene expression regulatory networks may be of profound clinical importance. Specifically these data suggest that

combinatorial therapy targeting both aberrant WNT signaling and additional oncogene and tumor suppressor signaling networks could provide an avenue for treating the most aggressive cancers.

Strategies for targeting WNT signaling pathways in cancer

Small molecules. A handful of U.S. Food and Drug Administration (FDA) approved drugs modulate WNT signaling in vivo, though these drugs have other cellular targets as well. For example, lithium chloride has been in clinical use for decades and activates CTNNB1 by inhibiting GSK3. Non-steroidal anti-inflammatory drugs (NSAIDs) and the selective COX2 inhibitor celecoxib can inhibit CTNNB1-dependent transcription in colorectal cells[193,194 195] and reduce polyp formation in FAP patients and in mouse models of colon cancer[196,197,198,199], suggesting that these drugs may act in part through the modulation of CTNNB1 signaling. A number of studies have employed high-throughput screening of WNT activated luciferase reporters to identify novel inhibitors of WNT signaling. For example, the drug IWP ("inhibitor of WNT production"), identified in a screen of a synthetic chemical library, was found to inhibit the activity of Porcupine, a membrane-bound acetyltransferase that modifies WNT ligands with a palmitoyl group required for their secretion and signaling activity[200]. Two recent studies have identified the small molecules XAV939 and pyrvinium, which enhance AXIN stability via Tankyrase inhibition, and promote CTNNB1 phosphorylation via casein kinase activation, respectively, using a reporter-based screening approach[201,202].

Since the activation and inactivation of WNT signaling pathways via mutations and differential expression of WNT pathway proteins can lead to unique responses in different cancers, the identification of small molecules targeting specific WNT signaling components may be therapeutically useful. Hyper-activation of the WNT/CTNNB1 pathway due to mutations in APC and AXIN limits the potential molecular targets for pathway modulation since factors acting upstream of the destruction complex are no longer necessary for pathway activation. To overcome these challenges several researchers have conducted screens to identify molecules that can disrupt the interaction between TCF7L2 and CTNNB1 and thus inhibit CTNNB1-dependent transcription[124,203–206]. Several of the identified compounds can inhibit the growth of colorectal carcinoma cells in vitro and in vivo[203–205], while additional compounds similarly prevented the initiation and growth of mammary tumors in mouse xenografts[124]. The development of specific inhibitors of DVL using protein-protein interaction screens and structure-based design algorithms has also been attempted with some success, and some of these small molecules have been shown to regulate WNT/CTNNB1 signaling[207–212]. It should be noted that these DVL inhibitors could theoretically inhibit DVL function in both CTNNB1-dependent and CTNNB1-independent pathways.

Direct targeting of WNT signaling has been historically difficult thus far largely due to the lack of pathway specific targets, and the potential redundancy of many pathway components. Despite these challenges, numerous small molecule inhibitors of WNT signaling pathways have been identified as described above. At present, far fewer published studies

describe either WNT signaling activators or synergists. It is unclear whether the scarcity of effective WNT activating compounds reflects the biology of WNT signaling, or whether this scarcity reflects longstanding concerns about the oncogenic effects of hyper-activated WNT signaling observed in selected cancers that might persuade researchers to focus their efforts on characterizing pathway inhibitors. For a more comprehensive overview of small molecules targeting WNT signaling pathways and their molecular targets please refer to Table 3.

Table 3: Modulators of WNT signaling and their effects on cancers

Class of compound and target	Molecular Target	Compound	Functional effects of drug in cancer model or in vivo	Reference
INHIBITOR	PORCN (O-acetyltransferase), WNT protein production	IWP	IWP did not regulate the proliferation of several different cancer cell lines in vitro.	[200,213]
	Stimulates CK1α to promote CTNNB1 degradation	Pyrvinium	Pyrvinium synergizes with 5-FU in mediating SW620 CRC apoptosis, and inhibits the proliferation of SW480 and HCT116 cells. It should be noted that Pyrvinium is not a specific inhibitor of WNT signaling.	[201]
	Tankyrase inhibitor that can stabilize AXIN	JW55	Reduces tumor growth induced by LGR5 in APC mutant mouse model of tumorigenesis	[214]
		IWR	Inhibits tailfin regeneration in zebrafish a WNT-dependent process	[200]
		XAV939	Inhibits colony formation in soft agar of DLD1 cells in an AXIN dependent manner, but does not inhibit colony formation of RKO cells in soft agar	[202]
	CTNNB1 interaction with CBP	ICG-001	Reduces polyp formation in APC^{min} mouse model and decreases xenograft growth of SW620 colon carcinoma cells	[215]
	CTNNB1 interaction with TCF7L2	iCRT3 , iCRT5, and iCRT14	iCRT3 reduced the growth of colorectal cancer cells derived from patient biopsies, iCRT3, -5, -14 inhibit WNT3A-dependent changes in mouse mammary cell morphology which have been previously associated with cellular transformation	[203]
		BC21	Not reported	[206]
		NC043	Inhibits SW480 cell tumorigenesis in a xenograft model, and inhibits Caco-2 and SW480 colorectal cancer growth in vitro	[204]
		PKF115-584, CGP049090 PKF222-310	PKF115-584, CPG049090, and PKF118-310 can inhibit the growth of HCC cells in xenograftss and axis duplication in frogs.	[124]

Class of compound and target	Molecular Target	Compound	Functional effects of drug in cancer model or in vivo	Reference
	CTNNB1 stability	Thiazolidinediones (Δ2TG and 28)		[216]
		Murrayafoline A	Reduces cell viability of DLD1, SW480, HCT116, and LS174T colorectal cancer cells	[217]
	CTNNB1/TCF dependent transcription	OSU03012	Inhibits the growth of a variety of medulloblastoma cell lines	[218]
		3,6-dihydroxyflavone	Inhibits MDA-MB-231 breast cancer cell proliferation	[219]
		CCT036477, CCT070535, CCT031374	Inhibits SW480, HCT116 colorectal cell line growth	[220]
		Non-steroidal anti-inflammatory drugs	Reduce polyp formation in FAP patients and tumor growth in mouse models of colorectal cancer	[193,194,196,197]
	DVL/FZD	NSC668036	Not reported.	[207]
		3289-8625	Inhibits growth of PC3 prostate cancer cells, yet it is unclear if this effect is due to a loss of WNT/beta-catenin signaling	[209]
		FJ9	Reduced the growth of H460 cancer cells in xenograft studies	[208]
		PEN-N3	Not reported.	[211]
	Reduced DVL2 and CTNNB1 protein	niclosamide	Inhibits the growth of HT29, HCT116, and CaCO2 colorectal cells in culture and reduces colorectal cancer growth in xenografts	[221]
	Unknown	Cardinogen-1, -2, -3		[222]
ACTIVATOR	GSK3β	indirubins (INO), SB-216763, BIO(6-bromoindirubin-3'-oxime),		[223–225]
	AXIN2 and CTNNB1 association	SKL2001		[226]
	SFRP1	WAY-316606		[227]
	CTNNB1	Deoxycholic acid	Enhances colorectal cancer proliferation and invasiveness	[228]
SYNERGIST	Unknown	Oxepane derived compounds		[229]
	metabotropic glutamate receptors	Riluzole	Decreases melanoma cell proliferation in combination with WNT3A, and decreases melanoma metastasis in a xenograft model	[230]

Blocking antibodies. WNT/CTNNB1 signaling is required for serial transplantation and self-renewal of both normal hematopoietic stem cells and subsets of leukemia stem cells [115–117,231,232], suggesting that general inhibition of CTNNB1 signaling in patients could have unwanted side effects on normal, adult stem cells. Targeting specific WNTs and WNT receptors aberrantly overexpressed in tumors may prove to be an attractive strategy for targeting WNT signaling pathways preferentially in cancer cells. Indeed, several WNT-blocking antibodies inhibit proliferation and to induce apoptosis in different cancers[233–235]. Promising results have also been obtained using WNT blocking antibodies in vivo. For instance, intraperitoneal injections of WNT3A neutralizing antibodies decrease proliferation and induce apoptosis in a mouse model of prostate cancer[82].

Other studies have utilized blocking antibodies targeting WNT receptors to inhibit growth and to induce apoptosis in cancer cells such as, FZD7 specific antibodies, which preferentially target colon and HCC tumor cells as opposed to normal tisue[236,237]. Similarly, LRP antibodies reduce the growth of allografts of tumor cells derived from MMTV-Wnt1 and MMTV-Wnt3 tumors[238]. It may also be possible to target WNT receptors lacking clear functional relevance to disease progression, as long as those receptors are preferentially expressed in tumors relative to normal tissue. In a mouse synovial sarcoma xenograft model, FZD10 antibodies have been used to target delivery of the radioisotope Yttrium90 to tumors, resulting in inhibition of tumor growth[239]. As ROR1 is highly expressed in embryonic tissues and blood

cancers, but not in normal adult cells[93], a similar approach might be useful in targeting these cancers. Several groups are currently developing ROR1 antibody derivatives or other techniques to target the ROR1 positive malignant cells for selective killing by the immune system[138,240,241].

Peptides. In addition to WNT and WNT receptor antibody-mediated therapies, a few studies have suggested the utility of WNT modulatory peptides. Supporting this approach, SFRP1 or SFRP1-derived peptides delayed HCT116 xenograft tumor formation in nude mice and reduced the fraction of mitotic, but not apoptotic cells in these tumors[242]. Similarly, injection of full-length SFRP1 protein in xenografted mammary tumors derived from transformed mesenchymal precursor cells reduced tumor growth[126]. Another study identified peptide ligands that bind to the PDZ domain of human FZD2 and used this approach to disrupt WNT/CTNNB1 signaling in cell lines[211]. Peptides may also provide effective for targeting CTNNB1-independent signaling, which can be challenging given that the activity of many downstream kinase such as PKC and JNK are important for normal homeostasis and metabolism. Full-length WNT5A, as well as a formylated hexapeptide of WNT5A inhibit HB2 normal mammary cell and MDA-MB-268 breast cancer migration in a transwell assay[243], which suggests that peptide mimetics of WNTs or other extracellular WNT pathway members might serve as a therapeutic strategy. Indeed, daily injections with a WNT5A -derived peptide reduced the number of lung and liver metastases formed in mice xenografted with 4T1 breast cancer cells[243].

Numerous groups have attempted with some success to generate peptides capable of blocking high levels of FZD receptor activity. FZD7 extracellular domain peptides can block TCF/LEF reporter activity and the expression of WNT/CTNNB1 target genes in HCC cells[97]. These FZD7 blocking peptides can also interfere with the growth of HCC cells, which express high levels of the FZD7 receptor, but do not interfere with the viability of normal hepatocytes lacking FZD7 expression[212,237]. FZD7 peptides derived from the domains that interact with DVL could similarly inhibit the growth of HCC cells[212], however it is not clear whether this peptide specifically blocks FZD7 function or perhaps more generally blocks DVL function in these cells. Like FZD7 extracellular domain peptides, a soluble WNT receptor consisting of a FZD8 cysteine rich domain fused to a human Fc domain exhibits activity against teratoma lines in vivo[244]. A crystal structure of Xenopus WNT8 bound to FZD8 extracellular domain has recently been solved[245] and this breakthrough may facilitate the design of future modulators of WNT signaling in cancer.

WNT signaling and combinatorial therapy. Given that deregulation of WNT signaling pathways is not sufficient to induce tumor formation, it is possible that inhibition or activation of WNT signaling pathways alone will be insufficient to curb cancer progression. In addition to regulating the normal proliferation and survival of cancer cells in a context-dependent manner, activation or inhibition of WNT signaling pathways can also either sensitize or desensitize cancer cells to toxic insults, which might be advantageous in the development of combination therapies. Supporting this idea, inhibition of the WNT/CTNNB1 pathway in a variety of cancer

cells increases cell sensitivity to chemotherapeutic agents. For example, WIF1 increases PC3 prostate cancer cell sensitivity to paclitaxil and etoposide, but has no effect on DU145 cell death[246]. DKK1 similarly increases the sensitivity of U87MG glioblastoma cells to various toxins including DNA the damaging agents and drugs targeting microtubules [133]. Conversely, increased activity of certain WNTs may sensitize cancer cells to chemotherapeutic agents. Overexpression of WNT5A sensitizes SKOV3 ovarian carcinoma cells to a variety of chemotherapeutic agents[70], while activation of WNT/CTNNB1 signaling can sensitize melanoma cells to inhibitors of BRAF/MAPK signaling in melanoma[85]. Collectively these studies indicate that activating or inhibiting WNT pathways in conjunction with more conventional chemotherapeutics might result in cooperative inhibition of tumor growth.

Conclusions

Based on early discoveries linking activation WNT/CTNNB1 signaling to breast and colon carcinomas, it has generally been assumed that elevation of WNT signaling promotes tumor initiation and progression. Subsequent studies have suggested that this initial assumption may be an oversimplification, and that instead WNT/CTNNB1 signaling, as well as CTNNB1-independent WNT signaling pathways can either promote or inhibit cancer progression in a context-dependent manner. Taken together, these studies argue that aberrations in WNT signaling cannot be targeted using one universal strategy, but rather that clinical decision-making should be informed by our increasingly detailed understanding of the context-dependent roles of WNT signaling in cancer. Future studies aimed at determining mechanisms controlling this context-dependency will be necessary to identify signaling nodes that could be targeted by therapeutic interventions. Given the interconnectedness of WNT signaling with other oncogene and tumor suppressor pathways, it will also be important for future research to focus on further unraveling the mechanisms of crosstalk between WNT pathways and related signaling networks.

In the past decade, we have witnessed an explosion in the development of strategies to targeting these WNT signaling. Many synthetic modulators of WNT signaling including, small molecules, peptides and blocking antibodies show great promise in animal models of several different cancers. Importantly, activating or inhibiting WNT signaling pathways alone is unlikely

to result in significant improvement in disease progression due to co-activation of numerous oncogenic pathways in most cancers. Further research is clearly necessary to not only optimize these reagents for applications in animals and eventually in human patients, but also to further explore the potential value of combinatorial therapies. Studies aimed at identifying genetic factors and biomarkers that can be used to predict response to treatment with WNT pathway modulators either alone or in combination with other therapies will be an important next step in determining the utility of these potential new therapies. Despite these future challenges, these pioneering studies suggest that targeting WNT signaling pathways in cancer patients will be possible in the near future.

Glossary

Constitutively active CTNNB1: Various forms of CTNNB1 with either N-terminal truncations or point mutations, which prevent phosphorylation and degradation by the proteasome.

Apc^{min} mice: An animal model with a mutation in APC leading to FAP and spontaneous colorectal tumors.

Tumor initiating cells: A subset of cancer cells often with stem-cell like expression profiles capable of generating new tumors.

Planar Cell Polarity (PCP): The collective orientation of cells within the epithelia plane.

feeder cells: Cells added to co-culture experiments intended to support the growth of other cells.

basal-like breast cancer: A subset of breast cancer characterized by a gene expression signature similar to that of the basal/myoepithelial cells of the breast.

mouse mammary tumor virus (MMTV): A species of retrovirus that can drive mammary adenocarcinoma development in susceptible strains of mice in the presence of steroid hormones.

scratch assays: An assay used to determine the motility of cells in vitro. In these experiments cell monolayers are scratched or wounded and the ability of cells to migrate and fill the resulting gap is measured.

Chapter 2: AGGF1 is a novel regulator of β-catenin-dependent transcription in colorectal carcinoma

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New regulators of WNT/beta-catenin signaling revealed by integrative molecular screening

M.B. Major, B.S. Roberts, J.D. Berndt, S. Marine, J. N. Anastas, N. Chung, M. Ferrer et. al (2008) Science Signaling. **1**, 45, ra12.

Abstract

Hyper-activation of β-catenin-dependent transcription is observed in majority of colorectal tumors and correlates with poor clinical outcomes. β-catenin activates transcription in cooperation with the TCF/LEF family of transcription factors and several chromatin modifying enzymes including histone acetyltransferases and the ATP-dependent chromatin remodeling complex, SWI/SNF. In collaboration with Rosetta-Merck, we have conducted a genome-wide siRNA screen in order to identify novel regulators of β-catenin signaling in colorectal carcinoma cells using a β-catenin-activated luciferase reporter. Using this high-throughput screening approach we have identified a novel activator of β-catenin-dependent transcription called AGGF1. Our further studies reveal that AGGF1 is a nuclear protein that associates with components of the SWI/SNF family of chromatin remodeling proteins. Using chromatin immunoprecipitation we then find that AGGF1 associates with putative TCF/LEF binding DNA sequences in β-catenin target genes, suggesting that AGGF1 can function as a novel transcription co-factor for β-catenin in the context of colorectal cancer.

Introduction

A majority of colorectal carcinomas harbor mutations in WNT pathway genes such as, APC, AXIN2, and CTNNB1 (the gene encoding β-catenin), which result in the stabilization of β-catenin and the hyper-activation of β-catenin-dependent transcription (Table 1). In the absence of β-catenin, members of the TCF/LEF family of transcription factors act as transcriptional repressors[247]. Stabilized β-catenin can convert TCF/LEF from transcriptional repressors to transcriptional activators by facilitating the recruitment of several chromatin modifying enzymes including histone acetyltransferase and the ATP-dependent chromatin remodeling enzyme complex, SWI/SNF to target genes[248–252]. Given that tumorigenesis in the colon requires the activation of β-catenin-dependent transcription[120,253,254], we reasoned that specific modulators of β-catenin dependent transcription in colorectal cancer might serve as novel therapeutic targets. In order to identify modulators of β-catenin-dependent transcription in colorectal cancer we have conducted near genome-wide siRNA screen using a β-catenin-activated luciferase reporter. This screen identified numerous proteins that can either enhance or inhibit β-catenin-dependent transcription in colorectal cancer cell lines including a protein called AGGF1.

Previous studies find that AGGF1 is mutated in Klippel–Trenaunay syndrome (KTS), a human vascular overgrowth disease characterized by capillary malformations and soft tissue hypertrophy[255–257]. Further mechanistic studies in endothelial cells reveal that disease-

associated AGGF1 mutations disrupt the function of AGGF1 as a pro-angiogenic, secreted protein[255,256]. In contrast to these previous results, several lines of evidence from our studies conducted using colorectal cancer cells support the surprising finding that AGGF1 can also function in the nucleus where it associates with chromatin to regulate the expression of target genes. First, we find that AGGF1 loss of function inhibits the WNT/β-catenin pathway downstream of the nuclear localization of β-catenin. Second, we observe that endogenous AGGF1 localizes to the nuclei of multiple cell lines. Third, AGGF1 protein complexes identified by affinity purification and mass spectrometry contain BAF57, a component of the SWI/SNF family of chromatin remodeling complexes[258,259] as well as other nuclear proteins. Finally, we used chromatin immunoprecipitation (ChIP) to show that AGGF1 associates with putative TCF/LEF responsive elements in cis regulatory regions for the β-catenin target genes AXIN2 and LEF1. Together these data suggest that AGGF1 and β-catenin cooperate at specific gene enhancer elements to regulate the transcription of target genes in colorectal cancer.

Results

siRNA-depletion of AGGF1 inhibits β-catenin-dependent transcription

We identified AGGF1 as a potential enhancer of β-catenin-dependent transcription in DLD1 colorectal carcinoma cells in a near genome-wide siRNA screen conducted in collaboration with Rosetta-Merck. The DLD1 cells used in this screen harbor inactivating mutations in APC and consequently display constitutively active WNT/β-catenin signal

transduction[7]. DLD1 cells were engineered to express a β-catenin– responsive firefly luciferase reporter (BAR) to enable high-throughput measurement of β-catenin-dependent transcription, as well as an EF1α-driven Renilla luciferase reporter for normalization purposes [260]. Using these cells, we screened 28,124 siRNA pools in triplicate, each consisting of 3 unique siRNAs, targeting 20,042 messenger RNAs (mRNAs). siRNAs targeting 3% of mRNAs fulfilled the hit-calling criteria of a normalized fold change of greater than 3 or less than 0.33 with a Student's t-test P value less than 0.01. With this stringent data restriction, the primary screen yielded 740 genes that regulate WNT/β-catenin signal transduction.

Given that off-target silencing effects are inherent to siRNA screens and can thereby produce high false-positive discovery rates , we implemented three validation screens, the first to increase the number of siRNAs tested, the second to eliminate cell type–specific hits, and a third to ensure that the hits were indeed regulators of endogenous β-catenin transcriptional target genes. In the first validation screen, we individually tested between three and nine non-overlapping gene-specific siRNAs. Of the 740 genes that passed the hit criteria in the primary screen, 268 were confirmed by a minimum of two independent siRNAs, suggesting that these genes were not a result of off-target effects. We then broadened the general applicability of our screening hits by eliminating cell line–specific effects. Specifically, we repeated the secondary screen by individually testing three to nine independent siRNAs targeting each of the hits from the primary screen in SW480 cells, another APC mutant colorectal adenocarcinoma cell line[261].

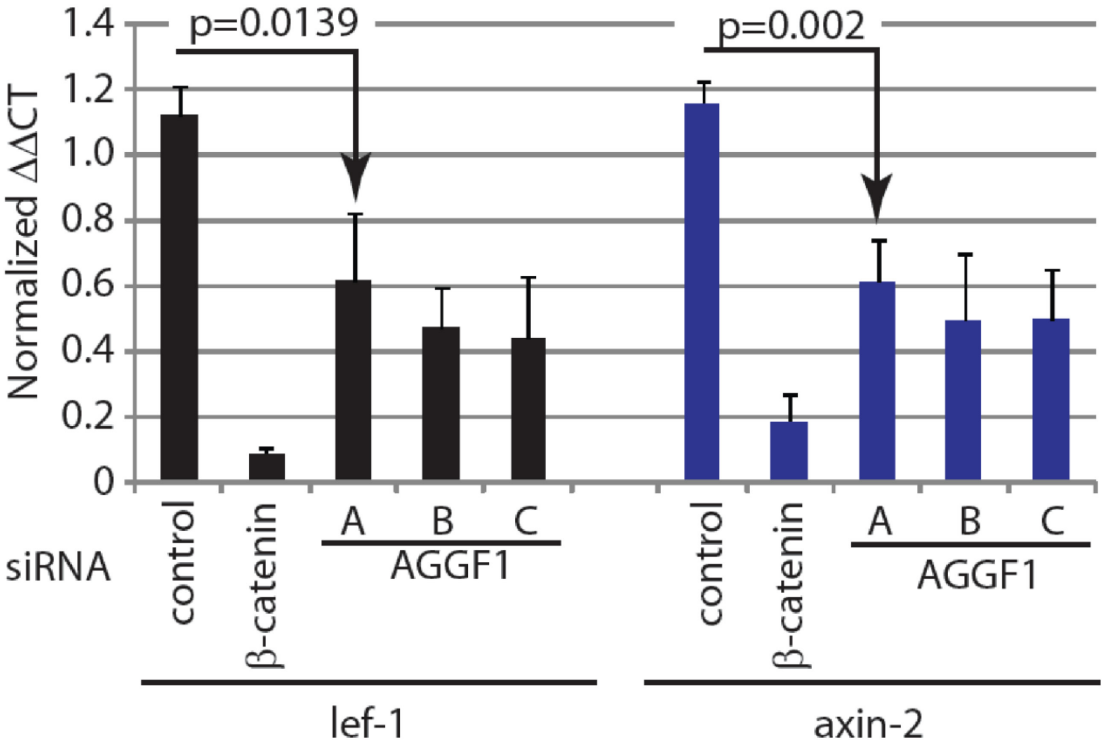

Figure 3: AGGF1 is required for the expression of both *LEF1* and *AXIN2*

qPCR for *LEF1* and *AXIN2* mRNAs conducted 72 hours following transfection of DLD1 cells with indicated siRNAs. p-values demonstrate statistical significance across 4 biological replicates (Students t-test).

Overall, 119 were identified at the intersection of secondary screen data sets for DLD1 cells and SW480 cells. Therefore, of the 28,124 siRNAs tested against the WNT/β-catenin signal transduction pathway, the secondary screen identified 119 genes that were confirmed with multiple siRNAs in multiple cell lines.

We selected AGGF1, one of these 119 high confidence hits from both the primary and secondary screens as a subject of future study based on previous studies reporting that AGGF1 mutations are associated with human disease (Online Mendelian Inheritance in Man database (OMIM) and references [255–257]). Since the original siRNA screen relied on the use of an artificial reporter construct we first validated the results of the siRNA screen by determining the consequences of AGGF1 depletion on the expression of endogenous β-catenin target genes. We find that three independent siRNAs targeting AGGF1 (A, B and C) reduce the expression of both AXIN2 and LEF1, two well-characterized β-catenin target genes in DLD1 colorectal carcinoma cells as measured by RT-PCR (Figure 3). Together, the results of the siRNA screen and these further validation experiments reveal that AGGF1 is a novel enhancer of β-catenin-dependent transcription in colorectal cells.

AGGF1 and β-catenin associate with components of the SWI/SNF family of chromatin remodeling proteins

In order to better understand the mechanisms responsible for AGGF1-dependent enhancement of β-catenin dependent transcription in colorectal cancer we isolated AGGF1

associated protein complexes by tandem affinity purification and identified associated proteins by mass spectrometry (Figure 4). Since AGGF1 was previously reported to act as a secreted factor that can promote angiogenesis[255,257], we expected to identify proteins associated with secretory machinery and the plasma membrane. However, we were surprised to identify several different nuclear proteins. The AGGF1-associated proteins identified in this analysis include proteins known to associate with the SWI/SNF family of chromatin remodeling complexes such as BAF57 (encoded by SMARCE1) (Figure 4, green box). AGGF1 was also found to associate with additional nuclear proteins such as XRCC5 and XRCC6[262], which are recruited to chromatin and participate in a variety of different processes such as DNA repair pathways, as well as NUP153, a component of nuclear pore complexes (Figure 4). Taken together, these data indicate that AGGF1 may have an as of yet unappreciated function in the nucleus in addition to its role as a secreted factor.

We focused our further studies on the interaction between AGGF1 and the SWI/SNF family protein BAF57 based on published studies linking SWI/SNF function to β-catenin-dependent transcription. Specifically, Brahma related gene, or BRG1 (encoded by SMARCA4) is an SWI/SNF family enzyme that both directly binds β-catenin and is required for β-catenin–mediated transcription[247,251,263]. By re-distributing nucleosomes along DNA, SWI/SNF family protein complexes creates a chromatin structure that can either facilitate or impede gene transcription depending on the specific genomic context[264,265] . .

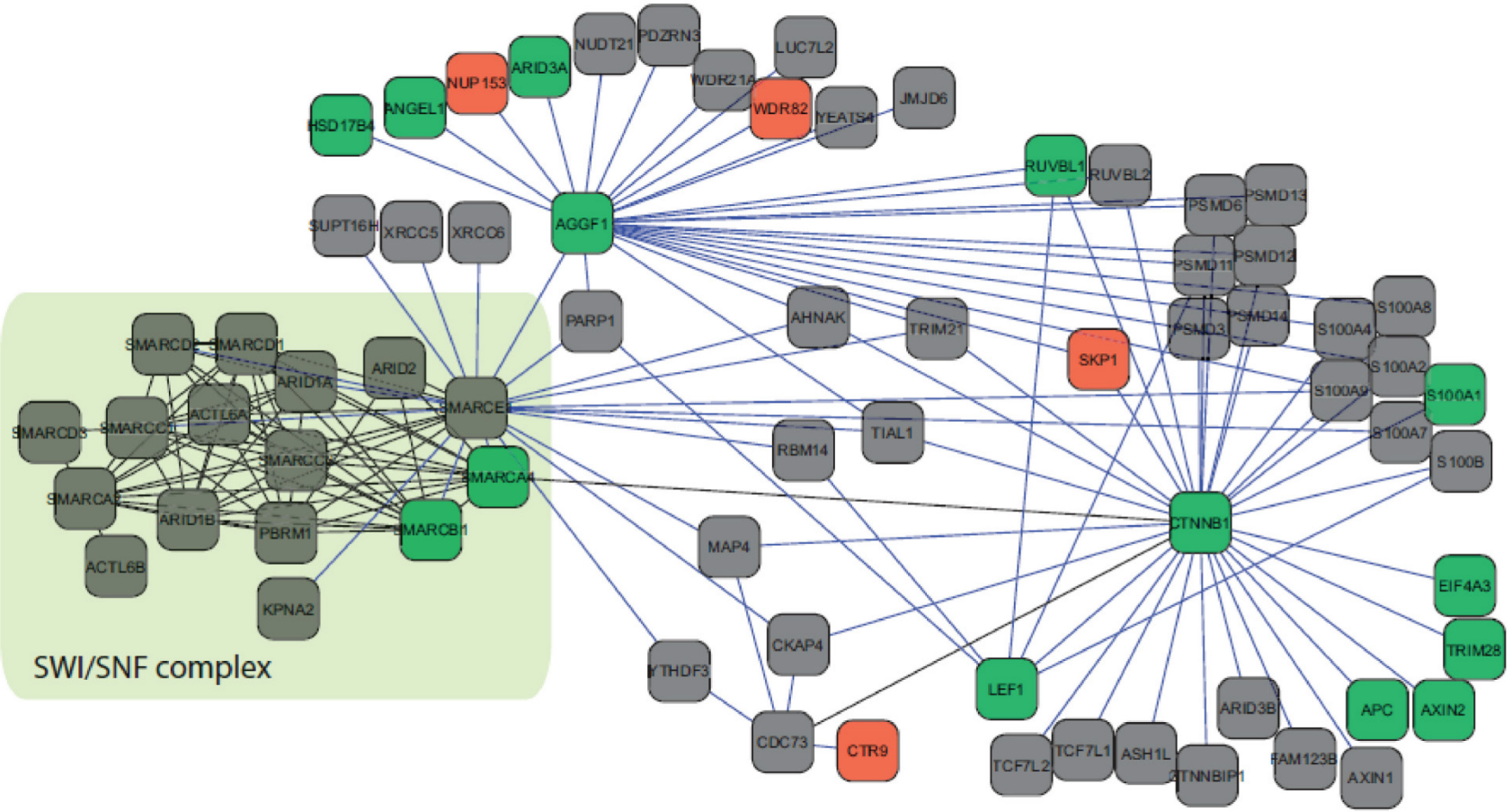

Figure 4: Protein-protein interaction network for AGGF1 and β-catenin reveals a shared association with SWI/SNF protein complexes

Diagram of protein-protein interaction network for AGGF1 and β-catenin (CTNNB1) reveals significant overlap particularly with proteins previously found to be associated with the SWI/SNF protein complex. Connecting lines were derived from mass spectrometry experiments (blue) and from bioinformatics resources (black). Red and green nodes denote protein inhibitors and activators of β-catenin-dependent transcription identified by genome wide siRNA screening, respectively.

The results of our siRNA screen validate these previous studies as siRNAs targeting transcripts encoding SWI/SNF family members, SMARCA4 and SMARCB1 inhibit β-catenin-dependent transcription[247]. These data suggest a model whereby AGGF1 regulates the transcription of β-catenin target genes by acting as a novel co-factor for SWI/SNF-dependent chromatin remodeling.

AGGF1 and AGGF1-associated proteins regulate β-catenin-dependent transcription

Since the results of our proteomic analysis of AGGF1 protein complexes suggest that AGGF1 might regulate β-catenin-dependent transcription via its interactions with SWI/SNF family of multi-protein complexes, we next validated results of our screen suggesting that SWI/SNF family proteins regulate β-catenin-dependent transcription in colorectal carcinoma cells as they do in other contexts. Consistent with our previous results we find that siRNAs targeting AGGF1 and β-catenin can inhibit the expression of a β-catenin responsive reporter and also effectively reduce their target proteins as determined by Western blotting (Figure 5a,b). As a control, we also transfected DLD1 cells with siRNAs targeting know negative regulators of β-catenin-dependent transcription and find that knockdown of APC and β-TRCP (encoded by the gene BTRC), two components of the destruction complex, can enhance reporter expression as expected.

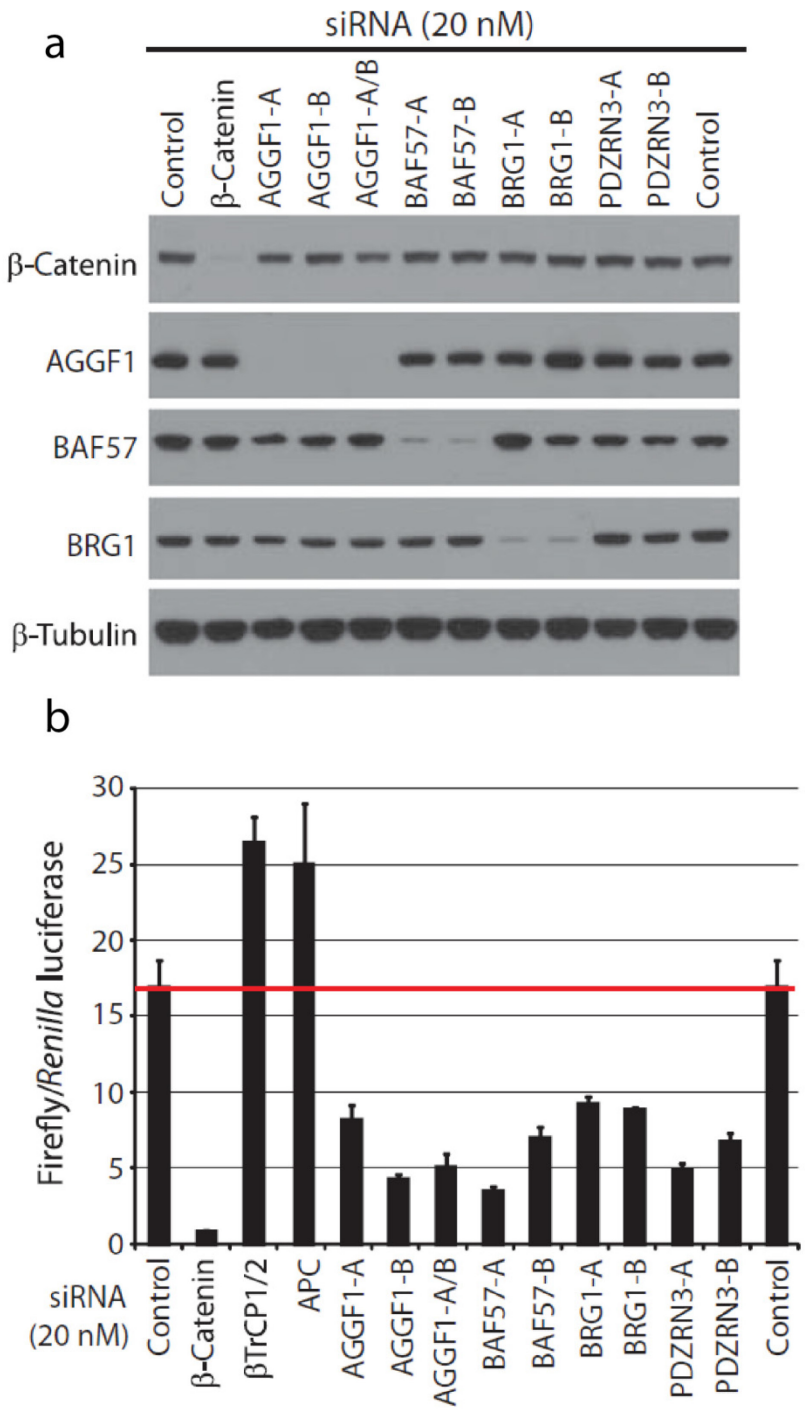

Figure 5: AGGF1 and AGGF1-associated proteins regulate β-catenin dependent transcription

(a) Validation of endogenous protein knockdown by Western blotting. siRNAs complementary to the indicated mRNAs were transfected into DLD1 cells 72 hours before protein isolation and Western blot analysis. (b) DLD1 colorectal carcinoma cells stably expressing β-catenin responsive luciferase reporter were transfected with the indicated siRNAs 72 hours before cell lysis and luciferase quantification. All values were normalized to a constitutively transcribed Renilla luciferase driven by an EF1alpha promoter sequence also expressed by these DLD1 reporter cells. Error bars represent standard deviation across biological quadruplicates and are representative of four independent experiments.

As we anticipated, multiple siRNAs directed against the SWI/SNF family members BAF57 and BRG1 also partially inhibit β-catenin-dependent transcription and reduce the abundance of their targets as assessed by Western blotting (Figure 5a,b). We also determined if BAF57 can regulate endogenous β-catenin target genes by analyzing the expression of LEF1 following knockdown of either AGGF1 or β-catenin (positive controls), or following knockdown of BAF57 and find that BAF57 is also required for optimum expression of LEF1 (Figure 6b).

AGGF1 and AGGF1-associated proteins regulate the transactivation function of β-catenin

Although APC mutant DLD1 cells exhibit constitutive expression of β-catenin target genes independently of active WNT/FZD receptor protein complexes, several possible mechanisms for regulating β-catenin-dependent transcription are still possible. First, β-catenin must be translocated and retained to the nucleus, a poorly understood process which involves modulation of both nuclear import and export machinery. Second, β-catenin must be recruited to specific promoter and enhancer sequences through its interaction with the TCF/LEF family of transcription factors and other proteins. Finally, β-catenin must actively recruit a number of different co-factors in order to transactive the transcription of these target genes. Since AGGF1 associates with SWI/SNF family proteins known to cooperate with β-catenin at target genes to promote transcription, we hypothesized that AGGF1 might similarly act at chromatin to enhance the ability of β-catenin to transactivate target gene transcription.

To test this hypothesis we determined whether AGGF1 and AGGF1-associated proteins can regulate the transactivation of luciferase reporter driven by an upstream activating sequence (UAS) following transfection with a β-catenin-GAL4 fusion protein. Specifically, this fusion protein consists of the C-terminus of β-catenin, which is required for its transcriptional activity[247], fused to the DNA binding domain of GAL4, which can bind to the UAS sequence in the reporter. Importantly, activation of the GAL4-luciferase reporter gene by the β-catenin-GAL4 fusion protein occurs independently of β-catenin recruitment to chromatin through its interaction with TCF/LEF transcription factors.

As expected, we find that siRNA-mediated knockdown of β-catenin reduces the activity of this GAL4 luciferase reporter (Figure 6a). We also find that siRNAs targeting both AGGF1 and the AGGF1-associated proteins BAF57 and PDZRN3 identified in our proteomics analysis similarly reduced the activity of this reporter (Figure 6a). As a negative control we also tested whether β-catenin, AGGF1, BAF57, and PDZRN3 siRNAs also regulated the GAL4 luciferase reporter driven by co-expression of a GAL4-VP16 fusion protein and observed no major enhancement of inhibition of reporter activity driven by VP16. These data suggest that AGGF1 and AGGF1-associated proteins are required for β-catenin to activate transcription, but are not generally required for the transactivation function of all transcription factors.

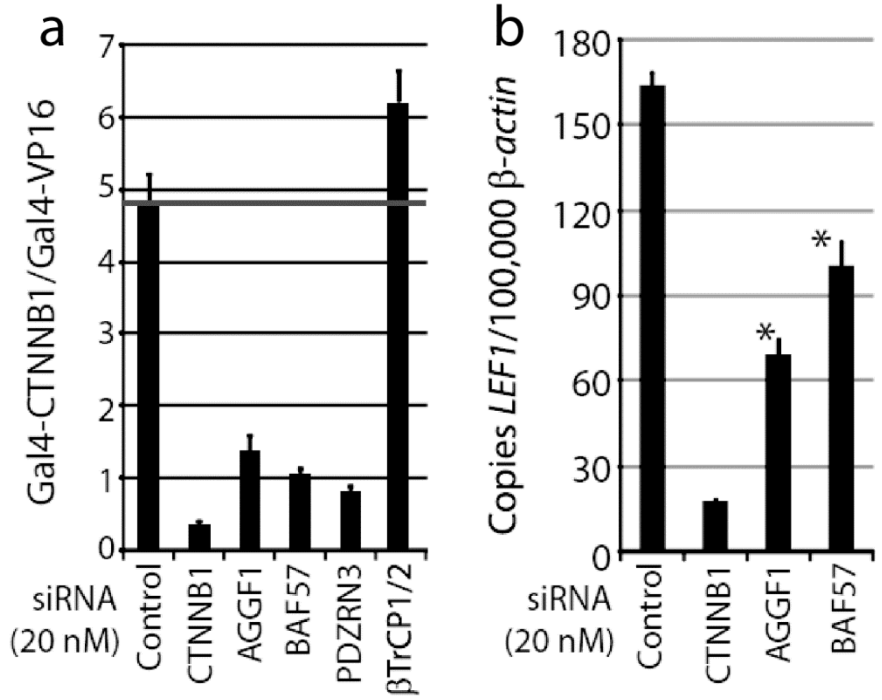

Figure 6: AGGF1 and AGGF1-associated proteins are required for the transactivation of β-catenin target genes.

(a) DLD1 cells stably expressing a UAS-luciferase reporter and either Gal4-β fusion protein (Gal4-CTNNB1) or a Gal4-VP16 fusion protein were transfected with the indicated siRNAs for 72 hours before luciferase quantitation. Error bars represent standard deviation across biological quadruplicates.(b) Quantitative PCR of *LEF1* and *β-actin* mRNA after siRNA-mediated silencing of β-catenin, AGGF1, or BAF57 in DLD1cells (*P < 0.05; Students t test).

AGGF1 partially localizes to cell nuclei

Although our results so far are consistent with the hypothesis that AGGF1 regulates β-catenin-dependent transcription through its interactions with SWI/SNF family proteins, it is not yet clear whether AGGF1 directly functions in the nucleus. While it is possible that AGGF1 directly associates with SWI/SNF associated protein complexes located at β-catenin target genes, it is also possible that AGGF1 might regulate SWI/SNF protein complexes outside the nucleus in a manner that indirectly enhances their ability to promote the transcription of β-catenin target genes. Since a nuclear function for AGGF1 has not yet been described, we next analyzed the subcellular localization of AGGF1 in colorectal cells by immunofluorescence staining using antibodies that recognize endogenous AGGF1.

We find that the AGGF1 signal localizes to both the cytosol as well as to the cell nuclei as marked by DAPI staining for DNA (Figure 7a). Specifically, AGGF1 localizes to euchromatic regions of the nucleus characterized by low intensity DAPI staining. We also analyzed the localization pattern of BAF57 in colorectal cells and find that BAF57 co-localizes with AGGF1 in DLD1 nuclei (Figure 7b). Finally, we tested whether overexpressed AGGF1 similarly localizes to cell nuclei. When we transfected HEK293T cells with HA-tagged AGGF1 (HA-AGGF1), and then stained cells using antibodies recognizing the HA tag we find that HA-AGGF1 similarly localizes to cell nuclei (Figure 7c). These data suggesting thatAGGF1 localizes to the nucleus are consistent with previous results where nuclear localization was also observed, but curiously not mentioned in the discussion of the data[255].

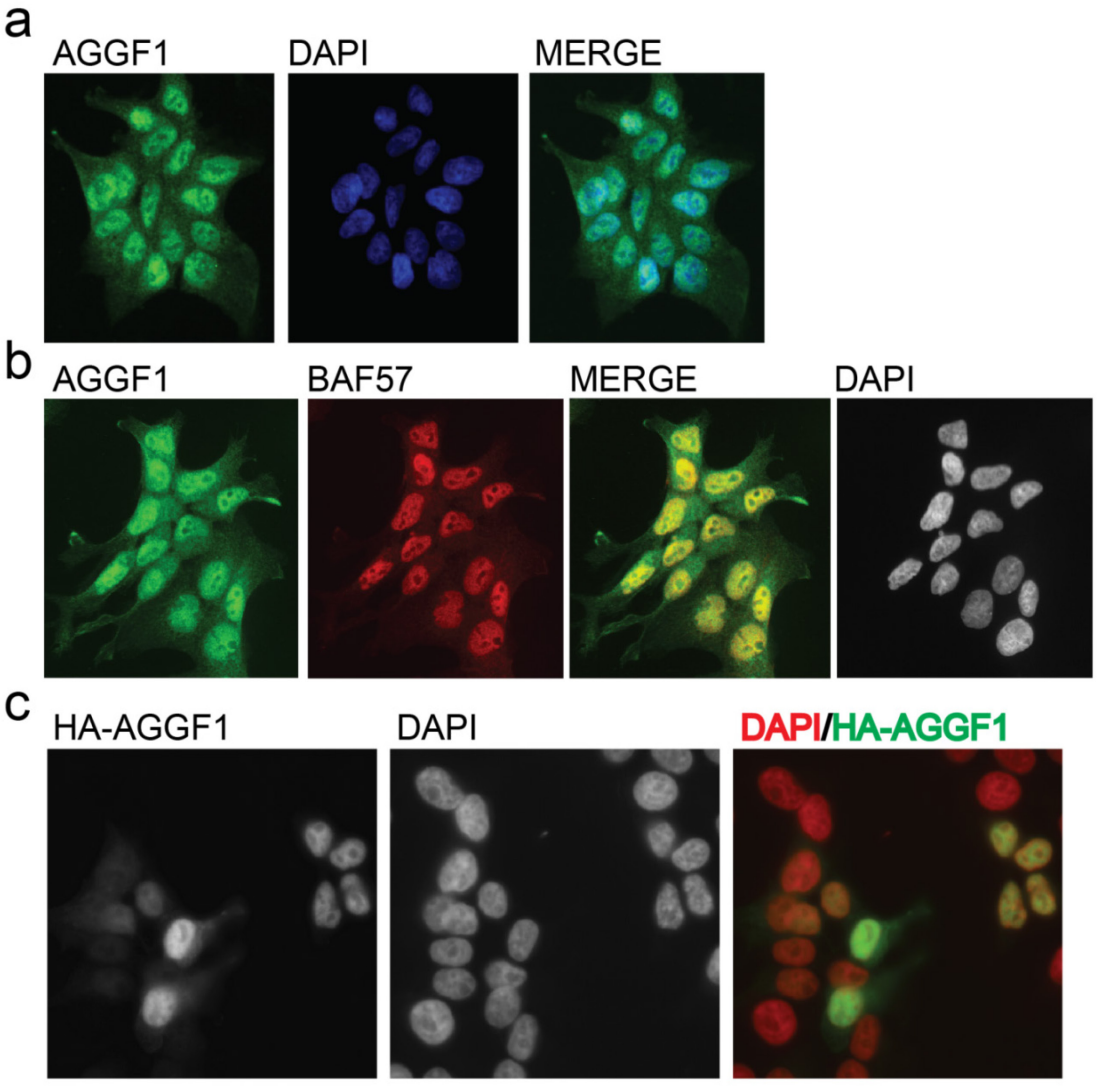

Figure 7: AGGF1 is a nuclear protein that co-localizes with BAF57 in the nucleus

(a) Co-staining of DLD1 colorectal carcinoma cells using an antibody recognizing AGGF1 (green) and DAPI (blue) to stain for DNA revealing localization of AGGF1 to both the cytosol and the nucleus. Merged image reveals areas of overlap for AGGF1 and DAPI (cyan). (c) Co-staining of DLD1 cells using antibodies to recognize AGGF1 (green) and BAF57 (red). Merged image reveals areas of overlap for AGGF1 and BAF57 (yellow). (c) Co-staining of HEK293T cells transfected with HA-AGGF1 stained for HA (green) and DAPI (red). Merged image reveals selected cells expressing AGGF1 in the nucleus (yellow).

Recombinant human AGGF1 (rhAGGF1) does not regulate β-catenin-mediated transcription.

Previous studies suggest that AGGF1 can act as a secreted factor to promote angiogenesis[255–257], whereas our results support the hypothesis that AGGF1 associates with nuclear proteins and may act as a transcriptional co-activator in colorectal cells. We next asked whether AGGF1 might also have a role as a secreted factor in colorectal cells. We predicted that adding AGGF1 to the cell culture media might enhance β-catenin dependent transcription if the secreted form of the protein was acting to mediate β-catenin responsiveness. To test this prediction we treated DLD1 cells harboring a β-catenin responsive luciferase reporter with increasing concentrations of recombinant human AGGF1 (rhAGGF1) at concentrations previously shown to enhance angiogenesis[255]. As a negative control we also treated additional DLD1 reporter cells with equivalent concentrations of bovine serum albumin (BSA). Neither rhAGGF1, nor BSA resulted in any significant change in reporter gene expression (Figure 8). It is possible that rhAGGF1 cannot promote β-catenin-dependent transcription due to the presence of abundant quantities of endogenous AGGF1 secreted by the cancer cells used in these studies. However, we were unable to detect any AGGF1 in media from DLD1 or other colorectal cell cultures even after concentrating the media (data not shown), which suggests that AGGF1 does not likely function as a secreted protein in colorectal cells. Together, these results indicate that DLD1 colorectal cells secrete very low levels of AGGF1 and that the addition of AGGF1 to cell culture media is not sufficient to regulate β-catenin-dependent transcription in colorectal cells.

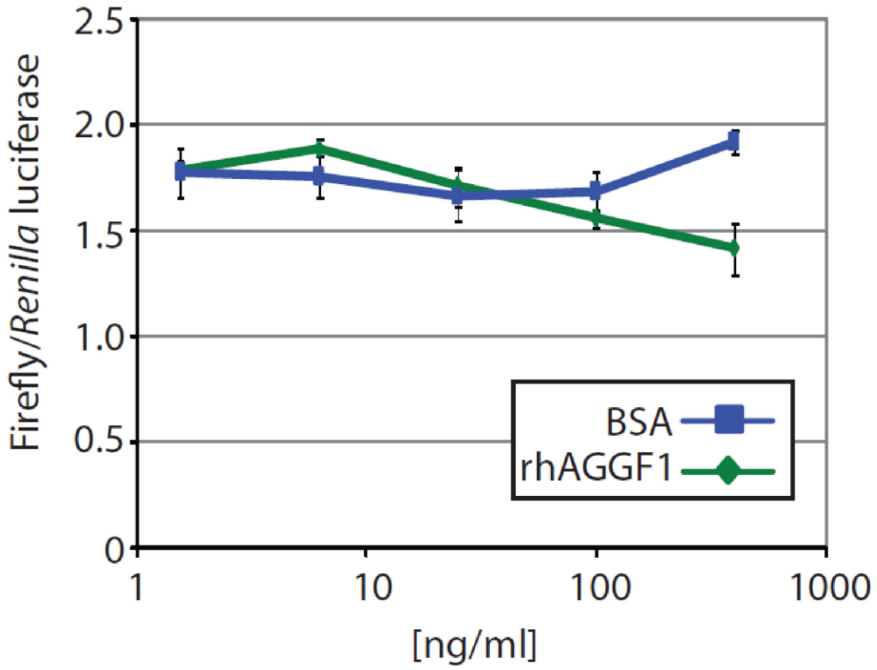

Figure 8: Recombinant human AGGF1 (rhAGGF1) does not regulate β-catenin-mediated transcription.

rhAGGF1 (R&D Systems) or bovine serum albumin (BSA) were added at the indicated concentrations to the growth media of the DLD1-pBAR/renilla cells for24 hrs prior to luciferase quantification.

AGGF1 and β-catenin associate with putative TCF/LEF binding sites in the LEF1 and AXIN2 genomic loci

Based on our finding that AGGF1 associates with BAF57, a chromatin associated protein, we next asked whether AGGF1 similarly associates with β-catenin target gene loci by chromatin immunoprecipiation. We used antibodies detecting either the trimethylated form of lysine 4 of histone 3 (H3K4Me3) (a positive control), IgG (a negative control), β-catenin, and two different antibodies recognizing AGGF1 (AGGF1a and AGGF1b) to enrich from chromatin associated with these proteins. Consistent with previous studies[266] we find that the H3K4Me3 antibody immunoprecipitates sequences located near the transcription start sites of the LEF1 and AXIN2 genes as assessed by semi-quantitative RT-PCR (Figure 9a,b). In contrast, β-catenin antibodies do not pull down sequences associated with the transcription start sites, but do enrich for sequences that include putative TCF/LEF binding sites (marked by red circles) as detected using primers centered around the region 2251 base pairs 5' of the LEF1 open reading frame (ORF) (Figure 9a), and located 966 base pairs 5' of the AXIN2 ORF (Figure 9b). We find that two independent AGGF1 antibodies similarly immunoprecipitate those sequences from the LEF1 and AXIN2 loci containing TCF/LEF binding sites, but do not enrich for more 3' regions located closer to the transcription start sites, which lack consensus TCF/LEF binding sequences (Figure 9a,b).

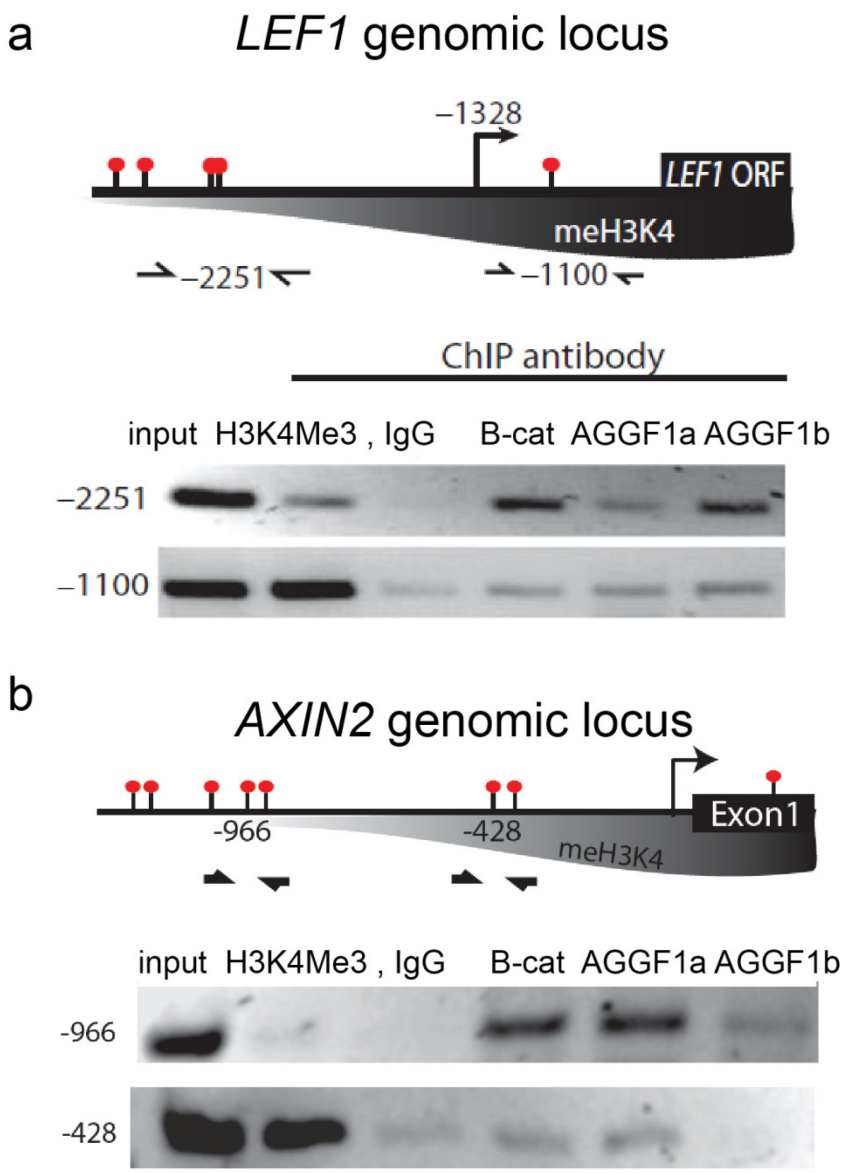

Figure 9: AGGF1 and β-catenin associate with putative TCF/LEF binding sites in the *LEF1* and *AXIN2* genomic loci

(a-b) Semi-quantitative PCR analysis of ChIP pulldowns using antibodies to enrich for chromatin associated with H3K4Me3 (positive control), β-catenin, IgG (negative control), and two different antibodies recognizing AGGF1 (AGGF1a and AGGF1b).Schematics of the *LEF1* (a) and AXIN2, (b) genomic loci, showing putative TCF/LEF DNA binding sites as red filled circles.

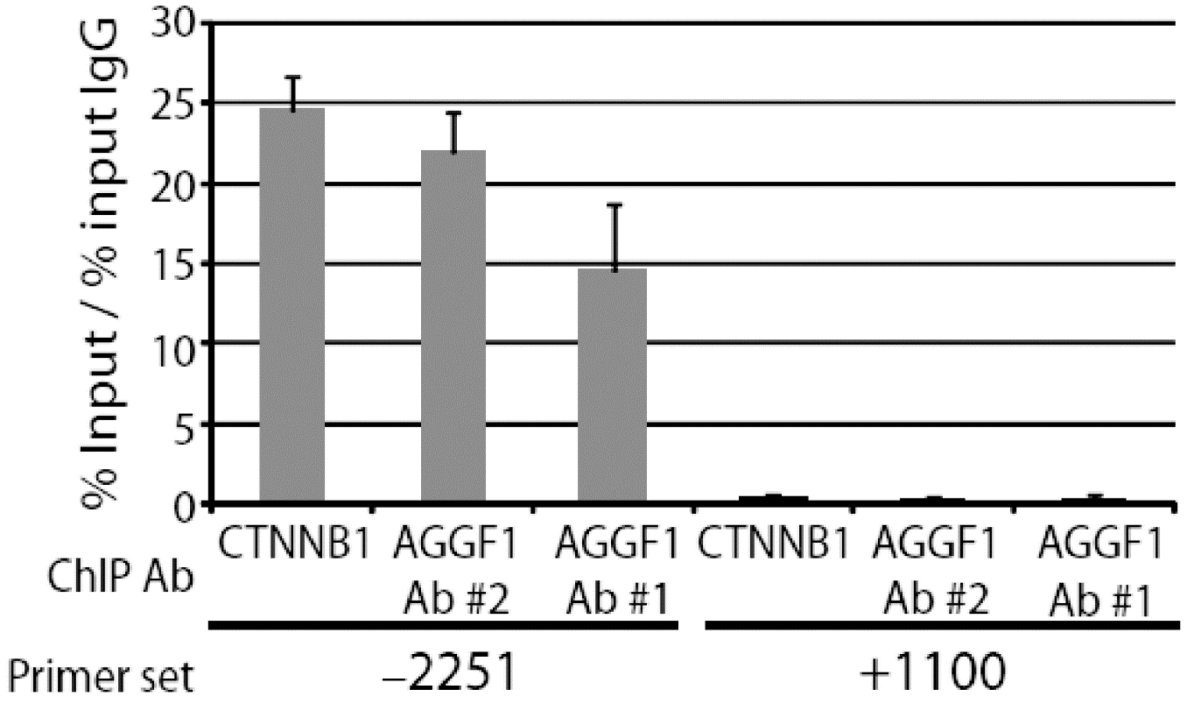

Figure 10: AGGF1 and β-catenin associate with putative TCF/LEF binding sites in the *AXIN2* promoter

qPCR for two regions of the LEF1genomic locus following ChIP analysis with the indicated antibodies. Data were normalized to the IgG ChIP signal and represented as percent of input and representative of three biological replicates. Error bars depict standard deviation from the mean.

To further validate these results we also used quantitative RT-PCR to detect DNA sequences enriched by these antibodies in in our chromatin immunoprecipitation experiments. Consistent with our analysis using semi-quantitative PCR (Figure 9a,b), we find that antibodies detecting β-catenin and AGGF1 pull down a region of the LEF1 genomic locus containing TCF/LEF binding sites (-2251), but do not pull down a control sequence located closer to the transcription start site (+1100) (Figure 10). In summary, these chromatin immunoprecipitation experiments reveal that both AGGF1 and β-catenin associate with DNA sequences containing TCF/LEF binding sites found at β-catenin target gene loci. These data suggest that AGGF1 may function at chromatin to regulate gene expression.

Depletion of AGGF1 reduces the recruitment of β-catenin to TCF/LEF binding sites in the LEF1 locus

The SWI/SNF chromatin remodeling complex can alter DNA accessibility to transcription factors and other chromatin associated factors by re-arranging chromatin structure[265]. Our data suggests that AGGF1 can associate with SWI/SNF complexes and β-catenin target gene loci. Based on these results, we hypothesized that AGGF1 might cooperate with other SWI/SNF components to regulate chromatin structure in order to facilitate the recruitment of β-catenin and other factors to target genes. We tested the hypothesis that AGGF1 might alter the ability of β-catenin to associate with DNA regulatory elements by performing chromatin immunoprecipitation experiments with either control (IgG) or β-catenin

antibodies following siRNA knockdown of various proteins. As a positive control we used siRNA to deplete β-catenin. As we expected, depletion of endogenous β-catenin dramatically reduces the amount of LEF1 promoter DNA that is chromatin immunoprecipitated using the β-catenin antibody (Figure 11). Consistent with our hypothesis, we find that knockdown of AGGF1 with pooled siRNA similarly reduces the amount of LEF1 promoter DNA that is chromatin immunoprecipitated with the β-catenin antibody (Figure 11). Since our previous results indicate that knockdown of AGGF1 does not alter β-catenin protein abundance (Figure 5a), these data suggest that AGGF1 is required for the recruitment of β-catenin to one of its target genes.

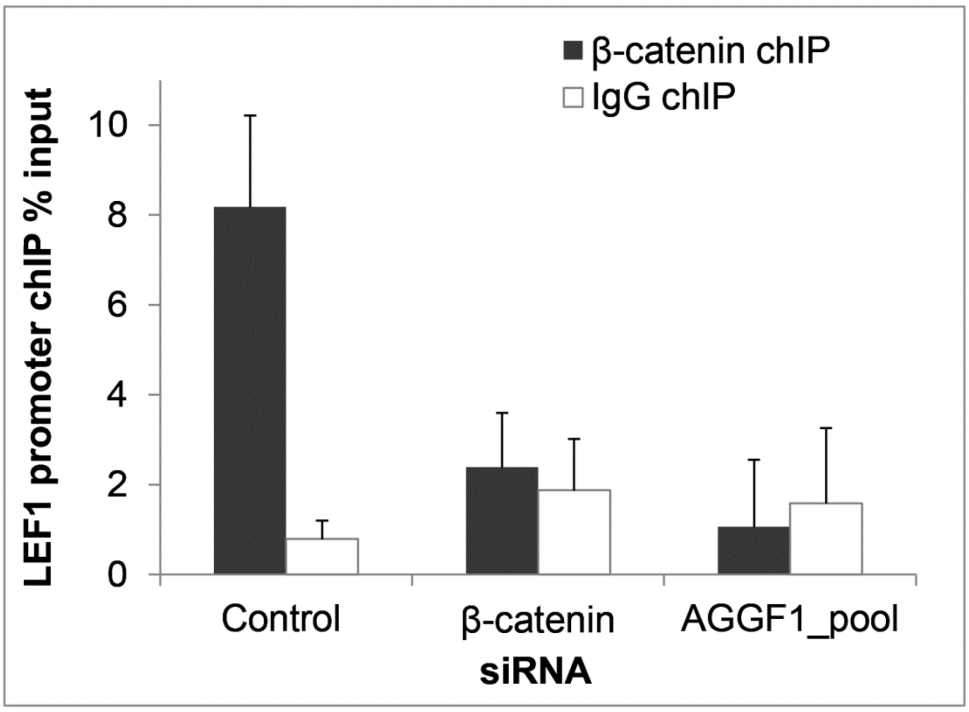

Figure 11: AGGF1 is required for β-catenin recruitment to *LEF1* promoter

DLD1 cells were transfected with the indicated siRNAs 48 hours before performing chromatin immunoprecipitation experiments with either beta-catenin or control IgG antibodies. RT-PCR was performed on extracted DNA to detect the genomic sequence containing TCF/LEF binding sites located 2251 base pairs 5' of the *LEF1* ORF.

Discussion

A vast majority of colorectal cancers harbor mutations resulting in the hyper-activation of β-catenin dependent transcription. Targeting factors necessary to that maintain this hyper-activation of β-catenin-dependent transcription observed in malignant cells and tissues, but which are dispensable for normal tissue homeostasis is an attractive strategy for therapeutic intervention in colon cancer. In this study we characterized a novel regulator of β-catenin dependent transcription in colorectal cancer called AGGF1. Our mechanistic studies reveal that AGGF1 associates with TCF/LEF consensus sites found at β-catenin target genes and regulates the expression these target genes.

Although previous studies characterized AGGF1 as a pro-angiogenic secreted factor[255,256], we were unable to detect any secreted AGGF1 from colorectal cells (data not shown) and find that adding AGGF1 to cell culture media has no effect on β-catenin-dependent transcription. These results suggested that AGGF1 might regulate β-catenin-dependent transcription independently of its secretion in colorectal cancer. Our further characterization of AGGF1 reveals that AGGF1 both localizes to cell nuclei in colon cancer cell lines and that AGGF1 associates with numerous nuclear proteins, raising the possibility that AGGF1 also functions in the nucleus in some cells. Other instances of secreted proteins also functioning in cell nuclei have been suggested by a handful of previous studies[267,268]. Moreover, numerous proteins contain both nuclear localization signals and signal sequences directing their translation in the

ER for secretion or expression as transmembrane proteins (reviewed in reference [269]). Our experimental results reveal that AGGF1 may also function as either a secreted factor or as a nuclear protein that associates with chromatin in a context-dependent manner. Determining whether the activities of the nuclear and secreted fractions of AGGF1 protein are either co-regulated or functionally coordinated in any way will be an important subject of future studies.

Our further studies explored mechanisms of AGGF1 function as a nuclear protein in colorectal carcinoma cells. Specifically, we characterized a functional role for nuclear-localized AGGF1 observed in colorectal carcinoma cells by performing chromatin immunoprecipitation experiments. These studies reveal that both AGGF1 and β-catenin associate with putative TCF/LEF binding sites at β-catenin target genes, which suggests that AGGF1 is a novel transcriptional co-factor for β-catenin . We go on to show that AGGF1 is required for the recruitment of β-catenin to one of its target genes, LEF1 and that AGGF1 regulates the transcription of several β-catenin target genes. Whether β-catenin recruitment to additional target genes is also regulated by AGGF1 is not yet clear. It is also not yet clear whether AGGF1 and β-catenin are found simultaneously or sequentially at target genes. For example, it is possible that SWI/SNF protein complexes containing AGGF1 can organize chromatin structure in a manner that will facilitate the future recruitment of β-catenin, which can then work to promote the expression of mRNAs encoded at these genomic loci.

A number of studies link changes in SWI/SNF protein expression or protein complex composition to cancer progression[264,270–276]. Specifically, alterations in the subunit composition

can direct patterns of gene expression regulated by various transcription factors and signaling pathways[277–280]. Taken together with our characterization of AGGF1, these findings raise the possibility that AGGF1 may also contribute to colorectal cancer progression by acting is a novel co-factor in SWI/SNF protein complexes that can enhance β-catenin-dependent transcription.

Materials and Methods

Cell culture

DLD1 and SW480 cells were obtained from American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. All siRNA transfections, irrespective of cell type, used RNAiMAX (Invitrogen, Carlsbad, CA).

High-throughput siRNA screens

The genome-wide siRNA screen was performed as previously described, with minor modifications (34). Briefly, cells were reverse-transfected in 1536-well plates, with a final concentration of pooled siRNA at 25 nM. Seventy-two hours after transfection, firefly luciferase and Renilla luciferase were quantitated. The pilot-scale screens were completed essentially as

described above, except that cells were reverse-transfected in 384-well plates and cell viability was controlled by AlamarBlue staining.

Affinity purification and mass spectrometry

TAP-MS experiments were performed as previously described[260,281]. The PINs for BAF57, APC, and CDC73 were determined by immunoprecipitation of the endogenous protein from DLD1 cells. Briefly, cells were harvested from 15 × 150-mm tissue culture plates, washed in PBS, lysed, and cleared by centrifugation. The supernatant was cleared two times with protein G agarose beads before immunoprecipitation with 10 µg of antibody. Antibody complexes were washed four times in lysis buffer, four times in ammonium bicarbonate (pH 7.0), and eluted in sodium citrate (pH 2.0). We provide in table S10 unfiltered mass spectrometry data for all baits analyzed, as well as PIN files generated in cytoscape (http://www.cytoscape.org/).

GAL4-luciferase reporter assays

A 5× UAS-luciferase reporter and an EF1α-driven Renilla luciferase reporter were introduced into DLD1 cells by lentiviral transduction. To these stable cells, we then introduced β-catenin-GAL4DBD or VP16-GAL4DBD fusion constructs by lentiviral transduction. (DBD, DNA binding domain). The resulting polyclonal cell lines were used to test AGGF1 and BAF57 siRNAs.

Immunofluorescence

DLD1 or HEK293T cells plated on poly-d-lysine coated coverslips were fixed with 4% PFA buffered to pH 7.5 in PBS for 20 minutes at room temperature. Samples were then permeabilized in PBS containing 0.1% Triton-X 100 for 30 minutes at room temperature and then blocked with 5% solution of BSA dissolved in PBST (PBS containing 0.1% Tween-20). Primary antibodies were diluted in blocking buffer and incubated with the coverslips for ~4 hours at room temperature. Following extensive washing species-specific fluorescent conjugated secondary antibodies (Invitrogen, Carlsbad, CA) similarly diluted in blocking buffer were incubated with the coverslips for 30 minutes at room temperature. After additional washes coverslips were stained with DAPI and mounted using Prolong Gold mounting reagent (Invitrogen, Carlsbad, CA). Stained samples were then imaged using a Nikon Eclipse E600 upright microscope equipped with a 60x oil objective. Images were imported into ImageJ software, which was used for all image analysis including false coloring and linear adjustments in brightness and contrast.

Chromatin immunoprecipitation

Chromatin immunoprecipitation experiments were carried out as previously described with the following modifications[282]. Briefly, cells were lysed in nuclei lysis buffer (1% SDS, 50 mM Tris-

HCl, pH 8.0, 10 mM EDTA) with proteinase inhibitors added and sonicated to an average DNA size of 300 to 1500 base pair. Sonicated DNA was then diluted fourfold in immunoprecipitation dilution buffer (16.7 mM Tris-HCl, pH 8.0, 11.2 mM EDTA, 0.01% SDS, 150 mM NaCl). Immunoprecipitations were performed overnight at 4^{o}C using β-catenin (Santa Cruz Biotechnology sc-1496), AGGF1 (Santa Cruz Biotechnology sc-47570, or AbCam ab14444), H3K4Me3, and IgG control antibodies. Antibody complexes were captured with protein G agarose slurry and eluted in 1% SDS + 0.1 M $NaHCO_{3.}$ After proteinase K treatment for 2 hours at 55°C, DNA was purified by Nucleospin PCR clean-up kit and the analyzed by gel or RT-PCR.

Chapter 3: A protein complex of SCRIB, NOS1AP, and VANGL1 regulates cell polarity and migration, and is associated with breast cancer progression

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A protein complex of SCRIB, NOS1AP, and VANGL regulates cell polarity and is associated with breast cancer progression.

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Abstract

By analyzing public datasets of gene expression in human breast cancers we observed that increased levels of transcripts encoding the Planar Cell Polarity (PCP) proteins SCRIB and VANGL1 correlate with increased risk of patient relapse. Experimentally, we found that reducing expression of SCRIB by shRNAs reduces the growth of human breast cancer cells in xenograft assays. To investigate SCRIB-associated proteins that might participate in the responses of breast cancer cells to altered levels of SCRIB we employed mass spectrometry and confocal microscopy. These studies reveal that SCRIB is present in at least two unique protein complexes: (1) a complex of SCRIB, ARHGEF, GIT, and PAK, and (2) a complex of SCRIB, NOS1AP, and VANGL. Focusing on NOS1AP, we observed that NOS1AP co-localizes with both SCRIB and VANGL1 along cellular protrusions in metastatic breast cancer cells, but does not co-localize with either SCRIB or VANGL1 at cell junctions in normal breast cells. We investigated the effects of shRNA-mediated knockdown of NOS1AP and SCRIB in vitro, and found that reducing NOS1AP and SCRIB slows breast cancer cell migration and prevents the establishment of leading-trailing polarity. We also find that reduction of NOS1AP enhances anchorage-independent growth. Collectively these data point to the relevance of NOS1AP and SCRIB protein complexes in breast cancer.

Introduction

The maintenance of tissue polarity requires the restriction of determinant proteins to specific subcellular regions. At a molecular level, localizing proteins to distinct regions involves the activity of scaffolds, including the PDZ-domain-containing protein SCRIB (Scribble). In Drosophila, deletions in scrib result in defects in apical-basal polarity[283–285], while simultaneous loss of scrib and van gogh (also known as VANGL1/2, or Strabismus in vertebrates) disrupts the planar cell polarity (PCP) of wing bristles and ommatidial cells[286]. Comparable PCP defects arise due to a loss of SCRIB orthologues during vertebrate development. Specifically, inactivating Scrib alleles lead to disorganized stereocilia in the mouse inner ear[287–289], and impaired convergent extension movements in zebrafish[290,291].

The evolutionarily conserved role of SCRIB in establishing or maintaining the three-dimensional organization of tissues may stem from its function as a molecular bridge between different members of multi-protein complexes. In epithelial tissues, SCRIB is recruited to sites of cell contact where it associates with junctional proteins[291–293]. The disorganization of epithelial tissues due to loss of function of SCRIB may in part result from the abnormal localization of junctional proteins such as E-cadherin and β-catenin[283,284,294–296]. In addition to acting at sites of cell-cell contact, SCRIB also participates in protein complexes necessary to establish leading-trailing polarity during cell migration. In motile cells SCRIB associates with a complex of ARHGEF7 (β-PIX), the closely related ARHGEF6 (α-PIX), PAK1-3 (p21-activated kinases), and GIT proteins[297–299]. This complex acts as a crucial regulator of cell migration and [160,295,298,300,301].

The ability of SCRIB to regulate both the three dimensional morphogenesis of tissues and the migration of cells in vitro[160,295,300] and in vivo[287,288,290,291] makes it a prime candidate for participating in cancer progression. This possibility is supported by the results of the present study in which we first demonstrate that altered expression of SCRIB correlates with poor clinical outcomes in breast cancer. We then demonstrate that SCRIB associates with at least two distinct protein complexes, and that a NOS1AP, SCRIB, VANGL1 protein complex modulates the migration, polarity, and growth of breast cancer cells.

Results

Expression of SCRIB is associated with adverse clinical outcomes in breast cancer

Although previous research indicates that SCRIB can act as a suppressor of tumor growth in a mouse model of breast cancer[302], the clinical relevance of SCRIB in human cancers remains unclear. We therefore evaluated the relationship between SCRIB expression and breast cancer relapse using data from a publically available multiple microarray dataset of 286 human breast tumors (GEO-GSE2034)[303]. Using the Kaplan-Meier method for risk-assessment we found that samples with the highest SCRIB expression (>65th percentile, n=100) were associated with a statistically significant increased risk of relapse (*P=0.0240) when compared to samples with the lowest SCRIB expression (<35th percentile, n=100) (Figure 12a). We again used Kaplan-Meier analysis to investigate the relationship between VANGL1 expression and clinical outcomes and found a similar association between high levels of VANGL1 and breast cancer relapse (**P=0.003) (Figure 12b). Finally, we analyzed a second multiple tumor microarray dataset (GEO-GSE3143)[304] to determine the relationship between SCRIB expression and overall breast cancer survival and found that samples with high SCRIB expression (>65th percentile, n=56) were associated with decreased overall survival probability (*P=0.0407) (Figure 12c). These data provided the impetus for the following initial inquiries into the effects of altered levels of SCRIB and associated proteins on breast cancer cells.

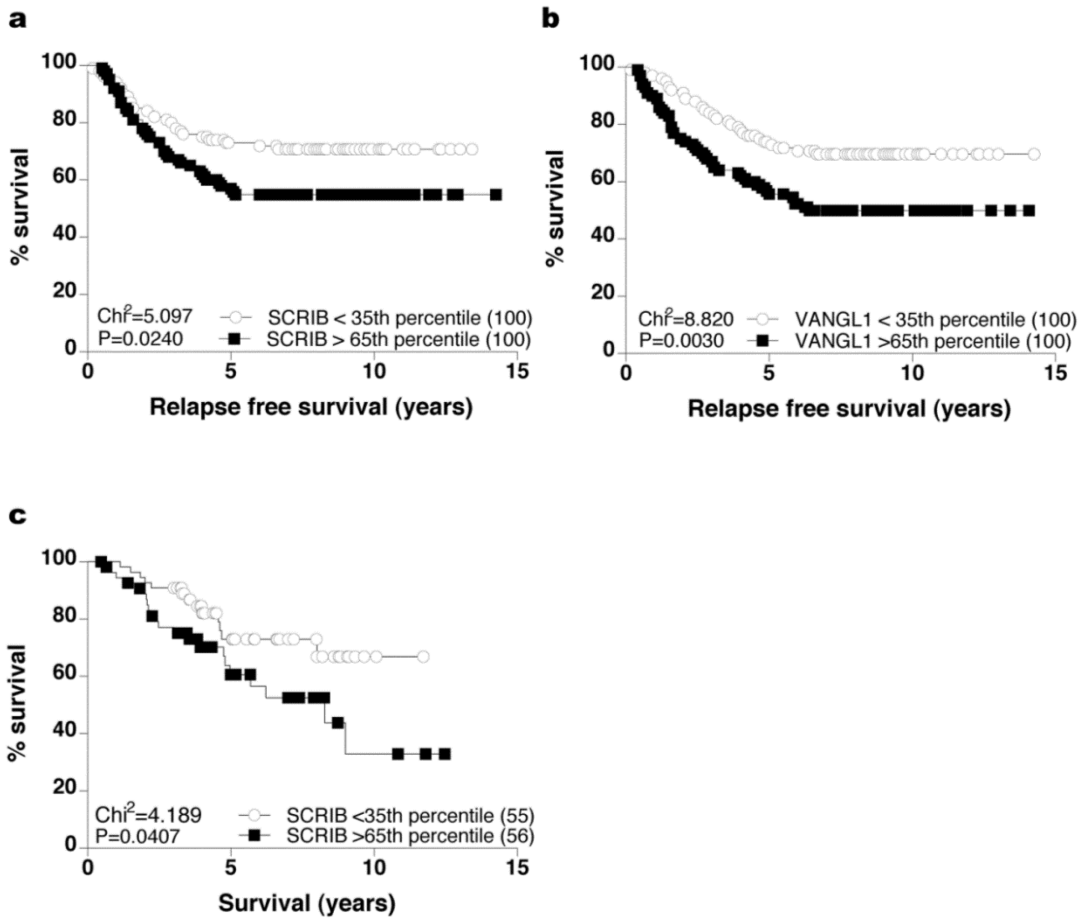

Figure 12: Expression of SCRIB is associated with adverse clinical outcomes in breast cancer.

(**a**) Analysis of publically available breast cancer multiple tumor microarray data set (Wang et al., 2005) for the association between SCRIB expression and risk of relapse. Samples were grouped into high SCRIB expression (>65th percentile, n=100) and low SCRIB expression (<35th percentile, n=100) and Kaplan-Meier curves were compared. *P=0.0240 (log-rank test). (b) VANGL1 expression data analyzed as for SCRIB using the Wang et al. (2005) dataset. **P=0.003 (log-rank test). (c) Kaplan-Meier survival analysis of a multiple tumor microarray dataset of breast cancer samples (Bild et al., 2006) for the association between SCRIB expression and overall breast cancer patient survival. Samples were grouped into high SCRIB expression (>65th percentile, n=56) and low SCRIB expression (<35th percentile, n=55) and curves were compared. *P=0.0407 (log-rank test).

Reduction of SCRIB inhibits the growth of human breast cancer cells in xenografts

We first asked whether manipulating SCRIB protein levels alters the growth of human breast cancer cells in xenograft assays. To obtain breast cancer cell lines with reduced levels of SCRIB protein we transduced MDA-MB-231 cells with two independent shRNAs complementary to SCRIB (SCRIB sh3 and SCRIB sh4). Western blotting of lysates of these cells established that levels of SCRIB were reduced when compared to cells transduced with PLKO.1 control shRNA (Figure 13a). We then pooled equal numbers of SCRIB sh3 and SCRIB sh4 cells and injected them into the mammary fats pads of immunocompromised NOG mice. We found that reducing SCRIB protein levels significantly slows tumor growth estimated by volume from caliper-measured dimensions (Figure 13b) and by final tumor mass (Figure 13c) when compared to cells transduced with PLKO.1 control shRNA. SCRIB shRNAs also inhibited xenograft tumor growth in athymic nude mice (Figure 13d), indicating that the effect of SCRIB shRNAs is not specific to the genetic background of the animal.

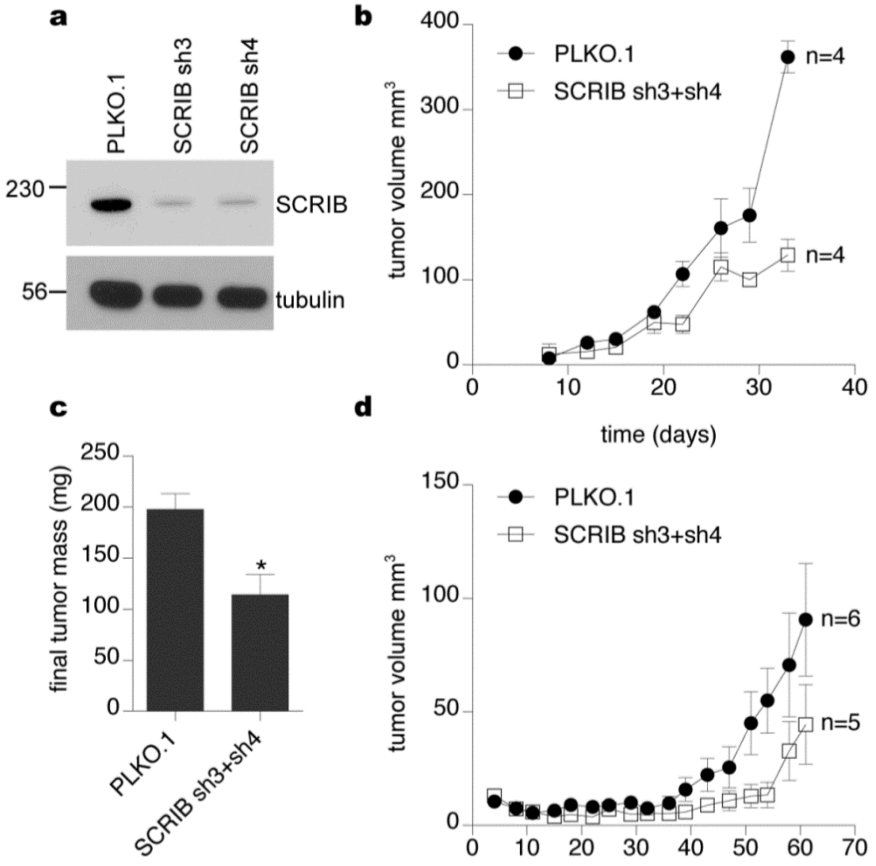

Figure 13 Reduction of SCRIB inhibits the growth of human breast cancer cells in xenografts.

(a) Western blot of lysates from stable MDA-MB-231 cell lines infected with PLKO.1 control shRNA, and two independent SCRIB shRNAs analyzed for SCRIB expression (upper panel), or β-tubulin loading control (bottom panel). (b) Tumor growth curves generated from mammary fat pad xenografts of shRNA-infected MDA-MB-231 cells in NOG immunocompromised mice (n=4). (c) Final mass of orthotopic xenograft tumors, which were removed after sacrificing the animals at the end of the growth experiment in (b)*$P<0.05$ (Student's *t*-test). (d) Tumor growth curves generated from mammary fat pad xenografts of shRNA-infected MDA-MB-231 cells in athymic nude immunocompromised mice (n=6).

As the consequences of altered SCRIB expression are likely to be mediated by its associated proteins, we characterized SCRIB protein complexes by mass spectrometry. Specifically, we immunoprecipitated both endogenous SCRIB and double epitope-tagged SCRIB, from HEK293T cells and analyzed the associated protein complexes by tandem mass spectrometry. First, the results confirm a recent report[299] that both endogenous and ectopically expressed SCRIB co-purify with a complex of ARHGEF7, PAK, and GIT proteins. Second, the mass spectrometry data indicate that several PCP pathway components co-purify with SCRIB, including the transmembrane proteins VANGL1, VANGL2, and CELSR2 (also known as Flamingo). Finally, we identified novel SCRIB-associated proteins including, KCTD3, and TJP1 (Figure 14a, Table 4,5). We next affinity purified ARHGEF7 protein complexes in order to determine if ARHGEF7 might also associate with some of the novel proteins identified in our SCRIB pulldowns. Our analysis of isolated ARHGEF7 protein complexes indicates only partial overlap with the SCRIB interaction network (Figure 14a, Table 7). Specifically, we did not co-purify any of the PCP complex components with ARHGEF7 (Figure 14a and Table 7).

Table 4: Summary of SCRIB-associated proteins identified by by Tap-tag pulldown and mass spectrometry

TAP-tag pull down. SCRIB protein complexes were isolated from HEK293T cells by tandem affinity purification with both a streptavidin and a calmodulin binding protein tag, trypsinized, and then analyzed by tandem mass spectrometry. Proteins are ranked in this table by the % coverage.

Gene symbol	% coverage	Number of peptides	Number of unique peptides
FLJ13137	54.4	20	13
SCRIB	51.5	616	112
GIT2	47.7	38	31
ARHGEF7a	44.4	53	33
GIT1	40.3	41	22
SHKBP1	38.3	26	21
ARHGEF7b	33.4	46	30
PPP1CA	31.8	26	11
NOS1AP	29.1	29	24
KCTD3	28	18	16
PPP1CG	26.9	23	9
VANGL1	19.7	8	7
PPP1CB	17.7	19	7
ARHGEF6	17.5	18	13
PAK1	17.2	6	6
SGEF	14	11	8
VANGL2	7.7	2	2
TJP2	6.2	3	3
PAK2	5.9	1	1
CELSR2	0.9	1	1

Table 5: Summary of SCRIB-associated proteins identified by immunoprecipitation of endogenous SCRIB protein complexes followed by mass spectrometry

SCRIB-associated protein complexes were isolated from HEK293T cells by affinity purification with anti-SCRIB IgG (Santa Cruz Biotechnolgy C20). Proteins are ranked in this table by the % coverage.

Gene symbol	% coverage	Number of peptides	Number of unique peptides
SCRIB	42.3	216	63
PAK1	29.4	26	13
ARHGEF7a	27.2	30	14
PAK2	26.9	20	10
GIT1	26.8	31	12
FLJ13137	23.9	4	4
GIT2	23.2	20	12
PPP1CA	18.8	7	4
SHKBP1	15.7	13	8
ARHGEF6	12.4	8	5
NOS1AP	12.1	11	6
PPP1CG	10.8	3	1
KCTD3	4	1	1

Table 6: Summary of NOS1AP-associated proteins identified by by Tap-tag pulldown and mass spectrometry

NOS1AP protein complexes were isolated from HEK293T cells by tandem affinity purification with both a streptavidin and a calmodulin binding protein tag, trypsinized, and then analyzed by tandem mass spectrometry. Proteins are ranked in this table by the % coverage.

Gene symbol	% coverage	Number of peptides	Number of unique peptides
NOS1AP	60.3	88	40
XP_378908	54.4	15	11
SCRIB	47.8	110	40
VANGL1	5	2	2

Table 7: Summary of ARHHGEF7-associated proteins identified by by Tap-tag pulldown and mass spectrometry

ARHGEF7 protein complexes were isolated from HEK293T cells by tandem affinity purification with both a streptavidin and a calmodulin binding protein tag, trypsinized, and then analyzed by tandem mass spectrometry. Proteins are ranked in this table by the % coverage.

Gene symbol	% coverage	Number of peptides	Number of unique peptides
GIT1	404	42	59.4
ARHGEF7a	989	60	55.9
GIT2 iso1	444	42	51.3
GIT2 iso 3	1	1	49.9
PAK1	276	27	47.2
PP2A regulatory subunit B	7	4	44.7
PAK2	70	22	44.5
ARHGEF7b	887	50	40.3
actin	13	5	24.8
SCRIB	52	25	23.2
PAK3	2	2	17.3
ARHGEF6	10	5	12

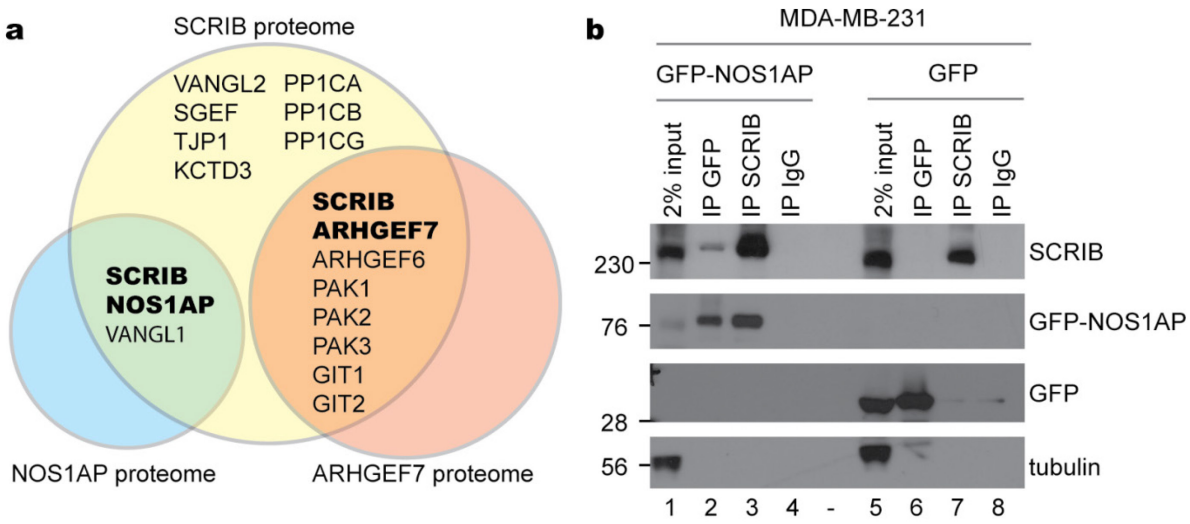

Figure 14: Mass spectrometry reveals multiple SCRIB protein complexes.

(a) Venn diagram of protein interaction network generated from SCRIB, NOS1AP, and ARHEGF7 mass spectrometry data. For peptide coverage information and detailed mass spectrometry results see Supplementary Tables S1-S4. (b) Analysis of protein complexes isolated from MDA-MB-231 breast cancer cells expressing either GFP (lanes 5-8), or GFP-NOS1AP fusion protein (lanes 1-4). Protein complexes were isolated by immunoprecipitation with the following antibodies: GFP (lanes 2 and 6), SCRIB (lanes 3 and 7), or control IgG (lanes 4 and 8), and associated proteins were detected by western blotting for endogenous SCRIB (top panel), GFP and GFP-NOS1AP (middle panels), and β-tubulin (bottom panel).

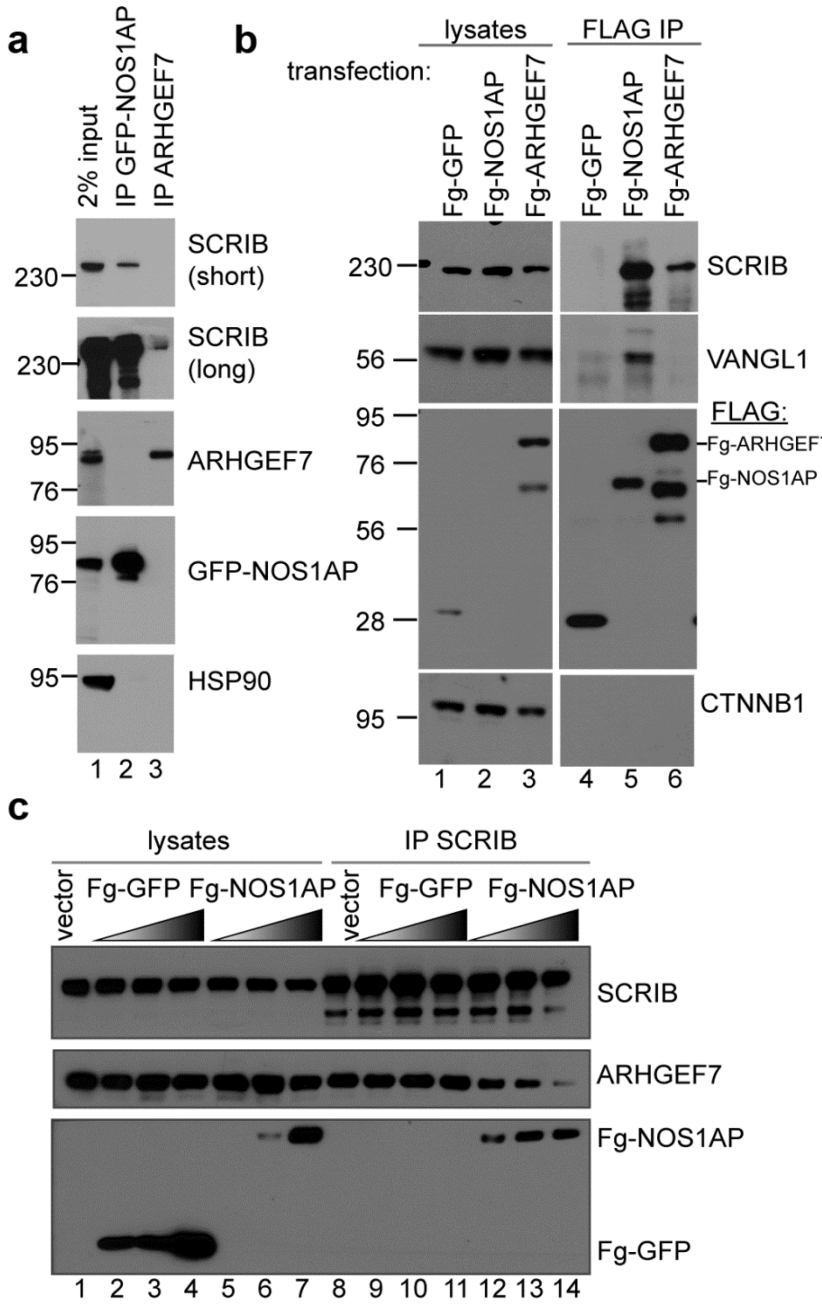

Figure 15: SCRIB associates with multiple protein complexes in breast cancer cells.

(a) Analysis of GFP-NOS1AP (lane 2), or endogenous ARHGEF7 protein complexes (lane 3) immunoprecipitated from MDA-MB-231 cell lysates. Endogenous SCRIB (top two panels), ARHGEF7 (third panel from top), HSP90 (bottom panel), and ectopic GFP-NOS1AP (fourth panel from top) were detected by western blotting. **(b)** Analysis of VANGL1 association with ectopically expressed FLAG (FLAG) fusion proteins in breast cancer cells. FLAG-GFP, FLAG-NOS1AP, or FLAG-ARHGEF7 protein complexes were immunoprecipitated from MDA-MB-231 cell lysates and the associated proteins were detected by western blotting for endogenous SCRIB, VANGL1, and CTNNB1, and for over-expressed FLAG. **(c)** Competitive binding experiment in HEK293T cells transiently transfected with increasing amounts of FLAG-GFP (bottom panel, lanes 2-4 and 9-11), or FLAG-NOS1AP (bottom panel, lanes 5-7 and lanes 12-14). SCRIB was immunoprecipitated from these cells (lanes 8-14) and the amount of ARHGEF7 (middle panel) associated with SCRIB (upper panel) in the presence of increasing amounts of FLAG-GFP (lanes 9-11) or FLAG-NOS1AP (lanes 12-14) was analyzed by western blotting.

We focused our further studies of SCRIB-associated proteins on NOS1AP due to high confidence in the mass spectrometry data and due to the known roles for NOS1AP in signal transduction[305–309]. Analysis of NOS1AP complexes isolated from HEK293T cells by mass spectrometry confirms that NOS1AP associates with SCRIB. During the course of these studies another lab also observed an association between NOS1AP and SCRIB in neuronal cells[306], suggesting that SCRIB and NOS1AP interact in other cells. Our proteome analysis of NOS1AP-associated proteins also and revealed a novel association between NOS1AP and VANGL1 (Figure 14a, and Table 6).

Next, we validated the protein interaction network discovered by mass spectrometry by expressing GFP, or a GFP-NOS1AP fusion protein in MDA-MB-231 breast cancer cells, and immunoprecipitating protein complexes with GFP, SCRIB, or IgG antibodies, followed by Western blotting (Figure 14b). We observed that GFP-NOS1AP, but not a GFP control co-purifies with SCRIB (Figure 14, lanes 2 and 3 versus lanes 6 and 7), but not with control IgG immunoprecipitates (Figure 14b, lanes 4 and 8).

NOS1AP interacts with SCRIB and VANGL1 but not with ARHGEF7 in breast cancer cells

Since a previous study reported that SCRIB acts as a molecular bridge that mediates the interaction between NOS1AP and ARHGEF7[306], we tested whether NOS1AP also associates with

ARHGEF7 in breast cancer cells. NOS1AP did not co-immunoprecipitate with endogenous ARHGEF7 in MDA-MB-231 cells (Figure 15a), which is consistent with our mass spectrometry data. Our proteomic analysis of SCRIB and NOS1AP-associated protein complexes also identifies a novel association between NOS1AP and the PCP protein, VANGL1, which was not observed in previous studies. (Figure 14a and Table 6). We performed additional co-immunoprecipitation experiments to confirm that NOS1AP is present in a protein complex with VANGL1 in breast cancer cells by showing that endogenous VANGL1 co-immunoprecipitates with FLAG-NOS1AP, but not with FLAG-GFP, or with FLAG-ARHGEF7 (Figure 15b, compare lane 5 with lanes 4 and 6).

SCRIB associates with multiple protein complexes in breast cancer cells

The partial overlap between the SCRIB, NOS1AP, and ARHGEF7 proteomes suggests that SCRIB may associate with multiple protein complexes in breast cancer cells. To further explore this possibility we fractionated whole cell lysates from MDA-MB-231 cells expressing GFP-NOS1AP using gel filtration chromatography. After analyzing collected fractions by western blotting we find that SCRIB protein is associated with both an early-eluting high molecular weight complexes (fractions 1-3) and a late-eluting smaller molecular weight complexes (fractions 6-12) (Figure 16). We find SCRIB, NOS1AP, and ARHGEF7 are each present in the lower molecular weight fractions. This result indicates that we are unable to separate the hypothetically independent SCRIB/ARHGEF7 protein complexes from SCRIB/NOS1AP protein complexes using size exclusion chromatography. I

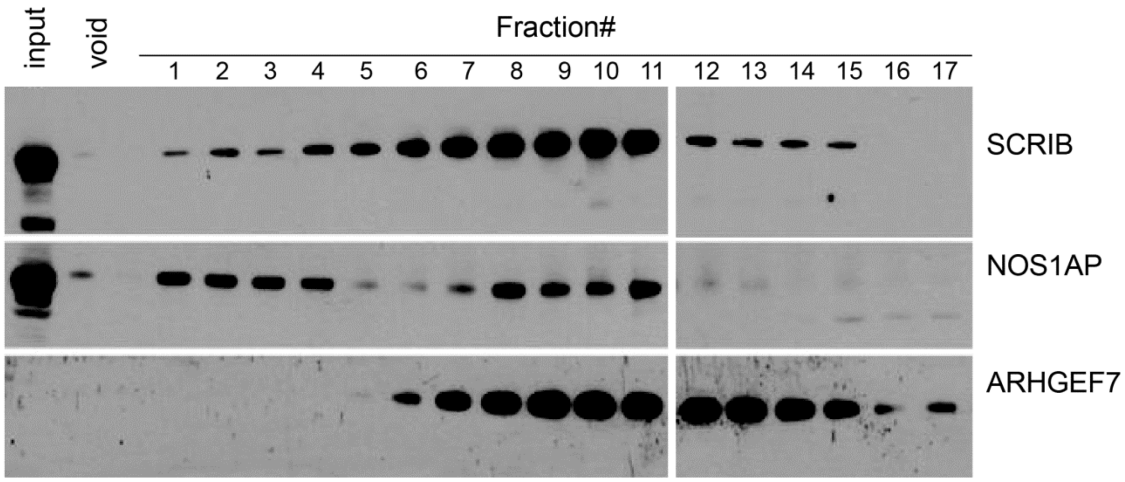

Figure 16: Gel filtration chromatography indicates that SCRIB associates with protein complexes of varying molecular weight.

It is possible that SCRIB/NOS1AP and SCRIB/ARHGEF7 protein complexes cannot be isolated using this approach because they are similar molecular weights. Another interpretation of these results is that SCRIB, NOS1AP, and ARHGEF7 associate in the same protein complex given the co-elution of these proteins in selected fractions, which contradicts the results of our mass spectrometry experiments indicating no overlap between the ARHGEF7- and NOS1AP-associated proteomes. Importantly, we also note that SCRIB and NOS1AP are both present in the higher molecular weight fractions, whereas ARHGEF7 is absent from these samples. These results further support our SCRIB associates with multiple protein complexes in breast cancer cells.

Given the potentially contradictory results of our proteomics and protein fractionation experiments, we then turned to alternative experimental approaches to better characterize SCRIB-associated protein complexes in breast cancer cells. In order to further test the hypothesis that SCRIB can interact with either ARHGEF7 or NOS1AP, but not both in the same complex, we performed a competition assay. For these experiments, we expressed different levels of FLAG-NOS1AP or control FLAG-GFP in HEK293T cells and found that as the levels of FLAG-NOS1AP, but not control FLAG-GFP, increased, there was a reduction in the amount of endogenous ARHGEF7 that co-immunoprecipitated with SCRIB. (Figure 15c, compare lanes 9-11 to lanes 12-14). These data suggest that NOS1AP competitively displaces ARHGEF7 from SCRIB complexes. Moreover, these co-immunoprecipitation experiments confirm our mass

spectrometry results, and support the hypothesis that SCRIB associates independently with ARHGEF-PAK-GIT protein complexes, and with NOS1AP-VANGL protein complexes.

Subcellular localization of the SCRIB protein interaction network

If SCRIB exists in multiple protein complexes as the above data suggest then confocal microscopy of intact cells should reveal domains of overlap between SCRIB and potentially associated proteins. We characterized the subcellular localization of SCRIB and SCRIB-associated proteins in MDA-MB-231 breast cancer cells to investigate this hypothesis. Consistent with our protein interaction data, we found that NOS1AP localizes in a punctuate distribution throughout the cytoplasm and partially co-localizes with SCRIB at cellular protrusions (Figure 17a). VANGL similarly localizes to cell protrusions and partially co-localizes with both SCRIB and NOS1AP (Figure 17c and d). Also consistent with our protein interaction data, we observed the co-localization of endogenous SCRIB and ARHGEF7 at lamellipodia in subsets of MDA-MB-231 cells (Figure 17b and Figure 18a). Consistent with the hypothesis that NOS1AP and ARHGEF7 interact with distinct pools of SCRIB, we observed minimal co-localization between ARHGEF7 and GFP-NOS1AP in breast cancer cells (Figure 18b).

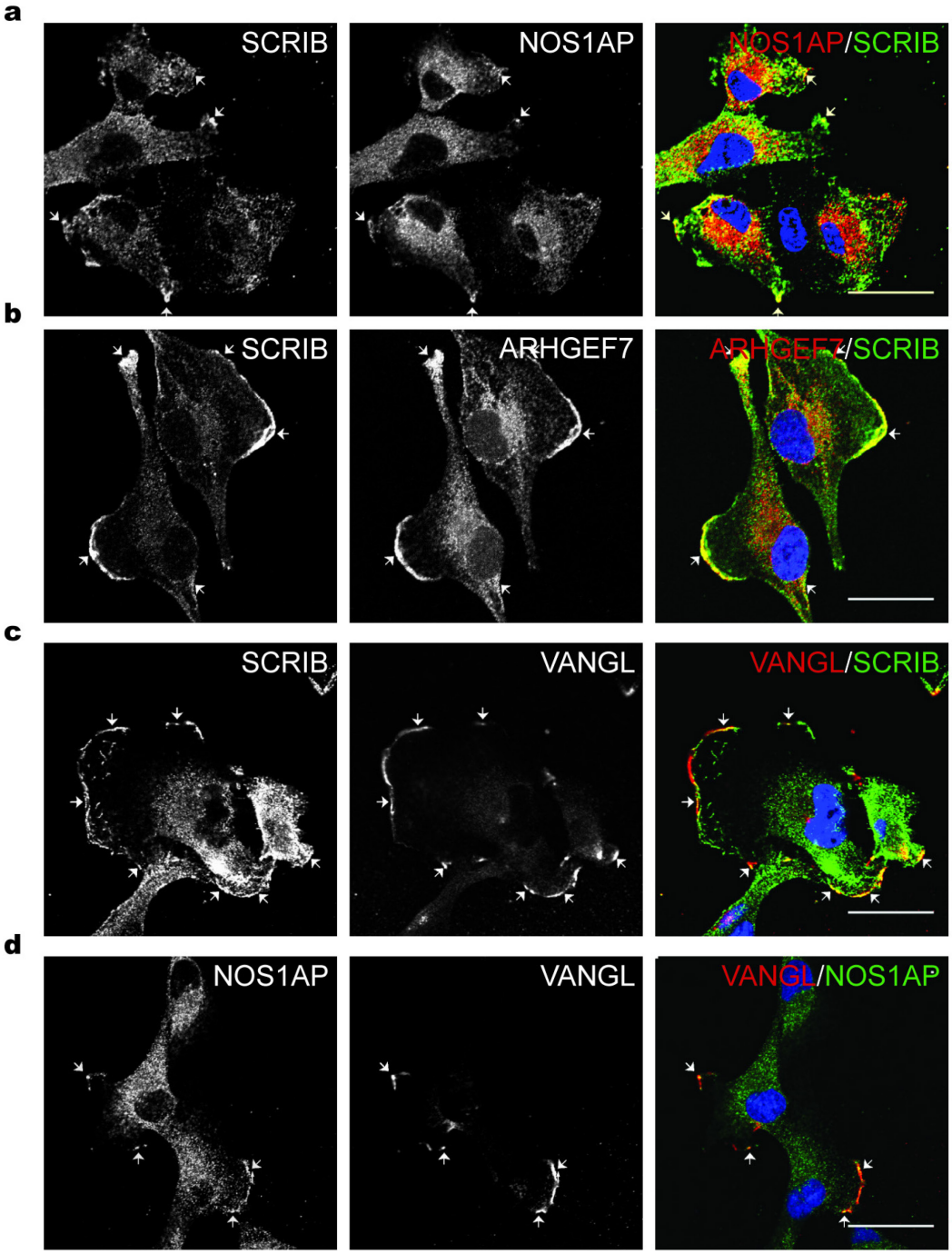

Figure 17: Subcellular localization of the SCRIB protein interaction network.

(a)-(d) Confocal images of MDA-MB-231 cells stained with the following antibodies to detect endogenous SCRIB-associated proteins: (a) SCRIB and NOS1AP, (b) SCRIB and ARHGEF7, (c) SCRIB and VANGL, or (d) NOS1AP and VANGL. Signals from individual antibodies are shown in the left and center columns, while a pseudo-colored merged image is included in the right column with DAPI DNA stain shown in blue. Areas of co-localization (yellow color) are indicated with white arrows. In all images scale bars are 30 µm.

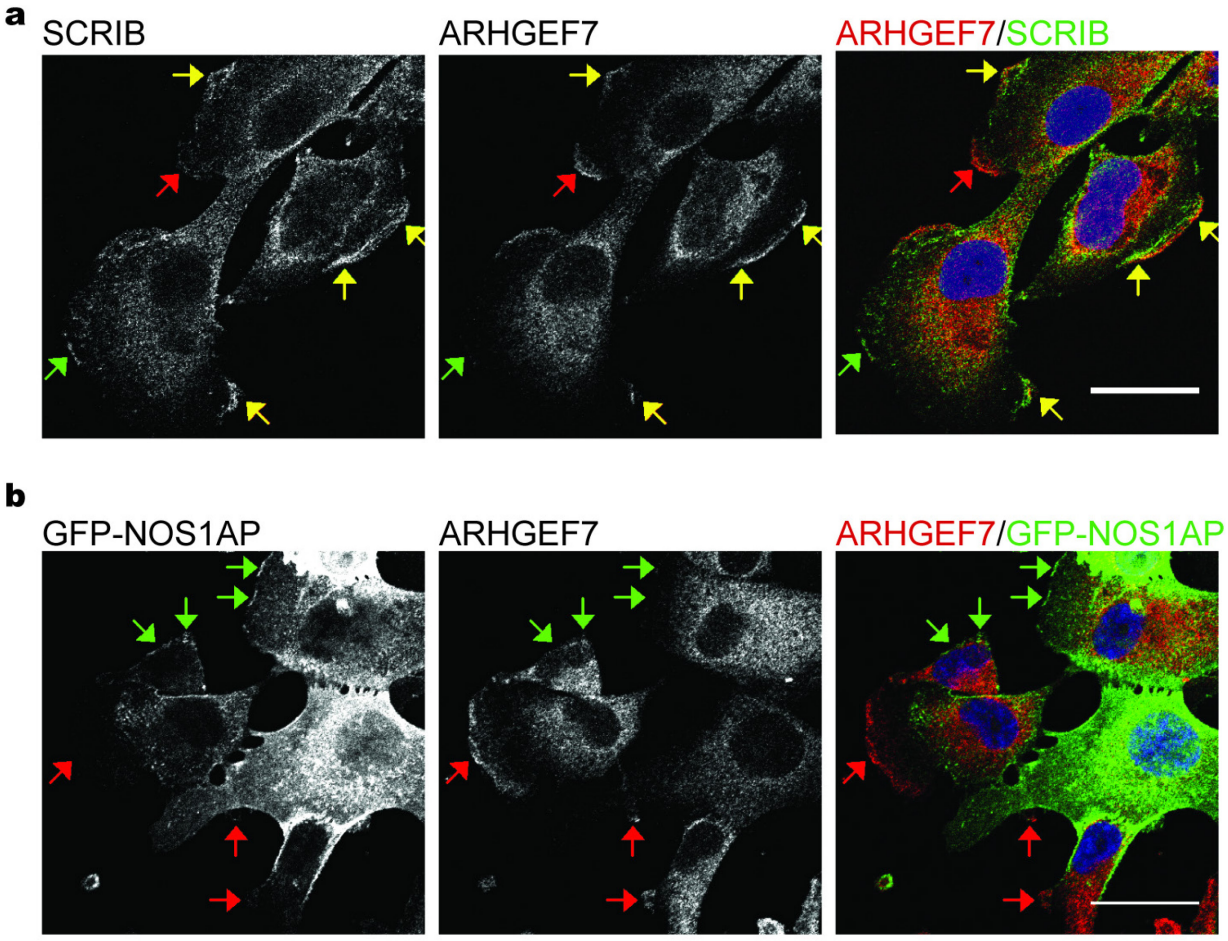

Figure 18: Minimal co-localization between GFP-NOS1AP and ARHGEF7 in MDA-MB-231 cells.

(a) Confocal images of MDA-MB-231 cells stained with antibodies detecting endogenous SCRIB and ARHGEF7. Green arrows indicate cellular protrusions where SCRIB localizes, red arrows indicate cellular protrusions where ARHGEF7 localizes, and yellow arrows mark areas where both SCRIB and ARHGEF7 localize. (b) Confocal images of MDA-MB-231 cells stained with antibodies detecting GFP-NOS1AP and ARHGEF7. Green arrows indicate cellular protrusions where GFP-NOS1AP localizes, red arrows indicate cellular protrusions where ARHGEF7 localizes, and yellow arrows mark areas where both GFP-NOS1AP and ARHGEF7 localize. Individual channels are shown in left and middle columns and merged images are included in the right column. Scale bars are 30 µm.

NOS1AP is undetectable at cell junctions in mammary cells

We next asked whether the association between SCRIB and NOS1AP was observed in normal mammary cells. Unlike invasive breast cancer cells, MCF10a cells form epithelial sheets with adherens junctions marked by CTNNB1 (β-catenin) (Figure 19). We found that SCRIB and VANGL1 localized to these sites of cell contact (Figure 19a and b), whereas NOS1AP remained mostly restricted to the cytosol (Figure 19c). We also assessed the localization of SCRIB and NOS1AP in MCF-7 breast cancer cells, which retain their epithelial characteristics and found that SCRIB again co-localized to cell-cell junctions with CTNNB1, while a majority of the NOS1AP signal remained in the cytosol (Figure 20).

We then used concanavalin-A beads to enrich for the plasma membrane and junctional complexes of mammary cells. We observed that a much greater proportion of SCRIB was bound to the concanavalin-A beads when we fractionated MCF10a cell lysates compared to when we fractionated MDA-MB-231 cell lysates (Figure 19d and e). These data are consistent with the confocal microscopy data that show localization of SCRIB to the plasma membrane at cell-cell junctions in MCF10a cells (Figure 19a-c). Furthermore, we found that most of the NOS1AP protein co-fractionates with SCRIB in the concanavalin-A flow through, which contains non-plasma membrane proteins from both MCF10a and MDA-MB-231 cells (Figure 19d and e). Taken together, these data suggest that NOS1AP localizes to the cytosol of both MDA-MB-231 breast cancer cells and in non-transformed MCF10a mammary epithelial cells.

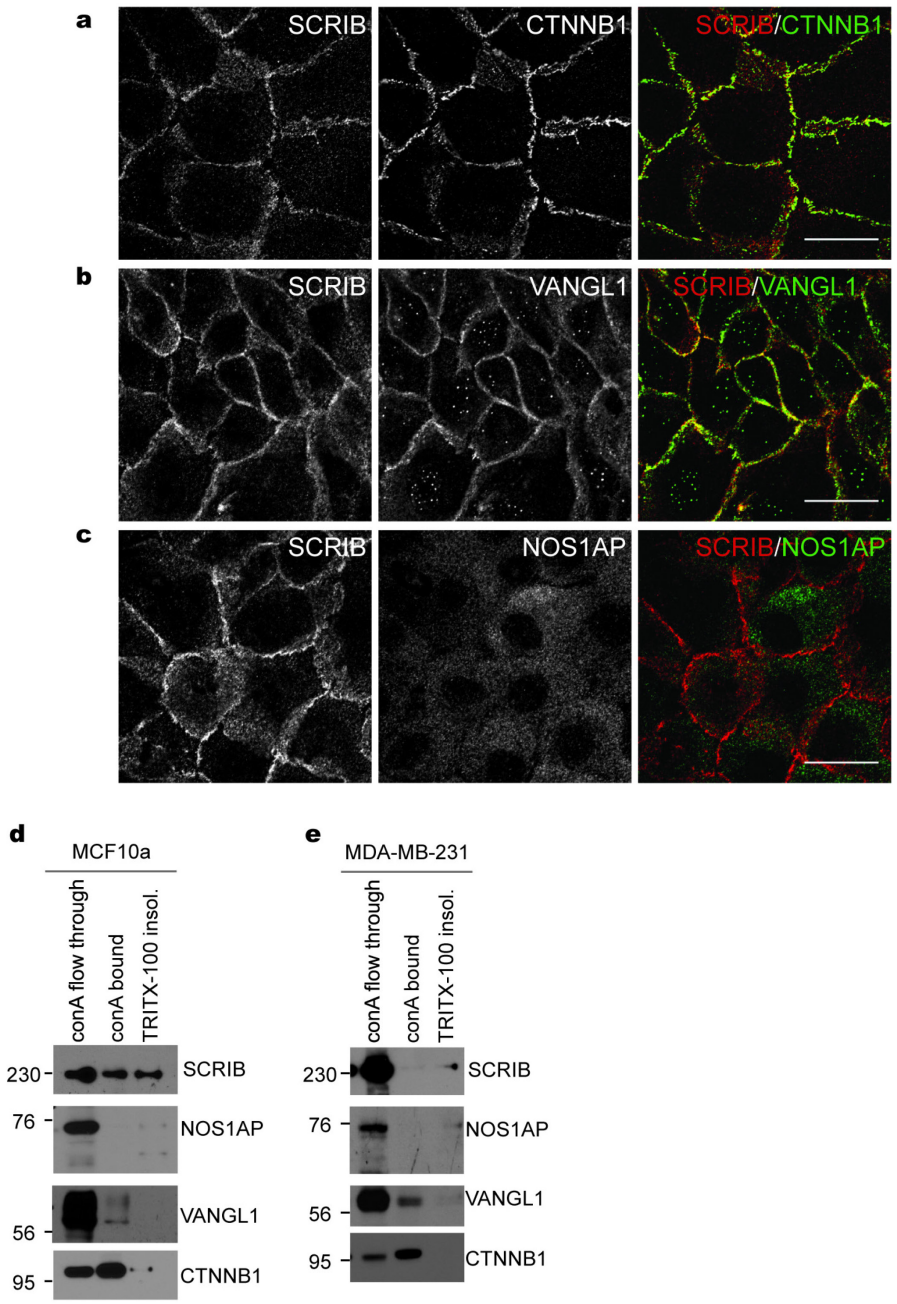

Figure 19: NOS1AP is undetectable at cell junctions in mammary cells.

(a)-(c) Confocal microscopy of confluent MCF10a cell monolayers stained with the following antibodies: (a) SCRIB and CTNNB1, (b) SCRIB and VANGL1, and (c) SCRIB and NOS1AP. Signals from individual antibodies are shown in the left and center columns, and a pseudo-colored merged image is included in the right column. Scale bars are 30 µm. (d)-(e) Concanavalin-A fractionation of (d) MCF10a, and (e) MDA-MB-231 cells lysed with Triton-X 100 buffer to identify: (1) proteins associated with the plasma membrane (lane 2), (2) proteins that were not associated with the plasma membrane under these conditions (lane 1), and (3) proteins insoluble in this lysis buffer, which were re-suspended in 6M urea (lane 3).

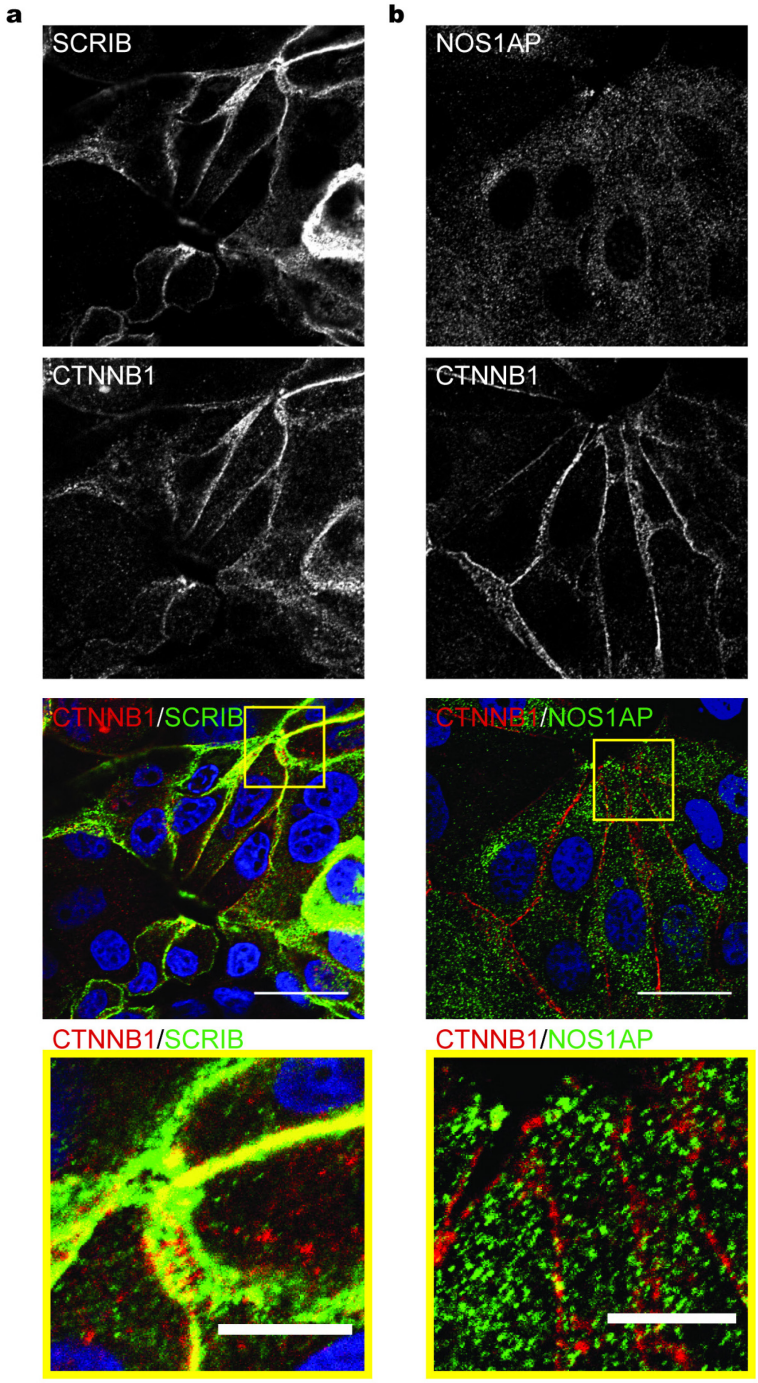

Figure 20: NOS1AP is undetectable at cell junctions in MCF-7 breast cancer cells.

(a) Confocal images of MCF-7 cells stained with SCRIB and CTNNB1 antibodies. (b) Confocal images of MCF-7 cells stained with NOS1AP and CTNNB1 antibodies. Top two rows show images of individual antibody channels, third row from top shows merged images (scale bars = 30 µm), and bottom row shows insets (marked by yellow box) from merged images (scale bars = 10 µm.)

NOS1AP is stably expressed in breast cancer cells, but not in non-transformed mammary epithelial cells

We then asked whether NOS1AP can interact with SCRIB in normal MCF10A mammary epithelial cells as it does in MDA-MB-231 breast cancer cells. Again, we find that endogenous SCRIB co-immunoprecipitates with FLAG-NOS1AP (Figure 21, lane 13) and that endogenous SCRIB also weakly co-immuniprecipitates with FLAG-ARHGEF7 (Figure 21, lane 14) when we express these proteins in MDA-MB-231 metastatic breast cancer cells. We attempted to perform similar experiments using MCF10A mammary epithelial cells, but were unable to express sufficient quantities of FLAG-NOS1AP for these experiments. In general, we find that NOS1AP is not expressed very robustly in comparison to either FLAG-GFP control protein or FLAG ARHGEF7 in either MDA-MB-231 cells (compare lysates in lane 10 to lanes 9 and 11) or in MCF10a cells (compare lysates in lane 3 to lanes 2 and 4) (Figure 21). However, after performing FLAG pulldowns we were able to enrich for both FLAG-NOS1AP-asssociated (lane 13) and FLAG-ARHGEF7-assoicated (lane 14) protein complexes from MDA-MB-231 cells, yet could only isolate FLAG-ARHGEF7 protein complexes from MCF10A cells (compare lanes 6 and 7). (Figure 21). Thus, we were unable to confirm that FLAG-NOS1AP associates with SCRIB in MCF10A cells because we were unable to detect any appreciable expression of FLAG-NOS1AP (Figure 21).

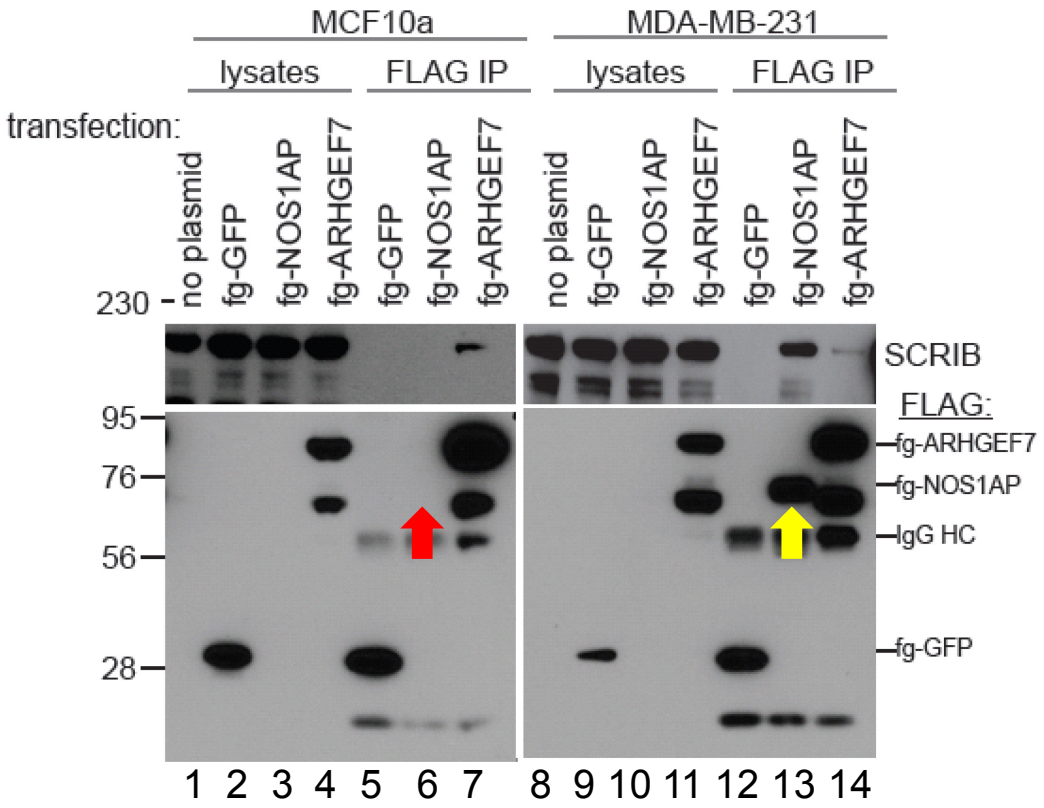

Figure 21: Co-immunoprecipitation of various FLAG constructs with endogenous SCRIB in either MCF10a (lanes 1-7) or MDA-MB-231 cells (lanes 8-14).

Both MCF10A cells (lanes 1-7) or MDA-MB-231 (lanes 8-14) cells were transiently transfected with plasmids to express FLAG-GFP, FLAG-NOS1AP or FLAG-ARHGEF7. 18-24 hours after transfection cleared whole cell lysates were obtained by lysing cells (1% Triton-X 100, 20 mM Tris HCL (pH 7.5), 150 mM NaCl) and FLAG pulldowns were performed using anti-FLAG mouse monoclonal antibodies. Both protein inputs (lanes 1-4 and lanes 8-11) and proteins isolated by FLAG pulldowns (lanes 5-7 and lanes 12-14) were analyzed by western blotting to detect FLAG-tagged proteins and endogenous SCRIB.

Given that FLAG-GFP and FLAG-ARHGEF7 were both robustly expressed in all cell lines tested and given that all of the expression constructs contain a viral CMV promoter to drive transcription of exogenous proteins, these results suggest that NOS1AP protein may be differentially regulated at the level of protein stability in different mammary cell lines.

SCRIB, NOS1AP, and VANGL1 regulate the migration of human breast cancer cells

In light of the roles for SCRIB in the polarized migration of a variety of cell types[160,295,298,300,301], and given the evidence above that NOS1AP is detected in complexes with SCRIB in some contexts, we next asked whether decreasing NOS1AP protein levels mimics the effects of decreasing SCRIB in altering the motility of breast cancer cells. We reduced NOS1AP by infecting MDA-MB-231 and MCF-7 cells with two independent shRNAs and verified knockdown by immunoblotting, and by quantitative RT-PCR (Figure 22a and Figure 23a and b). We then assessed the migratory capacity of SCRIB and NOS1AP knockdown cell lines using two different methods.

First, we monitored the migration of individual cells in a transwell assay and found that both SCRIB and NOS1AP shRNAs reduced the ability of MDA-MB-231 cells to migrate towards EGF provided as a chemoattractant cue (Figure 22b).

Transiently transfecting MDA-MB-231 cells with siRNA targeting NOS1AP similarly reduced migration in a transwell assay suggesting that this change in cell behavior was not due to nonspecific effects of long-term shRNA expression (Figure 23c and d). Second, we investigated the ability of stable shRNA cell lines to migrate in a wound-healing migration assay, and observed a consistent reduction in wound closure with SCRIB and NOS1AP shRNAs in both MDA-MB-231 and MCF-7 cell lines (Figure 22c-e). Since SCRIB, NOS1AP, and VANGL1 associate in a protein complex, we wondered whether VANGL1 might also regulate breast cancer migration. We therefore generated additional MDA-MB-231 cell lines with shRNAs targeting VANGL1 and verified knockdown by RT-PCR (Figure 22f). Like SCRIB and NOS1AP, knockdown of VANGL1 impaired MDA-MB-231 migration in a transwell assay (Figure 22g).

SCRIB and NOS1AP regulate cell polarity during wound healing migration

Having observed defects in the rate of cell migration with both SCRIB and NOS1AP shRNAs, we next evaluated whether reducing SCRIB and NOS1AP can impair the proper orientation of migratory cells. First, we assessed the establishment of leading-trailing polarity in MDA-MB-231 cells by scoring Golgi re-orientation in wound-healing assays.

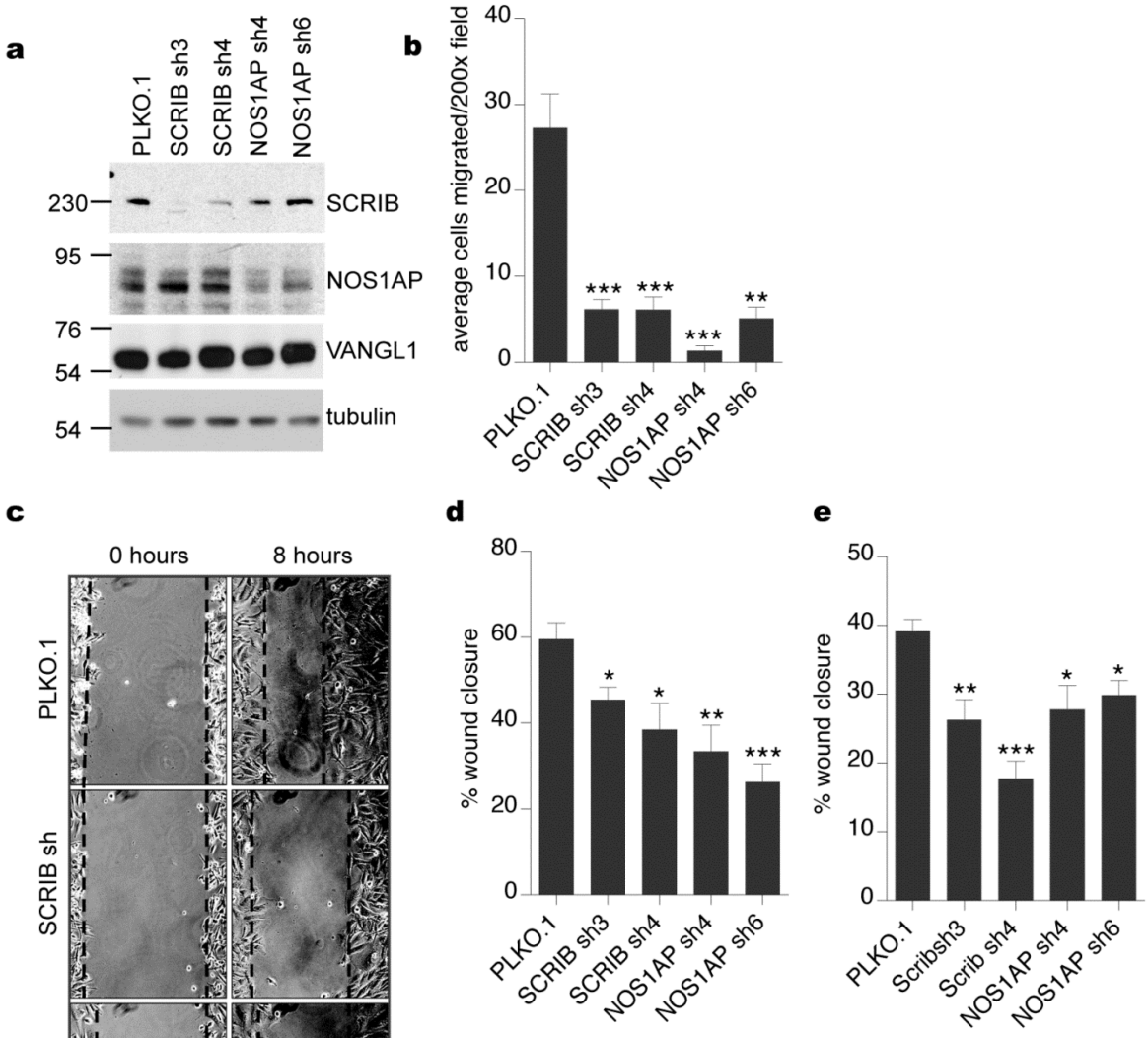

Figure 22: SCRIB and NOS1AP regulate the migration of human breast cancer cells.

(a) Western blot of lysates from MDA-MB-231 cells infected with various shRNAs probed with antibodies for SCRIB, NOS1AP, VANGL1, and β-tubulin. (b) Summary of transwell migration of MDA-MB-231 shRNA cell lines from low serum media to EGF media (20 ng/mL) for a period of six hours. Data represent average number of cells migrated per 200x microscopic field ± s.e.m. from four independent experiments.*P<0.05, **P<0.01, and ***P<0.001 (Student's *t*-test). (c) Representative images of MDA-MB-231 monolayers immediately and eight hours after wounding with a pipette tip. (d) Summary of MDA-MB-231 wound healing migration data from three independent experiments with duplicate scratches for each shRNA cell line. Data represent average wound closure ± s.e.m. *P<0.05, **P<0.01, and ***P<0.001 (Student's *t*-test). (e) Summary of MCF-7 breast cancer cell wound healing migration data from three independent experiments with duplicate scratches for each shRNA cell line.

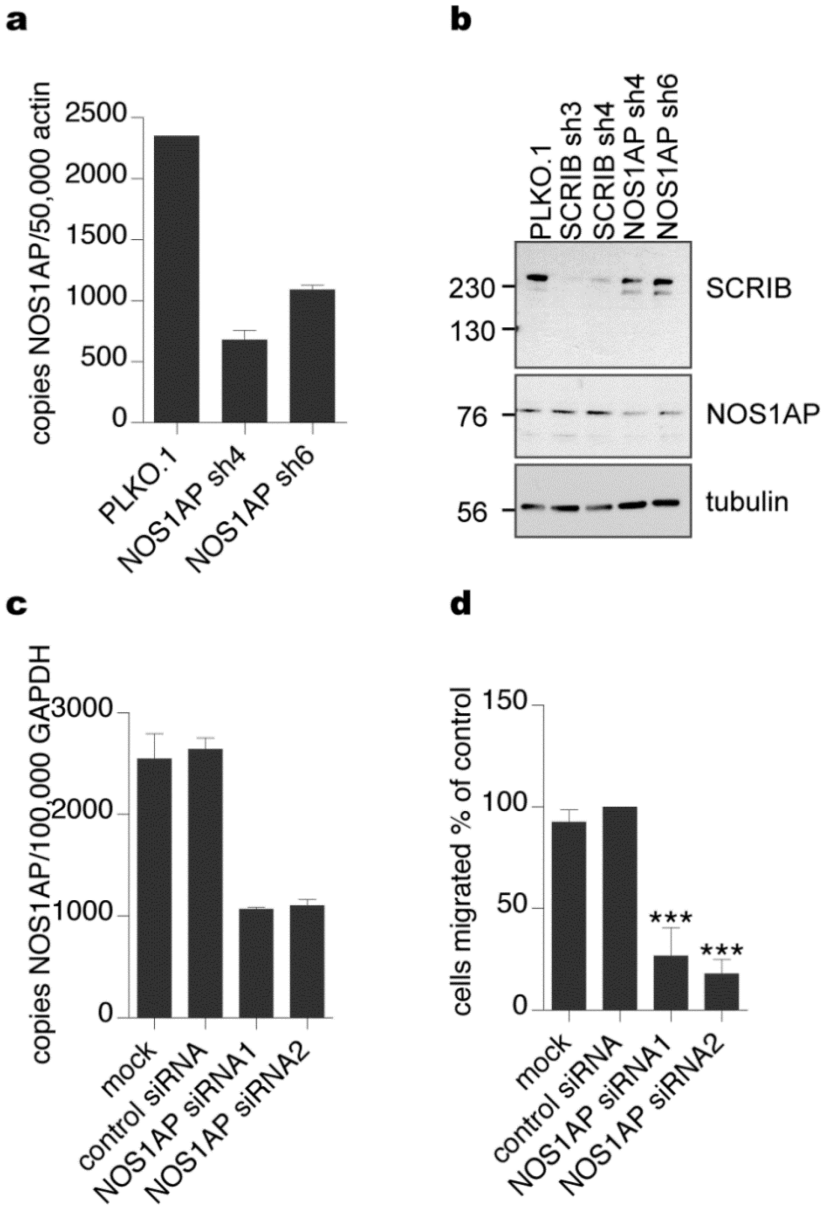

Figure 23: Short-term knockdown of NOS1AP inhibits the migration of breast cancer cells.

(a) Quantification of *NOS1AP* transcripts relative to *ACTIN* by RT-PCR from cDNA obtained from MDA-MB-231 cells expressing various shRNAs. **(b)** Analysis of protein levels in MCF-7 cell lysates expressing PLKO.1 control (lane 1), SCRIB shRNAs (lanes 2-3), or NOS1AP shRNAs (lanes 4-5). SCRIB, NOS1AP and β-tubulin were detected with appropriate antibodies by western blotting. **(c)** Confirmation of transient knockdown of *NOS1AP* transcripts by quantitative RT-PCR three days following siRNA transfection of MDA-MB-231 cells. *NOS1AP* transcript levels are normalized to *GAPDH* to control for differences in sample preparation. **(d)** Summary of transwell migration of MDA-MB-231 cells transfected with control siRNA, two independent *NOS1AP* siRNAs, or no siRNA (mock). Cells were loaded in the upper transwells in low serum media, provided with a stimulus of EGF-containing media (20 ng/mL) in the lower chamber, and allowed to migrate for a period of six hours. Data represent average number of cells migrated relative to control siRNA ± s.e.m. from four independent experiments. ***P<0.001 (Student's *t*-test).

Four hours after wounding, control shRNA infected cells displayed an increase in the fraction of properly oriented Golgi from ~30% to ~65%, and developed lamellipodia demarcated filamentous Actin running parallel to the wound edge as detected by phalloidin staining (Figure 24a). Quantification of Golgi re-orientation revealed that SCRIB and NOS1AP shRNAs significantly reduced the establishment of organelle polarity during wound healing migration (Figure 24b).

SCRIB, NOS1AP, and VANGL co-localize at the leading edge of breast cancer cells migrating in wound healing assays

To better understand the roles of SCRIB and NOS1AP in leading-trailing polarity, we used confocal microscopy to detect SCRIB-associated proteins in MDA-MB-231 cell monolayers after wounding to induce cell migration. We found that SCRIB, NOS1AP, and VANGL asymmetrically localize to the leading edge of cell lamellipodia extending into the wound (Figure 25a and b). When we analyzed the localization of SCRIB and NOS1AP in the knockdown cells, we found that SCRIB shRNAs disrupt the localization of NOS1AP to the leading edge of lamellipodia, resulting in a more uniform distribution of NOS1AP throughout the cytoplasm (Figure 26a). NOS1AP shRNAs also disrupted the sub-cellular distribution of SCRIB by similarly preventing the localization of SCRIB to the leading edge of lamellipodia (Figure 26b). Since SCRIB, NOS1AP, and VANGL associate in a protein complex, we wondered whether levels of SCRIB and NOS1AP might influence the subcellular localization of VANGL1.

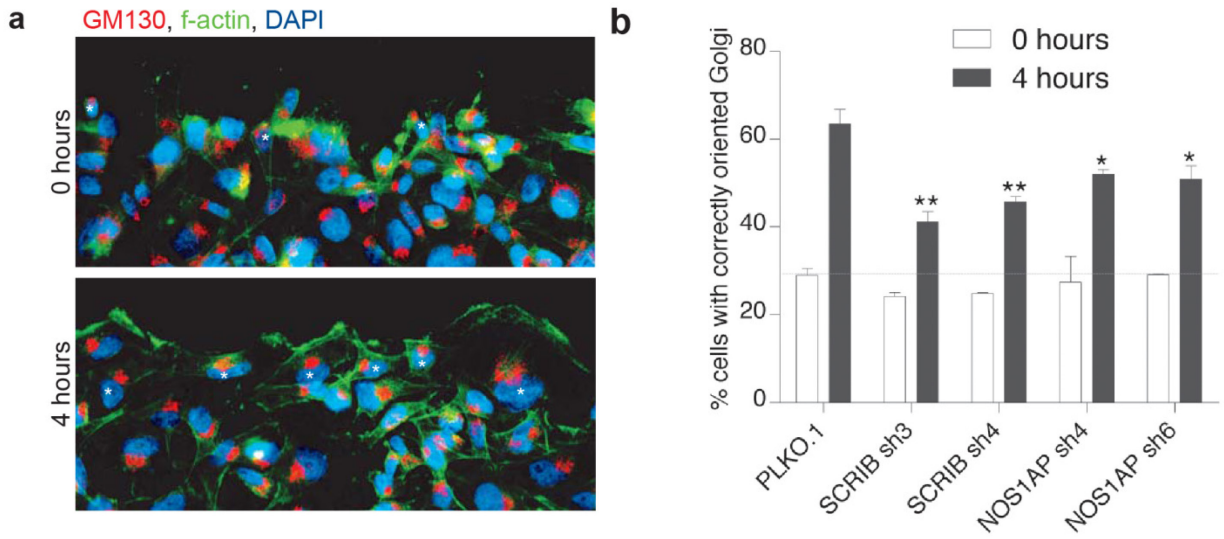

Figure 24: SCRIB and NOS1AP regulate organelle polarity during wound healing migration.

(a) MDA-MB-231 cells were fixed immediately, or four hours after wounding then incubated with GM130 antibody to visualize the Golgi apparatus, and phalloidin to visualize the ACTIN cytoskeleton. Cells with correctly oriented Golgi relative to the nucleus and the wound edge are marked with a white asterix. **(b)** Percent of MDA-MB-231 shRNA-expressing cells with proper Golgi orientation determined immediately, and four hours after wounding. The horizontal line indicates the level of Golgi polarization at the beginning of wound-healing migration. Data represents mean of four independent experiments ± s.e.m. *P<0.05, **P<0.01, and ***P<0.001 (Student's *t*-test). See Materials and Methods for more information on Golgi polarity scoring.

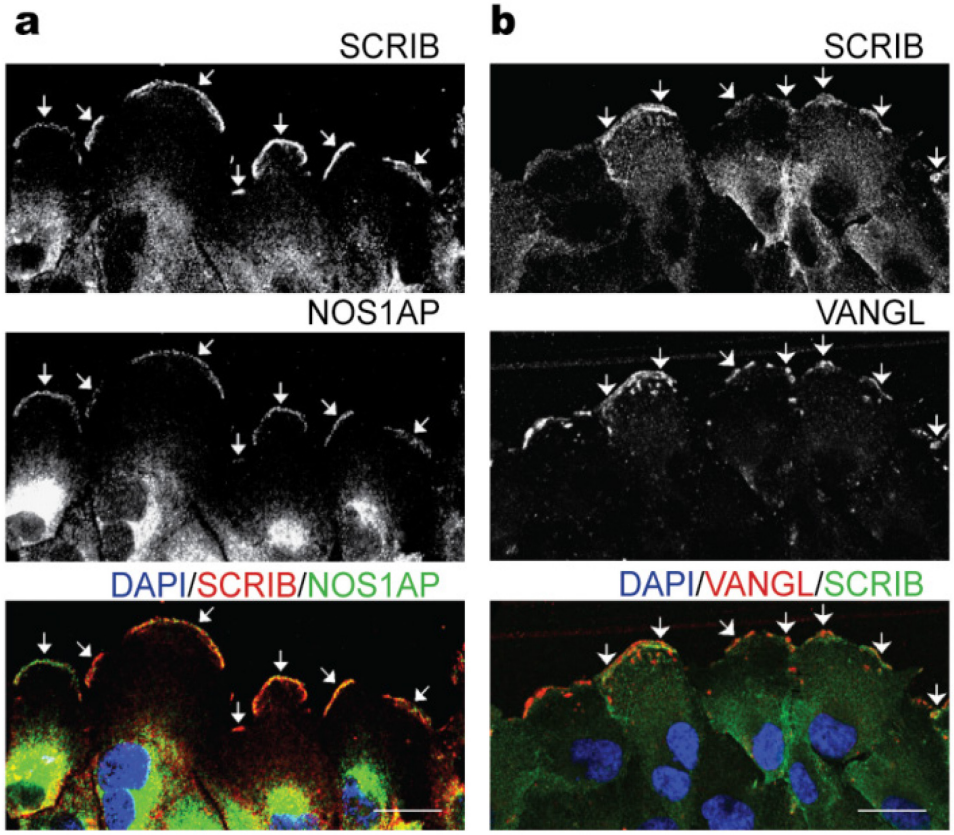

Figure 25: Co-localization of SCRIB, NOS1AP, and VANGL1/2 at the leading edge of wounded MDA-MB-231 monolayers.

Representative confocal images MDA-MB-231 wounded monolayers stained with various antibodies: (a) SCRIB and NOS1AP, (b) SCRIB and VANGL1/2. Areas of co-localization at the leading edge of cell lamellipodia are indicated by white arrows and scale bars are 30 μm.

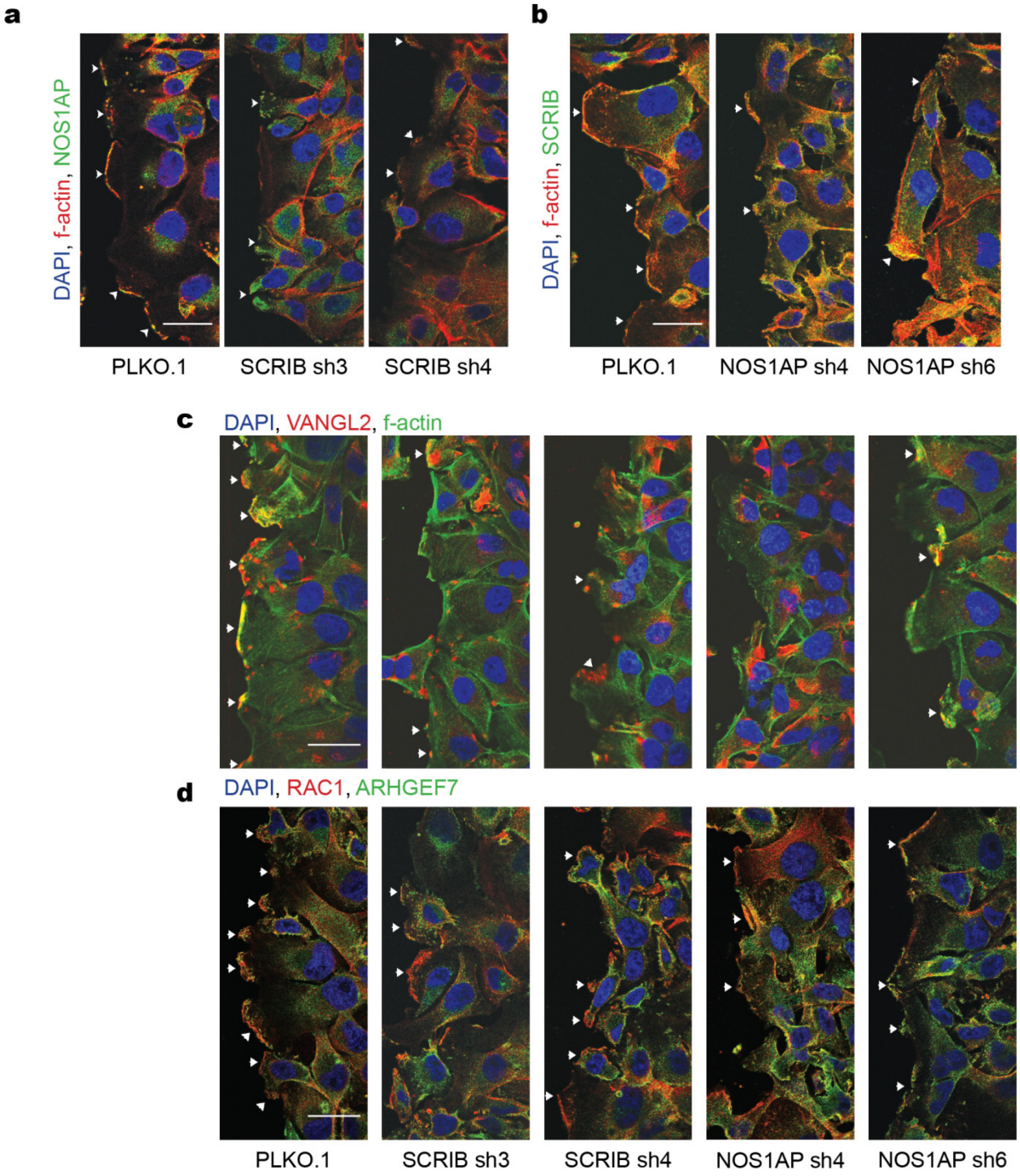

Figure 26: SCRIB and NOS1AP regulate the asymmetric localization of proteins to lamellipodia during would healing migration

(a-d) Confocal images of wounded MDA-MB-231 cell monolayers expressing either PLKO.1 (left panel), or SCRIB shRNAs. These cells were stained with the following antibodies to detect endogenous protein localization: (a) Co-stained with NOS1AP antibody and phalloidin (b) Co-stained with SCRIB antibody and phalloidin. (c) Co-stained with phalloidin and VANGL1/2 antibodies. (d) Co-stained with antibodies to detect RAC1 and ARHGEF7. Areas where SCRIB, NOS1AP, VANGL1/2, RAC1, ARHGEF7 asymmetrically localize to the leading edge are indicated with white arrowheads.

Compared to control, SCRIB and NOS1AP shRNAs also partially reduced VANGL1 localization to the leading edge (Figure 26c). These results indicate that SCRIB and NOS1AP regulate the recruitment of their associated proteins to the leading edge of migrating breast cancer cells. Given that our previous results The that NOS1AP, SCRIB, and VANGL associate in a protein complex, these studies raise the possibility that loss of either SCRIB or NOS1AP disrupts these protein-protein interactions which are potentially necessary for the maintenance of the asymmetric localization of factors along the leading-trailing axis.

Since SCRIB has been previously shown to regulate the localization of the small GTPases RAC1 and ARHGEF7 to the leading edge of migrating cells[160,300], we then asked if the localization of these proteins is also altered in these assays. Indeed, the localization of both RAC1 and ARHGEF7 to the leading edge was also partially disrupted in cells depleted of both SCRIB and NOS1AP (Figure 26d). These data suggest that NOS1AP generally regulates the asymmetric localization of polarity proteins to the leading edge. It is possible that NOS1AP regulates the localization of RAC1 and ARHGEF7 indirectly since we have not been able to detect any association between NOS1AP and ARHGEF7 by co-immunoprecipitation.

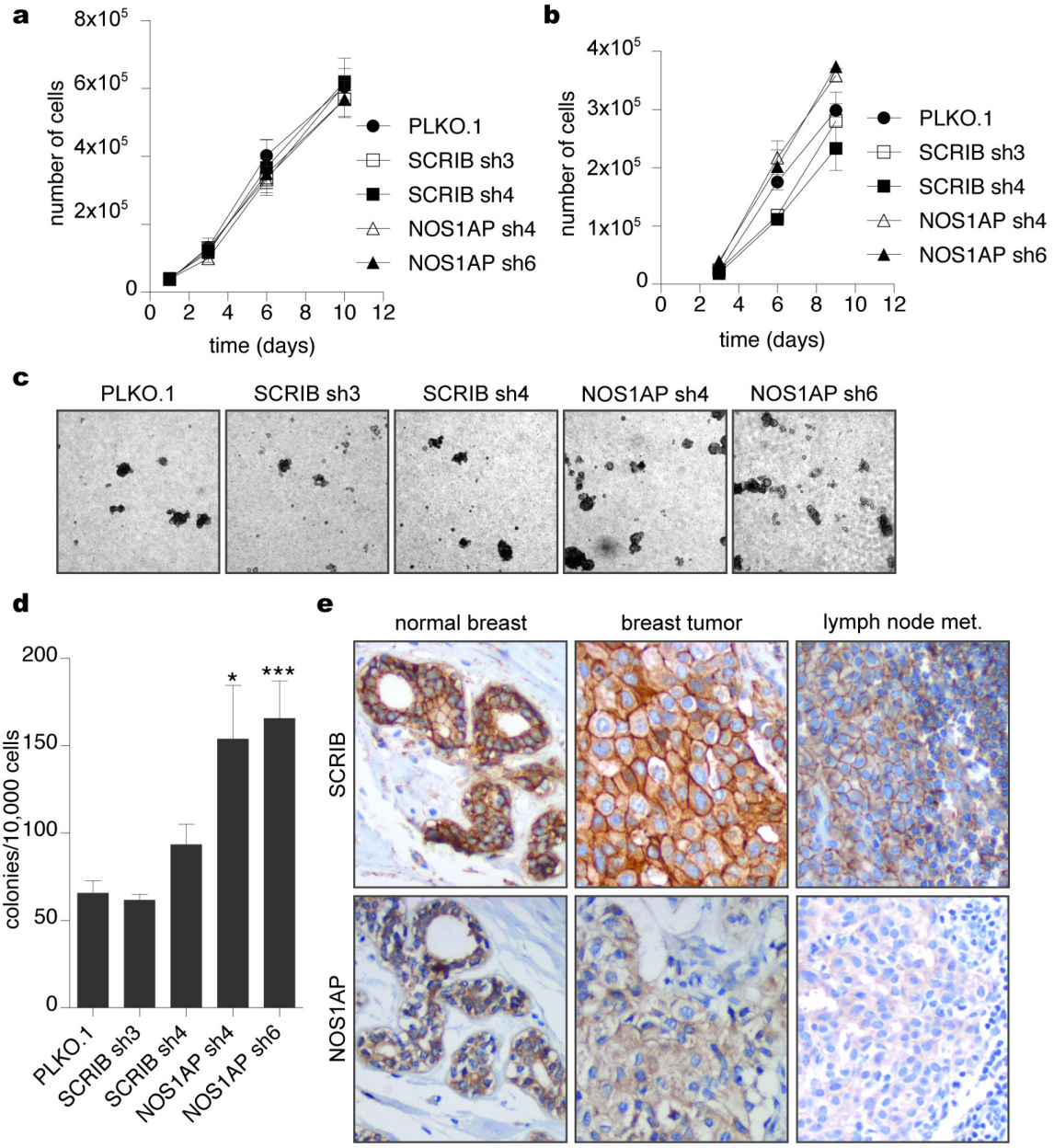

Figure 27: NOS1AP protein is reduced in breast cancer metastases from lymph nodes.

(a) Growth curves of DA-MB-231 shRNA cell lines grown in two dimensions (n=3). **(b)** Growth curves of MCF-7 shRNA cell lines grown in two dimensions (n=3). **(c)** Representative low-power microscopic images of MDA-MB-231 cells infected with various shRNAs growing as colonies in 0.3% agar for anchorage-independent growth assay. **(d)** Quantification of colonies formed per 10,000 MDA-MB-231 cells plated in 0.3% agar after three weeks. Data represents mean of three independent experiments done in triplicate. *P<0.05 and ***P<0.001 (Student's *t*-test). **(e)** Representative images of normal mammary tissue (left panels), breast cancer (middle panels), and lymph node metastatic lesions (right panels) stained for SCRIB and NOS1AP. Cell nuclei were detected by counterstaining with hematoxylin.

NOS1AP protein is reduced in breast cancer metastases from lymph nodes

We next asked if NOS1AP regulates other cellular behaviors related to breast cancer progression. First, we assessed the proliferation of cells expressing SCRIB and NOS1AP shRNAs in two-dimensional culture and found no significant difference in either MCF-7 or MDA-MB-231 cells (Figure 27a and b). Next, we assessed the anchorage-independent growth of MDA-MB-231 shRNA cell lines. In contrast to two-dimensional culture, we found that NOS1AP shRNA-expressing cells formed approximately twice as many colonies in soft agar compared to PLKO.1 controls, while SCRIB shRNAs did not significantly affect growth in this assay (Figures Figure 27c and d).

Since in vitro and mouse models of human cancers cannot fully recapitulate the complexities of human disease, we next analyzed human breast cancer specimens to determine if NOS1AP and SCRIB protein expression or localization patterns were altered. This study included a variety of different breast tumors: patient samples with and without DCIS (ductal carcinoma in situ), and patient samples with and without lymph node metastases. As others have observed[302,310]SCRIB was present in normal tissues, and was highly expressed in invasive tumors with a combination of membranous and cytosolic localization (Figure 27e and Table 9). SCRIB staining intensity did not correlate with ER/PR/HER2 status, the presence of metastatic lesions, or the grade of breast cancer samples, and was also strongly expressed in primary metastases taken from the lymph nodes (Table 9).

In order to investigate the relationship between NOS1AP expression and clinical outcomes in breast cancer we analyzed several multiple tumor microarray datasets from breast cancer patients. Unlike SCRIB and VANGL1 expression (Figure 1), there were no statistically significant associations between NOS1AP transcript levels and clinical outcomes (Figure 28). Next, we took additional sections of patient samples and performed immunohistochemistry to determine the level and localization of NOS1AP protein during cancer progression. In both normal and tumor tissue NOS1AP was localized to the cytoplasm, and was expressed at approximately the same level regardless of the breast cancer subtype, ER/PR/HER2 status, or presence of lymph node metastases (Figure 27e and Table 10).Significantly, when we analyzed primary metastatic lesions, we found that levels of NOS1AP were reduced or undetectable in half of the cases analyzed (Figure 27e and Table 10). This represents the first evidence that NOS1AP levels are altered in human breast cancer.

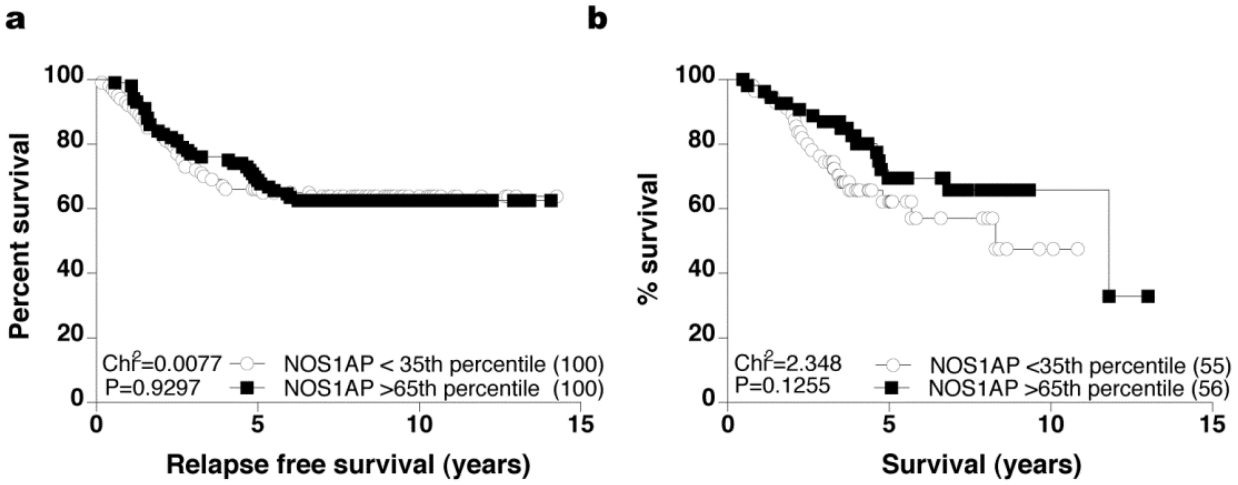

Figure 28: *NOS1AP* transcript level in primary tumors does not correlate with breast cancer relapse.

(a) *NOS1AP* expression data analyzed as for *SCRIB* using the Wang *et al.* (2005) dataset as described in Figure 1a. *P=0.9297* (log-rank test). **(b)** *NOS1AP* expression data analyzed as for *SCRIB* using the Bild *et al.* (2006) dataset as described in Figure 1c. *P=0.1255* (log-rank test).

Table 8: Histopathological characteristics of primary breast tumors used in this study.

Tumors were scored for grade using the Nottingham system. The estrogen receptor (ER), progesterone receptor (PR), and HER2/NEU status was determined by a single board certified pathologist as described in the Materials and Methods.

Study ID	Histological type	Nottingham grade	ER	PR	HER2/NEU	Lymph node status
			ALLRED score out of 8 possible			
A	DUCTAL	1	8	4	NEGATIVE	POSITIVE
B	LOBULAR	1	8	0	NEGATIVE	POSITIVE
C	DUCTAL	2	8	8	NEGATIVE	POSITIVE
D	DUCTAL	3	7	6	NEGATIVE	POSITIVE
E	DUCTAL	2	8	5	NEGATIVE	POSITIVE
F	DUCTAL	2	8	8	NEGATIVE	POSITIVE
G	DUCTAL	3	4	0	POSITIVE	POSITIVE
H	DUCTAL	3	7	8	NEGATIVE	POSITIVE
I	DUCTAL	1	8	8	NEGATIVE	POSITIVE
J	DUCTAL	1	8	2	NEGATIVE	POSITIVE
K	DUCTAL	2	8	5	NEGATIVE	POSITIVE
L	DUCTAL	2	8	8	NEGATIVE	POSITIVE
M	DUCTAL	3	0	0	POSITIVE	No lymph node
N	DUCTAL	3	0	0	POSITIVE	POSITIVE
O	DUCTAL	3	8	5	POSITIVE	NEGATIVE
P	DUCTAL	3	0	0	POSITIVE	POSITIVE
Q	DUCTAL	2	8	8	POSITIVE	POSITVE
R	DUCTAL	2	8	0	POSITIVE	NEGATIVE
S	DUCTAL	3	0	0	POSITIVE	NEGATIVE

Table 9: Analysis of SCRIB protein levels in normal tissue, invasive tumor, and breast tumors with DCIS (ductal carcinoma in situ).

SCRIB protein was detected by immunohistochemistry with anti-SCRIB IgG antibody (Santa Cruz C20), and samples were scored for the staining intensity and % of cells with positive signal by a board-certified pathologist.

Study code ID	**SCRIB IHC**					
	Normal		**Invasion**		**DCIS**	
	% Cells	**Intensity**	**% Cells**	**Intensity**	**% Cells**	**Intensity**
A	100	3+	100	2+	NO DCIS	
B	100	3+	100	2+	LCIS only	3+
C	100	3+	NO INV		NO DCIS	
D	100	3+	100	3+	100	3+
E	100	3+	100	3+	NO DCIS	
F	100	3+	100	3+	100	3+
G	100	3+	100	2+	NO DCIS	
H	100	3+	100	3+	100	3+
I	100	3+	100	3+	100	3+
J	100	3+	100	3+	100	3+
K	100	3+	100	3+	100	3+
L	100	3+	100	3+	100	3+
M	No residual tissue					
N	100	2+	100	2+	100	2+
O	100	2+	100	3+	100	3+
P	100	3+	100	3+	100	3+
Q	No normal		100	3+	NO DCIS	
R	100	3+	100	2+	100	3+
S	No normal		100	3+	NO DCIS	

Table 10: Analysis of NOS1AP protein levels in normal tissue, invasive tumor, and breast tumors with DCIS (ductal carcinoma in situ).

NOS1AP protein was detected by immunohistochemistry with anti-NOS1AP IgG antibody (Santa Cruz R300), and samples were scored for the staining intensity and % of cells with positive signal by a board-certified pathologist.

Study code ID	**NOS1AP IHC**					
	Normal		**Invasion**		**DCIS**	
	% Cells	**Intensity**	**% Cells**	**Intensity**	**% Cells**	**Intensity**
A	100	2+	100	1+	NO DCIS	
B	100	2+	100	1+	LCIS ONLY	1+
C	100	2+	100	NO INV	NO DCIS	
D	100	2+	80	1+	100	2+
E	100	2+	100	2+	NO DCIS	
F	100	2+	100	2+	100	2+
G	100	2+	0	0	NO DCIS	
H	100	2+	100	2+	100	2+
I	100	2+	100	2+	100	2+
J	100	2+	100	1+	100	1+
K	100	2+	100	1+	100	2+
L	100	2+	100	2+	100	2+
M	No residual tissue					
N	100	2+	60	1+	60	1+
O	100	2+	100	2+	100	2+
P	100	2+	100	2+	100	2+
Q	No normal		60	1+		
R	100	2+	80	1+	100	1+
S	No normal		100	2+	No DCIS	

Discussion

Since the majority of breast cancer deaths occur as a result of recurrent or metastatic disease, improving our understanding of the molecular mechanisms of cancer progression from primary tumor to the formation of metastases is of the utmost importance. The progression of breast cancer requires complex changes in cell behavior often involving a partial epithelial-mesenchymal transition. The planar cell polarity (PCP) protein SCRIB acts in a context manner to regulate cell-cell contacts in epithelial cells, or to regulate cell migration and front-rear polarity in mesenchymal-like cells, we reasoned that SCRIB protein complexes may act in a context-dependent manner in breast cancer cells.

Several studies that have analyzed SCRIB expression in tumors have led to somewhat conflicting results. Navarro et al., (2005) reported that SCRIB levels were reduced in lobular breast carcinomas, while levels were neither elevated nor consistently reduced in ductal carcinomas[310]. In an independent study, Zhan et al., (2008) found no difference in SCRIB protein levels in breast cancer samples, but found that siRNA-mediated reduction of SCRIB promotes the growth of mouse mammary tumors in vivo[302]. In the present study, we observed that reducing levels of SCRIB slows the growth of human breast cancer cells in mouse xenografts, and found that high expression of SCRIB in breast cancer patient samples correlates with relapse and decreased survival. Together, these data suggest that breast cancer progression may be sensitive to the levels of SCRIB.

We hypothesized that the ability of SCRIB to influence breast cancer progression might involve changes in the proteins with which SCRIB associates. We therefore characterized SCRIB protein complexes by mass spectrometry and found that SCRIB exists in multiple pools within cells, associating with a complex of SCRIB-ARHGEF-PAK-GIT proteins, and, also associating with a complex of SCRIB-NOS1AP-VANGL proteins. In invasive breast cancer cells we find that SCRIB, NOS1AP, and VANGL1 co-localize along the edge of cellular protrusions. These data support the mass spectrometry data by showing that proteins which associate with one another in cell lysates are normally found in overlapping domains within cells. In contrast, we find that SCRIB and VANGL1 localize to sites of cell-cell contact in normal mammary cells, whereas NOS1AP remains in the cytosol. The localization of NOS1AP at cell protrusions in invasive breast cancer cells led us to hypothesize that NOS1AP might cooperate with SCRIB, and VANGL1 to promote cell migration. Indeed, our results revealed a functional relevance for SCRIB-NOS1AP-VANGL1 protein complexes in breast cancer cells because loss of SCRIB, NOS1AP, or VANGL1 slows migration.

Several studies now suggest that SCRIB may promote cell migration by acting as a scaffold for the recruitment of ARHGEFs and PAKs to the leading edge, and by acting as a determinant of organelle polarity[160,300]. Since, NOS1AP analogously acts as a signaling adapter protein to localize active nNOS and RAC1 to the plasma membrane in neurons[305–309], we then asked whether NOS1AP might also play a role in the establishment of leading-trailing polarity. We found that SCRIB and NOS1AP are mutually-dependent in terms of their localization to the

leading edge of breast cancer cells. Furthermore, knockdown of either SCRIB or NOS1AP prevents the localization of VANGL at the leading edge, as well as the re-orientation of the Golgi apparatus. Alternatively, it is also possible that SCRIB and NOS1AP shRNAs indirectly result in defective leading-trailing polarity of SCRIB, NOS1AP, and VANGL due to a generalized loss of cell polarity. Collectively, these data indicate that tightly controlled levels of SCRIB, NOS1AP, and VANGL proteins are necessary to regulate cell polarity and motility.

Recent work demonstrates that expression of TNF (Tumor Necrosis Factor) and constitutively activated RASV12 can convert SCRIB from a tumor suppressor to enhancer of tissue overgrowth in in Drosophila epithelial tissues[311], supporting our hypothesis that SCRIB protein complexes may regulate cancer progression in a context-dependent manner. This study finds that other members of SCRIB protein complexes may also act in a context-dependent manner. Although we find that reducing NOS1AP slows cell migration as is typical of a metastasis suppressor, we paradoxically observe that NOS1AP-depleted cells outperform control cell lines in anchorage-independent growth assays. It is possible that NOS1AP may have a unique function in promoting three-dimensional growth that is independent of SCRIB, since SCRIB shRNAs did not modulate soft agar colony formation.

The development of metastases is thought to involve the delamination of cells from primary tumors through an epithelial-mesenchymal transition (EMT)[145,146,312,313]. It is possible that SCRIB acts to suppress tumor growth and to maintain epithelial integrity in tumors that retain their epithelial morphology, but conversely supports the migration of cancer cells that

have already lost their epithelial characteristics. If a distinct subset of SCRIB protein complexes promotes cell migration, whereas others act at sites of cell-cell contact, then it follows that pro-migratory SCRIB protein complexes would be over-expressed at the expense of junctional ones in during cancer progression. Indeed, one study found ARHGEF7 to be overexpressed in breast tumors[314] and another study found that overexpression of constitutively active forms of PAKs increases breast cancer cell invasiveness and anchorage-independent growth[315,316].

We also analyzed the expression of SCRIB and SCRIB-associated proteins in samples from a variety of breast cancer lesions, and note a reduction in NOS1AP in lymph node metastases compared to primary tumors and normal breast. Although we observed no significant difference between NOS1AP levels in normal breast tissue and tumors, we do note a loss of NOS1AP expression in metastatic lesions and enhanced malignant behavior in NOS1AP knockdown cells. It is unclear whether NOS1AP is lost as cells leave the primary tumor and invade the lymphatic system, or if loss of NOS1AP occurs as a result of different environmental conditions in the lymph node. Nevertheless, our results suggest that changes in the expression, composition, or activity of SCRIB- and NOS1AP-associated protein complexes may be involved in the progression of breast cancer.

Materials and Methods

Cell culture and generation of stable cell lines

MDA-MB-231, MCF-7, and HEK293T cells were cultured in 5% CO_2 incubator with DMEM with 10% FBS and antibiotics. In order to generate stable cell lines lentiviral particles were produced as previously described (ref) and MDA-MB-231 or cells were infected at low passage number with various constructs. NOS1AP and SCRIB shRNAs in PLKO.1 backbone were purchased from Open Biosystems (mature sense sequences as follows: NOS1APsh4; 5'-CGGATACGGTATGAGTTTAAA-3,' NOS1APsh6; 5'-GCTCCCTTCTTCTTCCTCGAA-3,' SCRIB shRNA3: , SCRIB shRNA4: CCGGCCTGAATGATGTGTCTCTGCACT).NOS1AP, ARHGEF7, and SCRIB were PCR amplified from pooled cDNA libraries, digested with Not1/Asc1 restriction enzymes and ligated into either a pcDNA3.1 backbone for transient transfection, or into a pPHAGE backbone for viral packaging.

Mass spectrometry and western blotting

For endogenous SCRIB pulldowns $1x10^9$ HEK293T cells were pelleted by centrifugation, rinsed with 1XPBS, and then lysed in standard TAP-TAG lysis buffer, subjected to two rounds of freeze-thaw cycle in liquid nitrogen, then incubated with gentle rotation at 4^oC for 30 minutes to

extract protein complexes. Lysates were cleared by centrifugation at 25,000 RCF in microfuge for 30 minutes, followed by incubation with protein G sepharose beads at 4^{o}C for 1 hour. 20 ug of SCRIB antibody were pre-incubated with protein G beads at 4^{o}C, then rinsed with lysis buffer to remove unbound antibody. Protein-G antibody complexes were then combined with the cleared lysates and incubated for 2 hours at 4^{o}C with gentle rotation. Immunoprecipitates were then washed with 3x1mL lysis buffer, followed by three additional washes with 50 mM Ammonium Bicarbonate pH 7.8 to remove any residual detergent. Antibody complexes were eluted with 50 mM sodium citrate pH 2.0 in 3, 100 µl fractions at 37^{o}C. All three elutions were combined and adjusted to pH 7.8-8.0 by adding approximately 35µL of 1M KOH before trypsin digestion as previously described[281].

Co-immunoprecipitation

0.5x10^{6}-1.0x10^{6} MDA-MB-231 cells in exponential growth phase were collected in ice cold PBS, and pelleted by centrifugation. Cell pellets were re-suspended in lysis buffer (50mM Tris 8.0, 150mM NaCl, 0.1% Na-doxycyclin, 1% NP40, with protease and phosphotase inhibitor cocktail (Roche) and incubated for ten minutes on ice, followed by clearing by centrifugation at 20,000 RCF for 15 minutes at 4^{o}C. Cleared cell lysates were combined with 0.5-1.0 ug of one of the following antibodies: GFP (AbCAM), ARHGEF7 (Cell Signaling), VANGL1 (Santa Cruz),SCRIB (Santa Cruz H300, or C20), or IgG control and incubated with gentle rocking at 4^{o}C for two

hours. Immune complexes were precipitated by incubation with protein G sepharose beads (Sigma) for 45 minutes. Immunoprecipitates were washed at least four times with 1mL with lysis buffer, and eluted with 1xNUPAGE loading buffer containing 2.5% β-mercaptoethanol. Immunoprecipitates were analyzed by western blotting as described above.

Concanavalin-A fractionation

MDA-MB-231 or MCF10a cells were lysed in a buffer containing 1% Triton-X 100, 20mM Tris-HCl 7.5, 150mM NaCl, and protease and phosphotase inhibitors. Proteins were solubilized on a rotator at 4^{o}C for one hour before clearing by centrifugation. The soluble fraction was incubated with concanavalin-A beads on a rotator at 4^{o}C for two hours at which point flow through fractions were collected. After washing with lysis buffer, concanavalin-A bound proteins were eluted with 1xNUPAGE loading buffer for western blotting.

Immunofluorescence and confocal microscopy

Cells were plated on FBS coated coverslips and fixed with 4% PFA for 5 minutes at room temperature, blocked with 4% w/v BSA in PBS + 0.2% tween before incubation with one or more of the following antibodies: 1:500 SCRIB (Santa Cruz H300), 1:250 SCRIB (Santa Cruz C20), 1:250 NOS1AP (Santa Cruz R300), 1:250 VANGL1 (Sigma), 1:2000 VANGL2, 1:250 ARHGEF7

(Chemicon), 1:100 RAC1 (BD transduction), 1:100 CDC42 (BD transduction), GM130 (Stressgen. Flurophore conjugagated secondary antibodies (Molecular Probes, Invitrogen) were used at 1:2000 dilution and coverslips were mounted with Prolong Gold (Invitrogen) with or without DAPI counterstain. For Golgi polarity assays cells were imaged on a Nikon widefield high resolution scope using a 40x objective and scored as polarized when the Golgi resided in the 120^{o} sector of the cell facing the wound. In order to determine the subcellular localization of other proteins ells were imaged using a Nikon imaging system with a 60x oil immersion objective. Images were imported into ImageJ analysis software and cropped and rotated for final presentation.

Cell migration assays

For trans-well assays, 50,000 cells were plated in the upper chamber of a transwell (8 µm pore) with 20 ng/mL EGF or 10% FBS provided in the lower chamber. Cells remaining in the upper chamber were removed, and cells that had migrated to the bottom of the membrane were fixed, stained with crystal violet, and counted. For wound healing assays, confluent MDA-MB-231 or MCF-7 cells were wounded with a pipette tip, and imaged at various time points on a Nikon Eclipse TS100 microscope. Wounds were measured at six or more locations in order to determine the width of each replicate wound.

Golgi orientation assays

Wounded MDA-MB-231 monolayers were stained with GM130 antibodies and phalloidin and imaged on a Nikon Eclipse widefield microscope. Images from four independent experiments were analyzed blinded to determine Golgi polarity essentially as described in Nobes and Hall (1999). Briefly, cells immediately adjacent to the wound were scored as polarized when the Golgi resided in the 120^{o} sector between the nucleus and the leading edge, and scored as un-polarized if the Golgi was either diffusely distributed, or was not localized to the 120^{o} sector between the nucleus and the leading edge. At least 150 cells were scored per shRNA per experiment in order to determine the percent of cells with polarized Golgi, which was calculated by dividing the number of polarized cells by the total cells scored for each shRNA.

Cell proliferation experiments

For two assays of two dimensional growth assays 30,000 MDA-MB-231 or MCF-7 cells were plated in 12-well plates and collected in PBS/EDTA after one, three, six, or nine days and counted on a hemacytometer. Experiments were repeated twice with triplicate wells per time point. For anchorage-independent growth assays, 1.5 mL of a single cell suspenstion of MDA-MB-231 cells expressing various shRNAs in 0.3% agarose-containing growth media was plated over a base 0.6% base agarose layer in a 6-well format plate. Cultures were fed every three days and analyzed after three weeks. Colonies with >25 cells were scored on a microscope and

the assay was repeated at least three times in duplicate. For xenograft experiments MDA-MB-231 cells were grown to sub confluence (75-80%) in 15 cm dishes, trypsinized and counted on a hemacytometer. Cells were resuspended at a concentration of $1x10^6$ cells/mL and 0.1 mL of cells were injected orthotopically using a 25 gauge needle into the inguinal mammary fat pads of NOG or athymic nude immunocompromised mice. The growth of primary tumors was monitored twice a week by measuring tumor diameters with calipers and calculating tumor volume using the formula: volume = $(\pi/6) \times a^2b$ where a is the short diameter and b is the long diameter. When tumors derived from control shRNA infected MDA-MB-23 cells reached >1 cm in diameter mice were sacrificed and gross necropsies were performed.

Xenografts

MDA-MB-231 cells were re-suspended at a concentration of $1x10^7$ cells/mL and 0.1 mL of cells were injected into the mammary fat pads of NOG or athymic nude immunocompromised mice (Harlan Labs). The growth of primary tumors was monitored biweekly using the formula: volume = $(\pi/6) \times a^2b$ where a is the short diameter and b is the long diameter.

Immunohistochemistry of breast cancer samples

Breast cancer patient samples were obtained from the University of Washington Department of Pathology. Tumor sections were de-paraffinized followed by antigen retrieval by microwaving slides in EDTA buffer (pH 8.0). Samples were then incubated with NOS1AP (1:60 dilution, Santa Cruz R300), or SCRIB antibodies (1:100 Santa Cruz C20) for 40 minutes, followed by incubation with biotinylated mIgG secondary antibody for 30 minutes, and detection using an ABC kit (avidin biotin complex with peroxidase) for 30 minutes. Color was developed with DAB and hematoxylin before dehydration through graded alcohols and mounting. Tumor grade was scored using a Nottingham system, and the ER/PR status of tumors was determined using the Allred scale as previously described (Harvey et al., 1999). The intensity (0-3+) and percent of SCRIB and NOS1AP positive cells was determined by a blinded board certified pathologist (K.H.A). All human studies were approved by an institutional review board and the Department of Health and Human Services.

Statistical analysis

All migration and growth assays were repeated at least three times in duplicate and significance was assessed using an unpaired Student's t-test. Error bars show S.E.M (*P<0.05, **P<0.01, and ***P<0.001). For Kaplan Meier survival analysis, significance of the association between

transcript expression and disease outcome was determined using Graphpad statistical software and the Mantel-Cox log-rank test.

Generation of VANGL antibodies

Peptide VANGL antibodies were generated by Aves labs Inc (peptide sequence: CZ RRR DNS HNE YYY EEA EHE RR) and validated by ELISA and western blotting (data not shown). Since this peptide is >90% identical to the amino acid sequence of VANGL1 we, therefore, refer to this antibody as VANGL throughout the text (Figure 29).

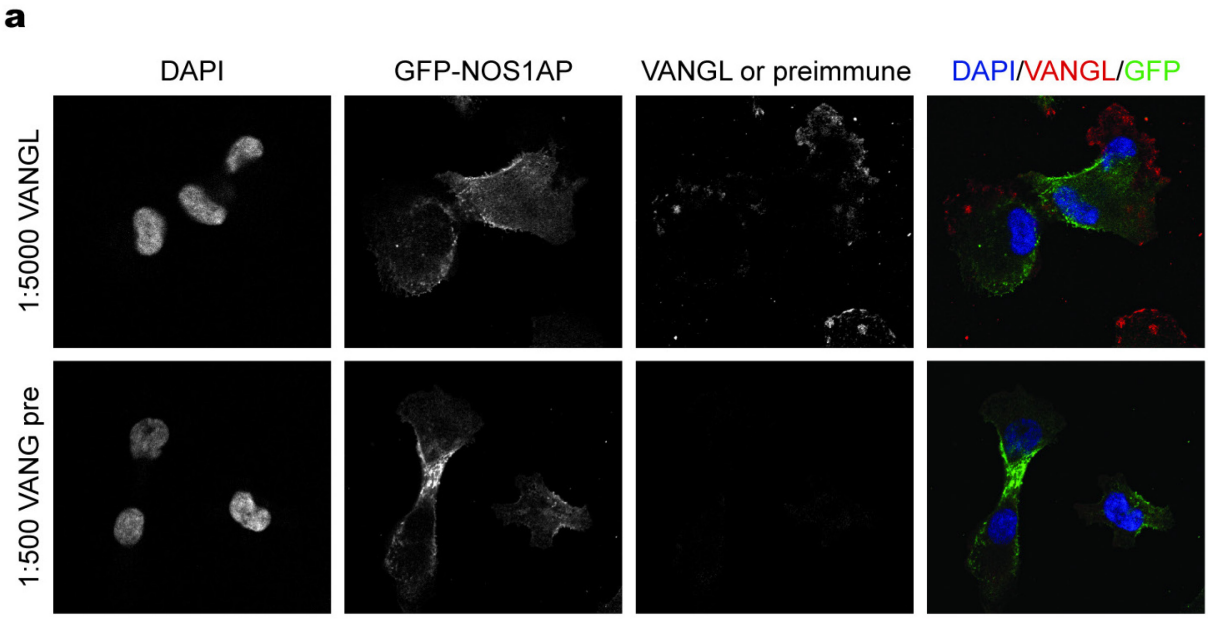

Figure 29: Characterization of VANGL antibody.

(a) Confocal microscopy of MDA-MB-231 cells expressing GFP-NOS1AP stained with chicken anti-hVANGL at 1:5000 dilution, or pre-immune serum at 1:500 dilution, showing specific signal with the purified antibody.

Chapter 4: WNT signaling in melanoma

Jamie N. Anastas and Andy J. Chien

An introduction to malignant melanoma

Malignant melanomas comprise a molecularly and pathological diverse group of deadly cancers that arise from epidermal melanocytes of the skin and other tissues. Melanoma incidence is skyrocketing at a rate that surpasses that of any other cancer worldwide[317]. Metastatic melanoma accounts for the vast majority of skin cancer-related deaths with a dismally low five-year survival rate ranging between 5-15% [318]. Clinically, melanoma is extremely resistant to available forms of chemotherapy and radiation therapy[319]. Given the rise of melanoma incidence and the current scarcity of effective therapeutic options, research efforts have focused intensively on understanding mechanisms of melanoma initiation and progression in order to develop novel treatment strategies.

Recent scientific advancements reveal that the development of metastatic melanoma involves a combination of genetic and environmental factors, most notably exposure to ultraviolet light[320]. The vast majority of melanoma tumors exhibit constitutive activation of the MAPK signaling pathway, mainly through activating mutations in the oncogenes BRAF or NRAS[320]. Other studies find that increased activity of the PI3K/AKT pathway similarly promotes melanoma tumorigenesis and progression [320,321].

One of the challenges for ongoing melanoma research will be to determine if and how

changes in the activity of additional signal transduction pathways such as those driven by WNTs can be leveraged to improve the diagnosis and treatment of melanomas. In addition to altered activation of kinase-dependent signaling pathways, numerous studies now implicate changes in the activity of in both β-catenin-dependent WNT signaling pathways and in β-catenin-independent WNT signaling pathways in the development and progression of melanoma. Basic and clinical researchers alike face several critical questions regarding potential roles for WNT signaling in melanoma:

1. Are WNT signaling pathways either activated or inactivated during different stages of melanoma progression and can we use evidence of changes in WNT signaling activity as prognostic indictors in a clinical setting?

2. Do WNT signaling pathways functionally contribute to melanoma tumorigenesis, progression and/or metastasis?

3. Mechanistically, what endogenous and exogenous factors determine the relative activity of WNT signaling pathways in melanoma?

4. Can we apply our emerging understanding of WNT signaling towards the development of new clinical strategies for therapeutically intractable melanomas?

WNT signaling pathways as prognostic indicators in melanoma

The WNT/β-catenin pathway

Numerous histological studies describe variations in staining for β-catenin, which acts as a surrogate indicator of active WNT/β-catenin signaling in melanoma patient samples. Specifically positive staining for cytoplasmic/nuclear β-catenin (encoded by CTNNB1 in humans and Ctnnb1 in the mouse) is observed in approximately one third of patient melanoma tumors[322–324] . Importantly, the presence of increased cytoplasmic or nuclear β-catenin in patient melanoma tumors correlates with improved patient survival in several studies[41,322,323,325]. Consistently, another study finds that increased nuclear phosphorylated β-catenin (phospho-β-catenin) detected using an antibody that recognizes phosphorylation at Ser33, Ser37, and Thr41 correlates with a poorer patient prognosis[326]. In the current paradigm of WNT/β-catenin signaling, the enhanced phosphorylation of β-catenin by GSK3β (encoded by GSK3B in humans) results in increased association between β-catenin and the destruction complex resulting in lowered β-catenin activity or abundance. As predicted by this model, tumors with increased nuclear phospho-β-catenin exhibit undetectable levels of total β-catenin[326]. Still other studies find that nuclear and cytoplasmic β-catenin abundance gradually decreases in later stage melanomas compared to primary tumors[323,324]. Collectively, these immunohistochemical studies employing either anti-(total) β-catenin or anti-phospho-β-catenin antibodies support a model in which inhibition of the stabilization of β-catenin correlates with

improved clinical outcomes for melanoma patients.

The prognostic significance of both total and phospho-β-catenin levels in melanoma raises questions regarding the mechanisms underlying the variable activity of the WNT/β-catenin signaling pathway observed in melanomas. In many cancers, the activation of WNT/β-catenin signaling occurs primarily through mutations in the pathway components (Table 1). While an initial report identified activating mutations in CTNNB1 in melanomas[327], further studies find that that mutations in neither CTNNB1 nor in genes encoding other WNT pathway components such as APC and AXIN1 are common in melanomas[328–331] . Importantly, elevated cytosolic/nuclear β-catenin staining is sometimes observed in melanoma tumors lacking mutations in WNT/β-catenin pathway genes. These observations suggest a model whereby activation of or inactivation of WNT/β-catenin signaling occurs independently of direct mutations in WNT signaling components. Moreover, these findings have important ramifications when considering possible strategies for targeting WNT signaling in melanoma. First, these results suggest that activating rather than inhibiting WNT/β-catenin signaling might curb melanoma progressions. Second, a pathway regulated by mutations resulting in either the total loss of or the hyper-activation of key signaling protein functions would be theoretically targeted using different strategies compared to a signaling pathway regulated by the differential activity or expression of endogenous factors.

Expression of WNT5A in metastatic melanoma

While most cancer research has focused on understanding potential roles for the WNT/β-catenin signaling pathway, numerous studies now point to possible roles for β-catenin-independent WNT signaling in melanoma, most notably those signaling pathways activated by WNT5A[332,333]. Interest in WNT5A-dependent signaling in melanoma was initially generated by large-scale efforts to characterize melanoma phenotypic subtypes by microarray analysis of gene expression[42]. By analyzing gene expression in 31 established patient-derived melanoma cell lines, one study finds that high levels of WNT5A expression correlate with a more invasive phenotype. While the number of clinical samples linked to corresponding patient outcomes in this study is too small to draw definitive conclusions, it is noted that 4 of 5 patients with a high WNT5A signature died, whereas only 3 of 10 patients with the low WNT5A signature died. Importantly, a correlation between increased expression of WNT5A and invasive melanoma phenotypes has been independently verified by another large transcriptional profiling study [334].

Several histological studies have also examined potential correlations between WNT5A expression and clinical outcomes. Bachman *et al* (2005) were the first to to report an association between increased nuclear/cytoplasmic β-catenin and increased patient survival also examined WNT5A expression in 312 tumors and notes the presence of cytosolic and nuclear staining for WNT5A in subset of the patient samples[322]. However, WNT5A staining levels do not predict patient outcomes in this dataset[322]. In contrast, another immunohistochemical study from Da Forno *et al* (2008) analyzing 102 patient tumors finds that

high cytoplasmic (but not nuclear) staining for WNT5Apredicts reduced time to metastasis and reduced time to patient death[335]. Both of these studies used the same commercially-available goat anti-WNT5A antibody (R&D Systems, Minneapolis MN), yet employed markedly different antigen retrieval procedures. Previous studies in other model systems have not reported nuclear WNT5A staining, nor have other studies characterized a role for WNT5A in the nucleus. At present, it remains unclear whether conflicting results from the Da Forno et al (2008) and Bachmann et al (2005) studies arise due to technical differences in the WNT5A staining procedures or due to underlying biological differences separating these two cohorts of patient tumors.

Transcriptional profiling of the WNT pathway in melanoma

Overall, genome-wide transcriptional profiling has proven disappointingly inconsistent in efforts to understand melanoma and, to date, researchers have failed to agree upon a consensus molecular signature that is applicable across multiple studies[336]. Whether microarray profiling studies provide a more accurate read-out of WNT signaling activity compared to histological studies also remains unresolved, so these data should be interpreted with caution. Further complicating matters, conclusions regarding the activation of WNT/β-catenin signaling derived from microarray profiling of gene expression often rely on the measurement of downstream transcriptional targets (including well-described β-catenin target genes such as

AXIN2, DKK1, LEF1 and MITF). Since these target genes exhibit variable responses to WNT/β-catenin stimulation in different cell lines, and can also act as inhibitors of WNT/β-catenin signaling via negative feedback loops, it is not clear whether expression of these genes can be interpreted as an accurate read-out if WNT signaling. One study incudes AXIN2, one of the most reliable WNT/β-catenin transcriptional targets, in a 45-gene signature that strongly correlates with improved prognosis[337]. However, numerous other studies have not associated AXIN2 expression with either good or bad patient outcomes. For instance, another study used hierarchical clustering of gene expression profiles to classify a set of >200 archived cutaneous melanomas as either high grade with very poor patient outcomes, or low grade with much better patient outcomes [338]. Although several genes encoding WNT signaling pathway proteins such as FZD4, FZD10, LEF1, TCF4, SFRP2, and BCL9 were differentially expressed in low grade compared to high grade tumors in this dataset, differences in AXIN2 expression were not observed[338]. Ongoing advances in genome-wide sequencing and in the annotation of the encyclopedia of DNA elements (ENCODE) will undoubtedly facilitate our understanding of both the gene expression changes that occur in melanoma cells and their relation to WNT signaling pathways.

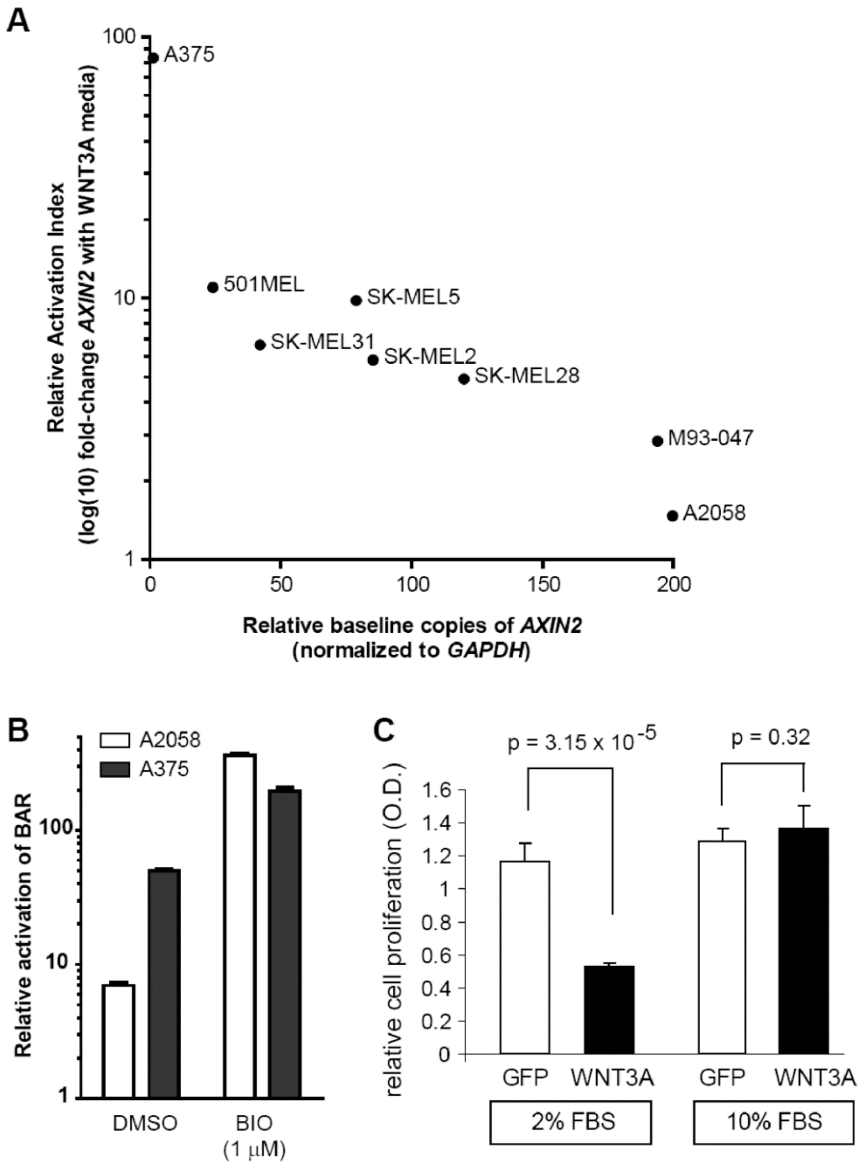

Figure 30: Melanoma cells exhibit a wide range of response to WNT3A.

(a)This plot reveals variation in both baseline *AXIN2* transcript levels as well as variations in the degree of response to exogenous WNT3A ligand in a panel of established human melanoma cell lines. The relative activation index represents (*AXIN2* levels with WNT3A/*AXIN2* levels with control media). Note that the y-axis is logarithmic in scale, showing a wide range of BAR activation across melanoma cell lines with WNT3A. **(b)** This graph shows two melanoma cell lines that exhibit markedly different responses to WNT3A as measured by a luciferase-based β-catenin-activated reporter (BAR). While the A2058 cells exhibit an almost ten-fold less activation of the reporter by WNT3A, both cell lines exhibit comparable levels of BAR activation with the small-molecule GSK3 inhibitor BIO. These data suggest that cell-specific determinants of WNT/β-catenin signaling are proximal to GSK3B and likely mediated at the level of ligand-receptor interactions, potentially through variations in the expression of FZD or other receptor isoforms. **(c)**This graph shows cellular proliferation measured using the colorimetric tetrazolium dye MTT. Reduction of MTT in proliferating cells is measured by an increase in O.D. Murine B16 cells expressing WNT3A exhibit a reduction in proliferation in 2% serum, paralleling the decrease in tumor growth observed in vivo. Increasing serum to 10% can overcome the effects of WNT3A, potentially due to the negative regulation of WNT/β-catenin signaling by MAPK signaling. This finding illustrates how variations in cell culture could account for some of the differences in phenotype reported in melanoma cells with WNT/β-catenin activation.

Functional relevance of WNT signaling pathways in melanoma

The role of β-catenin in transgenic mouse melanoma models

Transgenic mouse models have played an important role in characterizing the role of WNT/β-catenin signaling in melanoma initiation and progression. Researchers have generated transgenic mice engineered for melanocyte-specific expression (i.e. driven by the tyrosinase promoter) of a non-degradable constitutively-active form of β-catenin (β-cat^{STA}; containing alanine substitutions at Ser33, Ser37 and Thr41) to address the effects of β-catenin hyper-activation on melanoma formation and progression. Melanocyte-specific expression of β-cat^{STA} alone does not lead to spontaneous melanomas in mice (Delmas et al., 2007). However, double transgenic mice expressing both β-cat^{STA} and activated mutant Nras in melanocytes results in melanoma tumor formation at reduced latency and in enhanced tumor growth[177].
A second study similarly finds that melanocyte-specific expression of β-cat^{STA} alone fails to induce melanomas, whereas co-expresion of β-cat^{STA} in the presence of either a constitutively active mutant form of Braf or in the presence of inactivating mutations in Pten results in melanoma tumor formation[155]. Together, these studies suggest a model in which the hyper-activation of β-catenin promotes or enhances the ability of mutational perturbations in the BRAF/NRAS/MAPK and the PTEN/PI3K/AKT signaling pathways to drive melanoma tumorigenesis.

Although these mouse models of melanoma initiation and progression provide important insights regarding the role of β-catenin signaling, there are several potential caveats to these experimental paradigms. First, these genetic models rely on the expression of a β-cat^{STA} despite the rarity of these mutations in patient tumors[328,330,339]. Second, it is uncertain how closely the expression of a constitutively active mutant form of β-catenin (β-cat^{STA}) recapitulates the varied activity of the WNT/β-catenin signaling pathway observed in patients[340]. Finally, mice expressing melanocyte-specific β-cat^{STA} and mutant Nras develop melanomas arising from the bulge region of the hair follicle rather than from the interfollicular epidermis where most human melanomas originate[177]. Species-specific differences in the distribution of melanocytes may partially account for the contrasting pathological features observed in mouse models of melanoma compared to patient tumors [341]. Despite these caveats, the data from these mouse models provide important insights into the potential role of hyper-activated β-catenin signaling in melanomagenesis, particularly with regards to potentially synergistic effects of hyper-activated β-cat^{STA} and other major signaling networks.

Studies of WNT/β-catenin signaling using melanoma cell lines

There exists no consensus on which melanoma cell lines are appropriate representative laboratory models for this disease, so it comes as no surprise that studies in the literature use a broad variety of cell lines whose contextual similarities and differences are not yet fully defined, particularly in regards to WNT signaling. Cellular responses to exogenous WNT ligands can vary

widely between different melanoma cell lines (Figure 30a). The molecular origins of this variability remain unclear, but may involve differences in the expression of WNT receptors which include ten different FZD isoforms, the LRP5/6 co-receptors, and additional receptors such as, ROR1/ROR2, and RYK. Potential hetero-multimerization of various FZD isoforms with each other and with other co-receptors adds further complexity to the theoretical WNT ligand and WNT receptor interactions that may exist in different cell lines. While these hypotheses have not been directly tested using numerous melanoma cell lines, we have observed that melanoma cells exhibiting disparate responses to WNT3A can achieve similar levels of pathway activation using a GSK3 inhibitor (Figure 30b), which suggests that certain cell lines lack critical factors necessary for WNT3A-dependent signaling acting upstream of GSK3β and the β-catenin destruction complex. These studies raise the possibility that variable responses to stimulation by various WNTs may reflect different receptor expression levels or activity in different melanoma cell lines.

Keeping these limitations in mind, the studies to date using established melanoma cell lines have yielded interesting and unexpected results regarding the consequences of WNT/β-catenin activation. The B16 murine melanoma cell line represents far and away the most highly utilized and published melanoma cell line in the literature. Because they were derived from spontaneous tumors arising in the commonly used C57BL/6 mouse, B16 melanoma cells can be readily engrafted in an isogenic host background for in vivo studies of tumor growth and metastasis. Studies of WNT signaling using this model have found somewhat conflicting results. Forced overexpression of β-catenin enhances the proliferation of B16 cells, which can be

inhibited by expression of a dominant negative form of TCF7L2 that can inhibit the transcription of a subset of β-catenin target genes[342]. The enhancement of B16 proliferation induced by β-catenin also requires the expression of the transcriptional regulator Mitf, a WNT/β-catenin target gene in itself[342]. These data suggest that β-catenin can promote B16 proliferation by activating a gene expression network driven by the TCF7L2 and MITF transcription factors. Consistent with these initial observations, shRNA-mediated knockdown of Ctnnb1 inhibits the proliferation of B16 cells[343]. By contrast, forced expression of WNT3A in the B16 model leads to decreased cell proliferation in vitro and decreased tumor growth in vivo[41]. These studies reveal that activation of WNT/β-catenin signaling by either overexpressed β-catenin or by WNT ligand stimulation in melanoma can result in distinct cellular responses. One possible explanation for these seemingly contradictory results is that the use of overexpressed β-catenin circumvents the effects of WNT stimulation on GSK3β and other components of the destruction complex, which may have downstream effects on cellular process that are independent of β-catenin, but still activated by a WNT ligand. It is also possible that overexpression of β-catenin or WNTs can result in differences in either the amplitude of duration of pathway activation, which may result in distinct signaling outputs.

In contrast to the isogenic B16 melanoma model, the experimental assessment of WNT signaling in human-derived melanoma cell lines in vivo requires the use of immunocompromised mouse models for xenograft studies. The lack of immune function in host animals may affect how faithfully this model system recapitulates the development and treatment of human melanoma. Despite this important caveat, some studies suggest that

activation of WNT/β-catenin signaling may inhibit the growth of xenografted human melanoma cells. Specifically, forced expression of either WNT3A or a mutationally-stabilized β-catenin leads to decreased tumor size, paralleling results seen in vitro[85,344]. Whether and how these observations relate to the increased survival observed in patients with tumors expressing elevated cytosolic and nuclear β-catenin[41,322,325] is still ambiguous.

Recently, our group also found that activation of WNT/β-catenin signaling can enhance the apoptotic response of certain melanoma cell lines to targeted inhibitors of mutant active BRAF or MEK[85,345]. The ability of WNT/β-catenin signaling to enhance apoptosis in the presence of BRAF inhibitors involves the dynamic regulation of AXIN1 protein abundance. Although some melanoma cell lines do not respond to combined treatment with WNT3A and BRAF inhibitors, we find that knockdown of AXIN1 by siRNA can confer the apoptotic response. This unexpected finding raises the possibility that crosstalk between WNT/β-catenin and BRAF/MAPK signaling can provide an avenue for developing either prognostic or treatment strategies aimed at optimizing the targeted BRAF therapies that are currently used as a first-line therapy for patients with tumors harboring activating BRAF mutations[346]. We have also generated data suggesting that variations in serum levels in cell culture media can potentially inhibit or overcome the functional effects of WNT/β-catenin activation (Figure 30c). As different serum levels can affect the level of BRAF/MAPK and PI3K/AKT pathway activities, these results argue that potential interplay between WNT signaling and other kinase-dependent signaling pathways must be carefully considered in order to accurately interpret the results of experiments characterizing WNT signaling in melanoma cell lines.

WNT signaling pathways and melanoma metastasis

If cutaneous melanomas are identified and removed prior to metastatic dissemination patient survival is very high ordering on ~90%, which is in stark contrast to the extremely poor patient survival associated with the development metastatic melanoma[318]. Several independent studies find that β-catenin inhibits the migration of melanoma cell lines in vitro [153,154]. However, the presence of β-cat^{STA} (but not wild-type β-catenin or knock-out of β-catenin) was associated with increased metastasis to the lymph nodes and lungs in transgenic mouse models of melanoma[155]. Interestingly, one study directly compared the effects of β-catenin expression on melanoma cell behavior both in culture and in xenografts. This particular study finds that β-cat^{STA} inhibits the migration of melanocytic cells in culture, while at the same time promotes melanoma metastasis in mouse models when combined with mutationally-activated Nras[154]. These results indicate that changes in cell motility mediated by activated β-catenin do not always directly correlate with metastatic potential. One possible explanation for these discrepancies is that WNT/β-catenin signaling mediates complex cell-cell interactions necessary for metastatic progression in animal models but not for the regulation of cell migration and invasiveness in vitro.

Studies indicating that WNT5A expression is increased in metastatic melanoma cell lines and tumors compared to primary tumors [42,43,334] implicate WNT5A-dependent signaling in the regulation of the metastatic process. During embryogenesis, WNT5A regulates the

establishment of cellular polarity and cell motility in many different tissues and developmental contexts [347–350]. Consistent with studies observing elevated expression of WNT5A in metastatic melanoma and with studies characterizing a functional roles for WNT5A in promoting cell migration, follow-up studies have shown that WNT5A can correspondingly increase melanoma cell motility in vitro [43,351,352]. Importantly, one study finds that systemic injections of recombinant WNT5A (rWNT5A) results in the increased engraftment and growth of B16 melanoma cells injected through the tail vein, providing the first in vivo evidence that WNT5A may regulate the initiation or progression of metastatic lesions [353].

The molecular events required for WNT5A-dependent induction of cell motility and metastasis are not yet entirely clear, but likely involve the activation of various kinase-dependent signaling pathways. WNT5A-induced invasiveness of melanoma requires the phosphorylation of PKC [43]. Recent studies also highlight an important role for the tyrosine kinase-like WNT receptor ROR2 in mediating metastatic cell behaviors. Specifically, siRNA-mediated reduction of Ror2 in B16 mouse melanoma cells reduces the frequency and severity of lung metastases in mice [166]. Collectively, these observations suggest that increased WNT5A can enhance cellular motility and experimental metastasis, providing a possible model to explain why elevated levels of WNT5A are observed in late-stage metastatic melanomas.

WNT signaling and melanoma heterogeneity

Studies in developmental models find that activation of WNT/β-catenin signaling plays a critical role in regulating the differentiation of neural crest stem cells in a time and cell-type dependent manner [354–356]. In melanoma, activation of WNT/β-catenin signaling by either WNT3A or by GSK3β inhibition leads to enhanced pigmentation accompanied by the up-regulation of cellular genes associated with melanocyte differentiation such as Silv, Mlana and Kit, suggesting that WNT/β-catenin pathway activation can result in a fundamental change in the differentiation state of a melanoma cell population[41,357]. The expression of these differentiation-associated genes in B16 cells can be antagonized by WNT5A[41,353], suggesting that melanoma cell fate may be regulated by a balance between WNT/β-catenin and β-catenin-independent WNT signaling (discussed further below).

In heterogeneous tumors, it is likely that regulation of WNT/β-catenin and β-catenin-independent signaling is temporally and spatially complex. Several gene expression profiling studies now suggest that melanomas can be categorized into various subtypes (i.e. proliferative, metastatic, melanocyte differentiated, neuronal-like etc.) based on hierarchical clustering of gene expression profiles obtained from patient samples and from melanoma cell lines[23,38,39]. For example, a two-state model as proposed by Hoek and colleagues suggests that melanomas dynamically transition between more proliferative and more invasive phenotypes (Hoek et al., 2008). In this analysis, more invasive cells exhibited a gene signature with relatively higher levels of WNT5A and lower levels of genes suggestive of WNT/β-catenin signaling

activation and melanocyte-like differentiation. A related study finds that changes in gene expression profiles corresponding with either a more differentiated/proliferative state or a more motile/invasive state are regulated by the abundance of the β-catenin transcriptional co-regulators LEF1 and TCF7L2, respectively[360]. One potential problem with this two-state model is that it predicts that cells with elevated WNT/β-catenin signaling might be more proliferative. Unfortunately, this prediction is not supported by functional studies showing that activation of the WNT/β-catenin pathway can inhibits human melanoma cell proliferation in vitro and in vivo[41,344]. Together, these studies highlight some of the difficulties in unifying the existing data on WNT/β-catenin signaling in melanoma into a model which is remains pertinent to multiple contexts and model systems.

More recently, transcriptional profiling studies revealed that melanoma cell resistance to the clinically-used targeted BRAF inhibitor PLX4032 (marketed clinically as Vemurafenib) correlates with relatively higher levels of WNT5A expression, whereas the expression of CTNNB1 (which encodes β-catenin) and several WNT/β-catenin target genes negatively correlate with PLX4023/Vemurafinib resistance [361]. Given that β-catenin-independent WNT signaling can antagonize WNT/β-catenin signaling, the consistent observation of increased WNT5A expression in later-stage, more invasive melanomas may indicate a potentially complex interplay between known arms of WNT signaling[362]. In summary, these studies suggest that complex changes in the expression of components of both β-catenin-dependent and β-catenin independent signaling pathways may result in complex phenotypic changes in melanomas, which may be clinically relevant given the heterogeneous and beguilingly unpredictable

responses of melanomas to current therapies.

Cell non-autonomous effects of WNT/β-catenin signaling in melanoma

WNT/β-catenin signaling can also regulate other elements of the tumor microenvironment, with important consequences for tumor formation and host response. Overexpression of WNT1 in human melanoma cells leads to decreased lymphangiogenesis in mouse xenograft tumors, likely through the regulation of VEGF-C expression[363]. Interestingly, this regulation is independent of both GSK3β and β-catenin, suggesting that WNT ligands can functionally regulate tumor cell phenotypes outside of the existing canonical pathway model. In another study, forced activation of WNT/β-catenin signaling in human melanoma cells inhibits the ability of dendritic cells to activate T cells, suggesting that the presence of active WNT/β-catenin signaling in tumors may be immunosuppressive[344]. The regulation of the immune-modulating molecule CTLA4 in melanoma cells by WNT/β-catenin signaling further demonstrates the potential impact that activation of this pathway in melanoma cells can have on the overall tumor environment [364]. As our models for melanoma become more sophisticated, we will undoubtedly uncover other aspects of tumor biology that help clarify how the regulation of WNT/β-catenin signaling in laboratory studies relates to the observed associations between WNT/β-catenin signaling and patient prognosis.

Conclusions

The potential importance of WNT/β-catenin signaling as well as β-catenin-independent signaling in melanoma is reminiscent of these signaling pathways' context-dependent roles in regulating cell fate and migration during embryogenesis. Accumulating data from mouse and cell line models continue to refine our understanding of how WNT signaling pathways analogously regulate melanoma cell growth, cell fate, and metastatic spread in a context-dependent manner. Numerous studies indicate that altered expression of WNT pathway components correlate with patient outcomes in melanoma, raising the possibility that targeting WNT signaling could be advantageous in the clinic. However, targeting WNT signaling in melanoma is still challenging as neither the functional relevance of WNT signaling pathways, nor the mechanisms of activation and inactivation of these signaling networks are completely understood. Overall, current evidence suggests that any attempts to target WNT signaling in melanoma and other disease models will have to take into account the effects of cellular, temporal and spatial contexts on the regulation of this pathway, as well as the interplay between WNT signaling pathways and other signaling networks.

Chapter 5: A WNT5A/FZD7/RYK pathway promotes AKT activity and the growth and survival of both naïve and BRAF inhibitor-resistant melanoma cells

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Abstract

Inhibitors of constitutively active mutant $BRAF^{V600E}$ kinases have recently shown some promise in treating patients with metastatic melanomas. Treatment with BRAF inhibitors (BRAFi) such as Vemurafinib (PLX4032) can result in short-term improvement characterized by a dramatic reduction in tumor burden. Unfortunately, a vast majority of patients relapse within six months. We report that chronic treatment of melanoma cells with BRAFi results in dramatic increases in levels of a secreted signaling protein, WNT5A in both resistant cells lines derived in vitro and in patient samples following relapse. We find that endogenous WNT5A promotes the growth and survival of both naïve melanoma and BRAFi-resistant melanoma cells and that exogenous WNT5A can enhance melanoma tumorigenesis in xenografts. Mechanistically, this increased cell proliferation and survival correlates with WNT5A enhancing the activity of AKT, a critical growth factor in melanoma. We then find that the WNT5A receptors FZD7 and RYK similarly promote the growth of both naïve and BRAFi-resistant melanoma cells as well as AKT phosphorylation. Together, our results identify a WNT5A/FZD7/RYK/AKT pathway as a key determinant of the viability of both naïve and therapy-resistant melanoma cells.

Introduction

Melanoma cells rely on the hyper-activation of the RAS-RAF-MEK-ERK signaling pathway for their growth and survival. Activation of this signaling pathway is traceable to a highly specific mutations in a single protein kinase, $BRAF^{V600E}$, and $BRAF^{V600K}$ in approximately 50% of melanomas[365,366]. Vemurafinib, (also known as PLX4032), specifically targets mutant $BRAF^{V600E}$ and blocks the activation of ERK[367,368]. In clinical trials treatment with BRAF inhibitors (BRAFi) initially reduces tumor burden and increases the survival of melanoma patients[369–371]. Unfortunately, these patients invariably develop resistance to BRAFi and subsequently relapse[346,369–371]. Mechanistically, BRAFi-resistant melanoma cells often exhibit re-activation of MAPK signaling, which can be driven by somatic mutations in RAS-RAF-MEK-ERK pathway proteins such as, MEK and N-RAS[372,373], and by splice variants in BRAF[374]. In addition to the presence of additional somatic mutations, BRAFi-resistant melanoma cells often display aberrations in the expression or activity of mitogenic proteins such as the receptor tyrosine kinases, PDGFRβ,VEGFR, EGFR, and IGFR[372,375,376], as well as changes in the expression of pro- and anti-apoptotic proteins[377–379].

Previously, high levels of WNT5A have been shown to correlate with metastasis and patient deaths in melanoma[42–44,380]. In this study we explore a novel role for WNT5A-dependent signaling in mediating melanoma cell proliferation and viability. We find that WNT5A protein and transcript levels are dramatically increased in both BRAFi-resistant melanoma cells that have been chronically treated with PLX4720 and in patient tumors following relapse occurring

after BRAFi treatment. We go on to characterize the functional relevance of WNT5A signaling and find that endogenous WNT5A promotes the growth and survival of both naïve melanoma cells that have not been treated with BRAF inhibitors and similarly promotes the proliferation of melanoma cells which have acquired resistance to the BRAFi PLX4720. WNT5A also promotes the clonogenic growth of single melanoma cells and delays the onset of tumorigenesis in xenograft models.

In pursuing the underlying mechanisms that might account for these effects of WNT5A on melanoma cells, we find that WNT5A likely promotes melanoma cell viability through the enhancement of PI3K/AKT signaling, another critical pathway in melanoma proliferation and survival[381,382]. Furthermore, we find that WNT5A likely promotes melanoma growth and survival via its receptors RYK and FZD7. We find that RYK and FZD7 associate in a protein complex, enhance AKT signaling, and support the growth and viability of both naïve and BRAFi-resistant melanoma cells in culture.

Results

Chronic inhibition of $BRAF^{V600E}$ with PLX4720 results in increased WNT5A expression

Previous expression microarray profiling studies find that melanoma cell lines that are inherently insensitive to low doses of the BRAF inhibitor PLX4032 express high levels of WNT5A[361]. In the present study, we asked whether WNT5A expression is similarly increased in melanoma cells exhibiting de novo resistance to chronic $BRAF^{V600E}$ inhibition. We chronically treated both A375 and MEL624 cells with 2 μM PLX4720 for more than eight weeks to generate resistant cell lines (subsequently referred to as A375-R and MEL624-R). We validated the resistance of these cells to $BRAF^{V600E}$ inhibition by treating equal numbers of parental or resistant cells with increasing doses of PLX for three days and then determining cell viability. PLX4720 dose-dependently decreases the viability of parental cells as previously reported, yet does not affect the viability of either A375-R or MEL624-R cells confirming that these cells are indeed resistant to $BRAF^{V600E}$ inhibition (Figure 31a).

We determined the expression of WNT5A protein in both parental and resistant cell lines by western blotting and observe a dramatic increase in WNT5A abundance in the resistant cells (Figure 31b, compare lanes 1-3 to lanes 4-6, and lanes 7-9 to lanes 10-12).

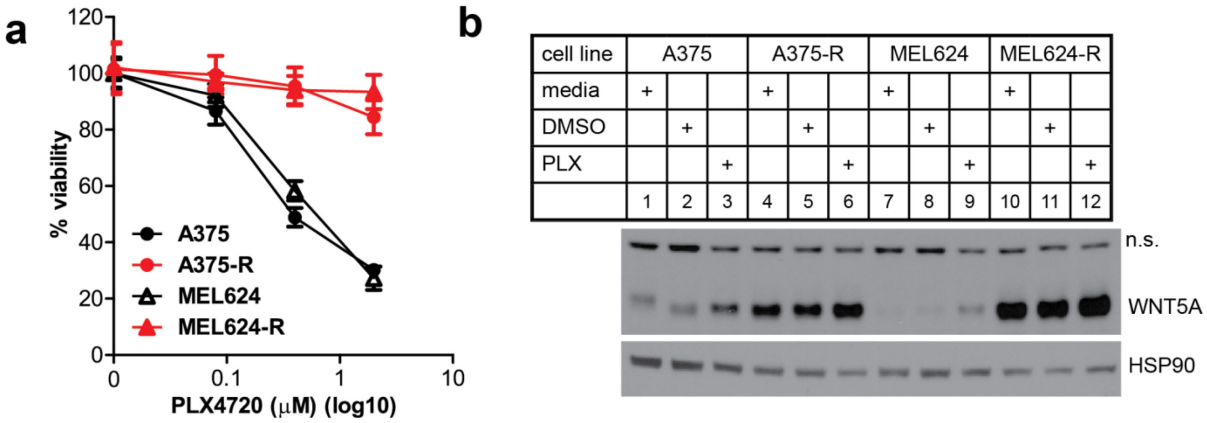


Figure 31: WNT5A protein is increased in response to chronic inhibition of BRAF^{V600E} with PLX4720.

(a-b) Normalized viability of (a), A375 and (b), MEL624 parental and PLX-resistant cells (-R) as determined by rezaurine viability dye after treatment with either media, DMSO, or increasing doses of PLX4720 for 48 hours. Resistant cell lines (A375-R and MEL624-R), were derived by treating melanoma cells with 2μM PLX4720 for more than 6 weeks. (c) Western blots of lysates from A375, A375-R, MEL624, and MEL624-R cells after culturing in either standard growth media or treatment with DMSO or 2 μM PLX4720 for two days.

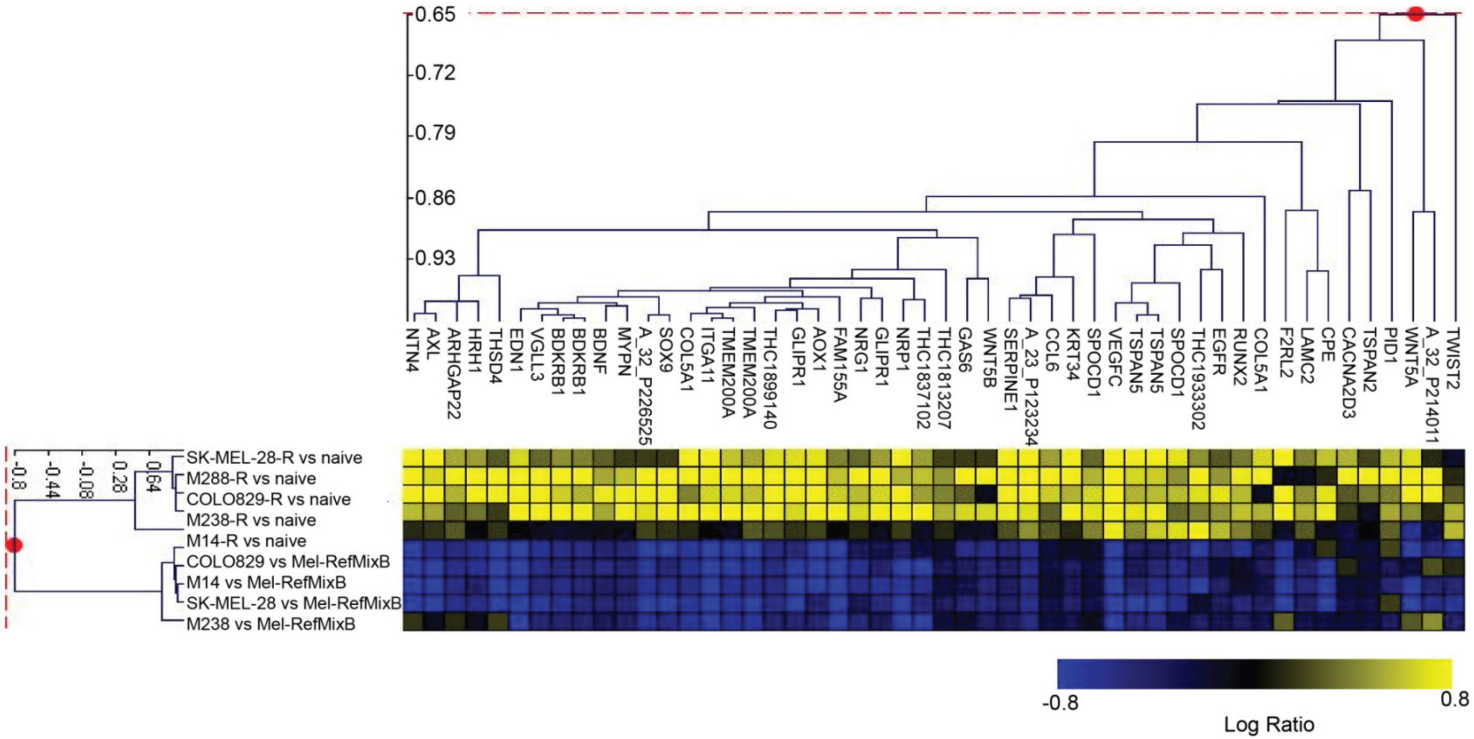


Figure 32: Heatmap representation of genes with similar expression patterns to WNT5A in BRAFi-resistant melanoma.

Hierarchical clustering of gene expression in PLX-resistant (R) and PLX naïve cells displaying the subset of genes that significantly correlate with *WNT5A* expression. The top five rows represent relative expression changes in naïve cell lines compared to BRAFi-resistant (-R) cell lines generated by chronic treatment with PLX4032. The bottom five rows display the relative expression of these genes in naïve melanoma cells in comparison to a reference dataset determined as an average of the expression profiles of 47 different melanoma cell lines (Mel-RefMixB).

Returning the resistant cells to either normal media or media containing vehicle (DMSO) does not diminish the observed high WNT5A protein abundance, suggesting that increased expression of WNT5A in resistant cells does not require continual presence of BRAF inhibitors (Figure 31b, compare lane 6 to lanes 4,5 and lane 12 to lanes 10,11). WNT5A protein is similarly increased following knockdown of BRAF with siRNA, which indicates that loss of BRAF activity rather than some off target effect of PLX4720 treatment results in the up-regulation of WNT5A (Figure 35d).

Since cancer cells respond heterogeneously to drug treatment we then asked whether WNT5A levels were altered in additional melanoma cells as a consequence of long-term $BRAF^{V600E}$ inhibition using microarray profiling to analyze expression changes in COLO829, SK-MEL-28, M288, M233 and M14 melanoma cells chronically treated with PLX4032 (Figure 32). The microarray analysis of gene expression reveals that WNT5A expression is low or undetectable in the naïve cell lines (Figure 32), which is consistent with our analysis of WNT5A expression in selected cell lines such as COLO829 and SK-MEL-28 cells by RT-PCR (Figure 33a). In contrast to these naïve cell lines, we find that WNT5A expression is strongly induced in four out of five additional BRAFi-resistant cell line derivatives (COLO829, SK-MEL-28, M288, and M233, but not M14 cells) (Figure 32). Our other results indicate that WNT5A expression is further increased in BRAFi-resistant derivatives of melanoma cells that had expressed relatively high levels ofWNT5A prior to BRAFi treatment such as MEL624 and A375 cells (Figure 33 and Figure 31b).

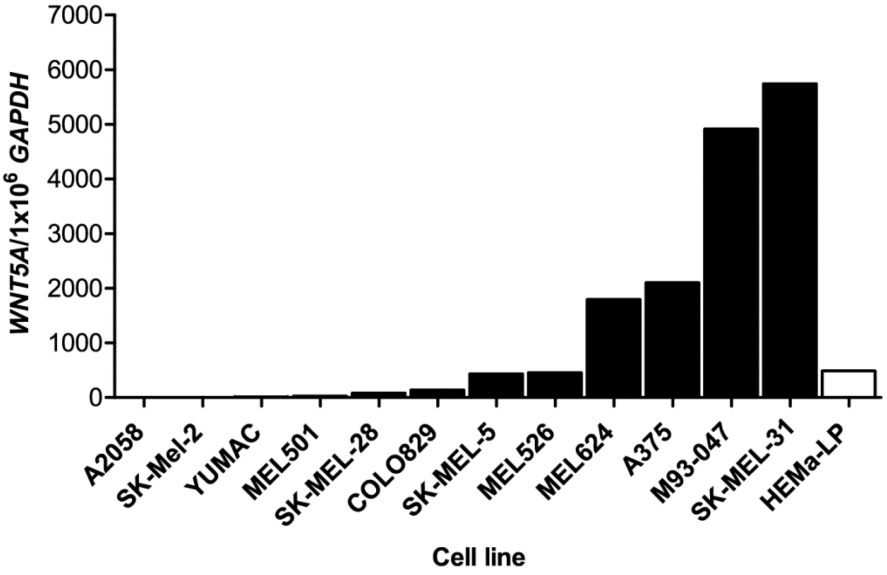


Figure 33: Expression of endogenous *WNT5A* in melanoma cells.

WNT5A transcript expression normalized to *GADH* in various melanoma cell lines (black bars) and HEMa-LP (melanocytes) determined by RT-PCR.

Here, we find that WNT5A expression is similarly increased in additional BRAFi-resistant melanoma cell lines such as SK-MEL-28 and COLO829 cells (Figure 32) that had expressed low levels of WNT5A prior to chronic treatment with treatment with BRAFi (Figure 32 and Figure 33).

We further analyzed the gene expression profiles of these BRAFi-resistant cells in order to identify additional transcripts with expression profiles similar to WNT5A. The top 50 genes significantly correlating with WNT5A expression include transcription factors 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. These results suggest that WNT5A is up-regulated as part of a unique gene expression signature observed in multiple BRAFi-resistant human melanoma.

It is formally possible that WNT5A expression is increased in these melanoma cell lines following chronic treatment with BRAFi due to some artifact of in vitro cell culture. We then asked if WNT5A expression is similarly increased in melanoma tumors from patients who have developed resistance to $BRAF^{V600E}$-targeted therapies. Indeed, WNT5A expression is increased in a subset of BRAFi-resistant samples (7/11 analyzed) (Figure 34b). Taken together, these studies reveal that WNT5A expression is induced in both melanoma cell lines and in patient tumors chronically treated with BRAFi. These data raise the possibility that WNT5A may play an important role in the biology of therapy-resistant melanomas.

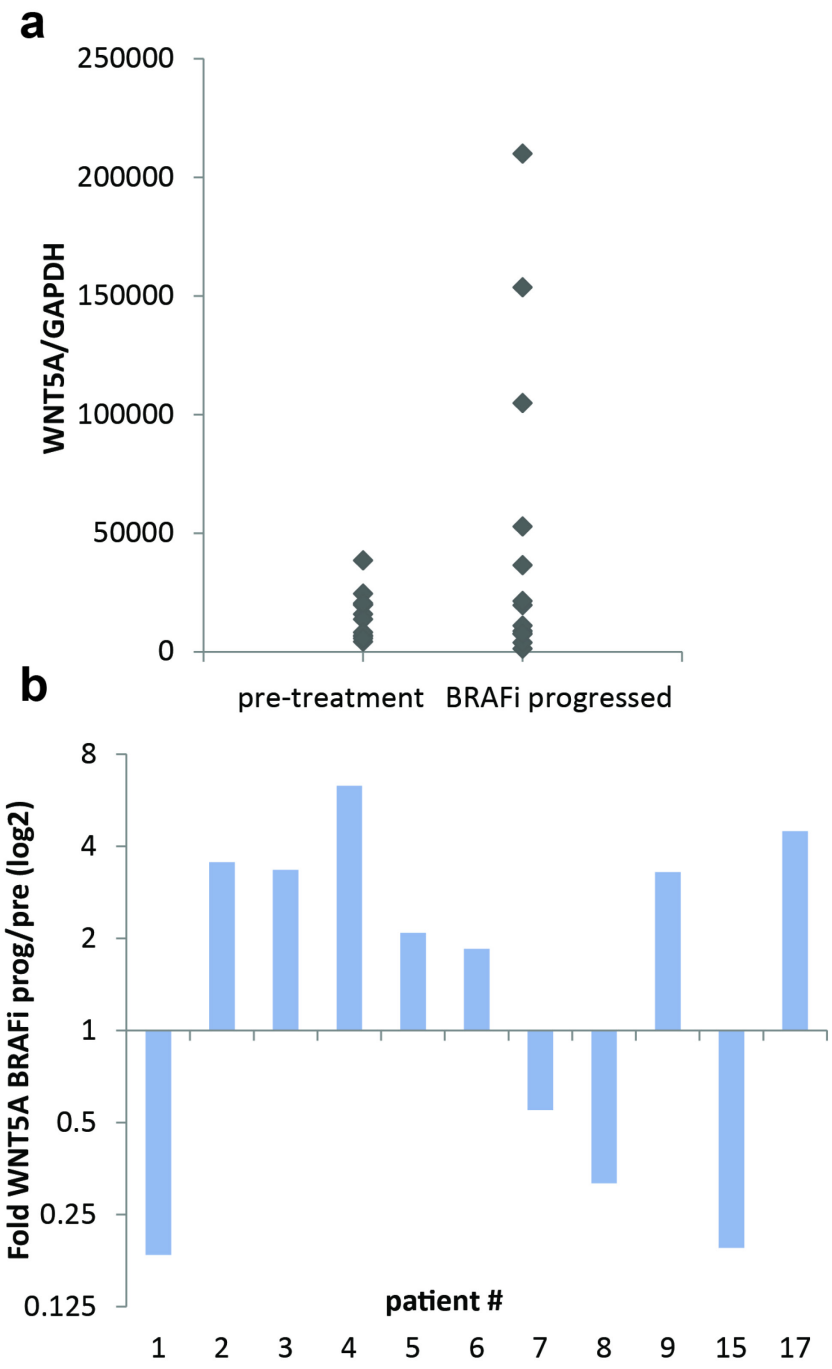


Figure 34: Normalized expression of WNT5A in clinical specimens from PLX4720 trial.

The expression of WNT5A in pre-treated patient tumors (Pre), and in tumors with progressive disease following relapse after long term treatment with the BRAFi, PLX4720 (BRAFi Prog). (**a**) Normalized expression of WNT5A in unpaired melanoma samples. (**b**) Fold change in WNT5A expression in BRAFi progressed samples compared to tumor samples taken prior to treatment initiation.

WNT5A expression is not induced due to apoptosis, but is induced by WNT3A, an activator of β-catenin-dependent transcription

So far our results indicate that WNT5A expression is induced in six out of seven human melanoma cell lines analyzed as well is in a subset of BRAFi resistant patient tumors. We then sought to determine possible factors leading to increased expression of WNT5A in these resistant cells. We reasoned that apoptosis induced in a subset of BRAFi resistant cells might result in the increased expression of WNT5A. To test this hypothesis we asked whether WNT5A expression is also following short term treatment with various factors that can lead to robust apoptosis. Our previous study finds that co-treatment with PLX4720 and WNT3A, which activates β-catenin-dependent transcription, can synergize to induce high levels of apoptosis in selected melanoma cell lines as indicated by an increased percentage of cleaved PARP1+ cells, an apoptotic maker (data reproduced in Figure 35b)[85]. Our analysis of WNT5A expression following treatment with PLX4720, WNT3A, or combinations of these factors indicates that WNT5A is not robustly increased following 24 hour treatment with PLX4720 alone (Figure 35a, black bars), but was increased in several cell lines (A375, SK-MEL-28, SK-MEL-5, and MEL624) following treatment with WNT3A alone (Figure 35a, green bars). Importantly, WNT5A expression remained low when cells were treated with both WNT3A and PLX4720 (Figure 35a, blue bars).

We also analyzed WNT5A protein levels in A375 (lanes 1-7) and MEL624 cells (lanes 8-14) following treatment withPLX4720 and WNT3A alone, or in combination (Figure 35c). Again, we find that co-treatment with PLX4729 and WNT3A induces rampant apoptosis as indicated by high levels of cleaved PARP1 (lanes 7 and 14), yet WNT5A protein abundance is not altered by these treatments (Figure 35c, compare lane 4 to lane 7 and compare lane 11 to lane 14). Consistent with our analysis of WNT5A mRNA expression (Figure 35a, compare white and green bars), we also note that WNT5A protein is induced by short term treatment with WNT3A. These findings are consistent with previous studies indicating that WNT5A is regulated by β-catenin and TCF/LEF-dependent transcription[362].

In summary, these studies reveal that combined treatment of melanoma cells with WNT3A and PLX4720 induces the greatest percentage of apoptotic cells (Figure 35b,c), yet does not induce increased expression of WNT5A (Figure 35a,c) suggesting that WNT5A is not up-regulated as a general consequence of apoptosis. One possible interpretation of these results is that increased levels of WNT5A observed in BRAFi-resistant cells (Figure 31 and Figure 32) occurs due to the selection of a small subset of BRAFi-insensitive cells from a heterogeneous starting population following chronic treatment with PLX4720 (see schematic in Figure 36). Unexpectedly, we also find that WNT5A can be induced by WNT3A treatment alone. Whether WNT/β-catenin signaling is also required for the induction of WNT5A observed in BRAFi-resistant cells and patient tumors has not yet been determined.

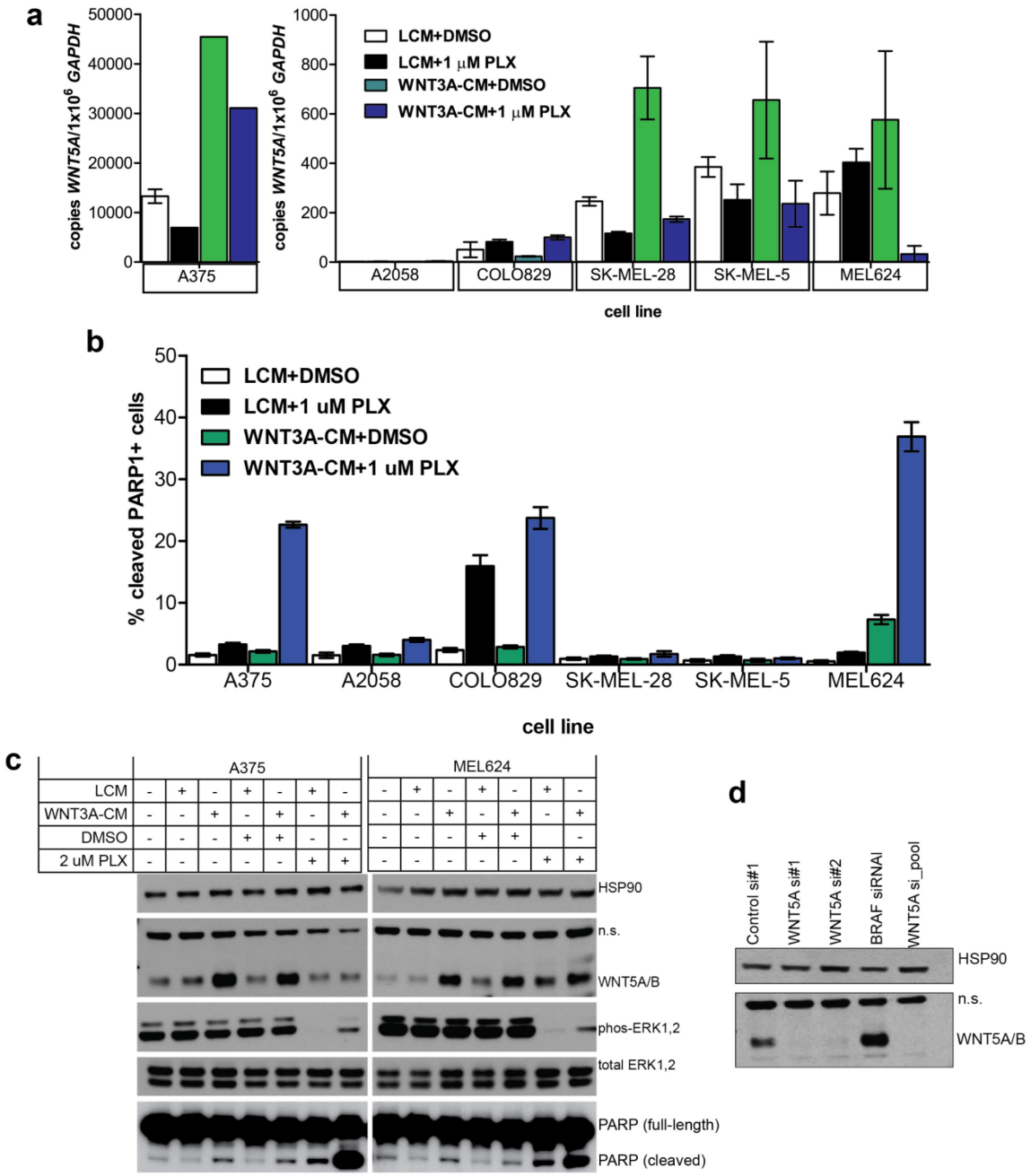


Figure 35: WNT5A expression is not increased due to apoptosis in melanoma cells

(a) Expression of *WNT5A* in multiple melanoma cell lines treated with either DMSO, control conditioned media (LCM), WNT3A conditioned media (WNT3A-CM), and 1 μM PLX4720. (b) Percentage of cleaved PARP+ apoptotic cells observed in multiple melanoma cell lines treated as described in (a). (c) Immunoblot analysis of lysates from A375 (lanes 1-7) and MEL624 cells (lanes 8-14) following treatment with combinations of WNT3A-CM and PLX4720. These protein lysates were probed with antibodies to detect HSP90, WNT5A, phospho-ERK1/2, total ERK1/2, and PARP, which is robustly cleaved following co-treatment with WNT3A and 1 μM PLX4720. (d) Immunoblot analysis of whole cell lysates collected three days following transfection with either control, WNT5A siRNAs, BRAF siRNA, or pooled WNT5A siRNAs.

Figure 36: Schematic for selective process that occurs following long-term treatment with BRAFi resulting in the outgrowth of a subset of drug-resistant cells (shown in purple).

WNT5A loss of function reduces melanoma cell growth

One important question posed by our results indicating that WNT5A is up-regulated in BRAFi-resistant melanomas is whether WNT5A functionally contributes to the growth and viability of melanomas. To this end, we determined if knockdown of endogenous WNT5A regulates melanoma growth and viability. For these studies we used two independent WNT5A siRNAs that consistently reduce endogenousWNT5A protein and mRNA expression (Figure 37a,b). WNT5A knockdown inhibits the growth of both A375 and MEL624 cells to a similar degree as BRAF knockdown, which serves as a positive control (Figure 37c,d). To test for the specificity of these siRNAs we performed a rescue experiment by treating siRNA transfected cells with WNT5A conditioned media (-CM). WNT5A-CM partially rescues growth inhibition induced by WNT5A knockdown (Figure 37). Since the growth factor dependence of cancer cells often differs in three-dimensional compared two-dimensional cell culture[383], we then asked if endogenous WNT5A also enhances melanoma growth in anchorage independent growth assays. WNT5A siRNAs reduce colony formation in soft agar by more than 80% in both A375 and MEL624 cells (Figure 38a,b) and similarly reduce the size of melanoma cells grown in 3D as melanospheres (Figure 38c,d).

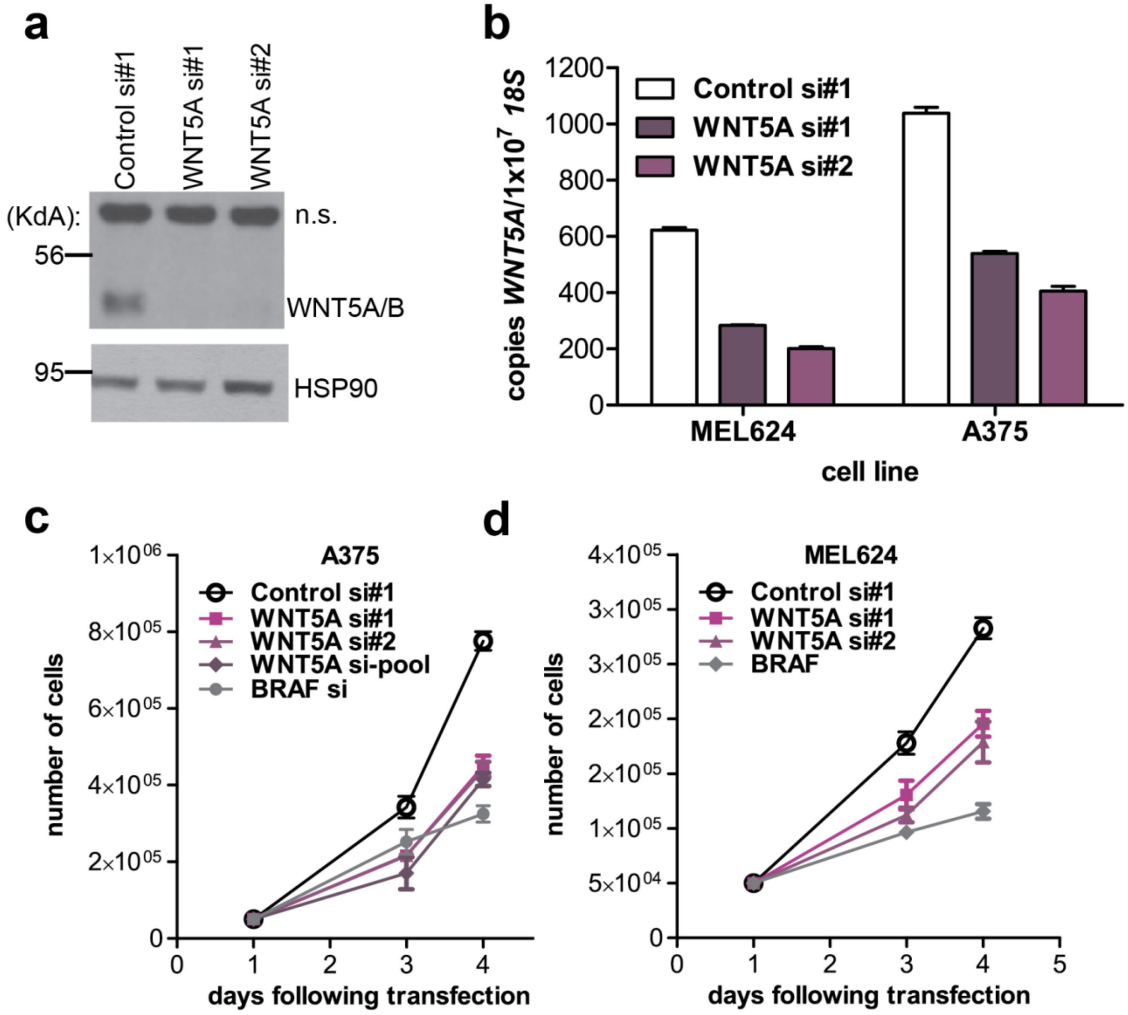


Figure 37: WNT5A loss of function reduces melanoma cell number.

(a) Western blots of A375 cell lysates probed with antibodies to detect WNT5A, and HS90 expression. The WNT5A/B antibody detects a non-specific bad designated as, n.s. (b) *WNT5A* transcript expression normalized to GAPDH in A375 and MEL624 as determined by RT-PCR following transfection with siRNAs (c-d) Growth curves of (c), A375 and (d), MEL624 melanoma cells transfected with Control si#1, WNT5A siRNAs #1, #2, and BRAF siRNA as a positive control. Error bars indicate standard deviation calculated from three independent experiments with triplicated wells counted for each time point. (e) A375 cell number following siRNA transfection and treatment with WNT5A conditioned media (-CM). A375 cells were transfected with siRNAs and then treated with either control or WNT5A-CM two days later for a period of three days and then counted. (***p>0.001, ANOVA).

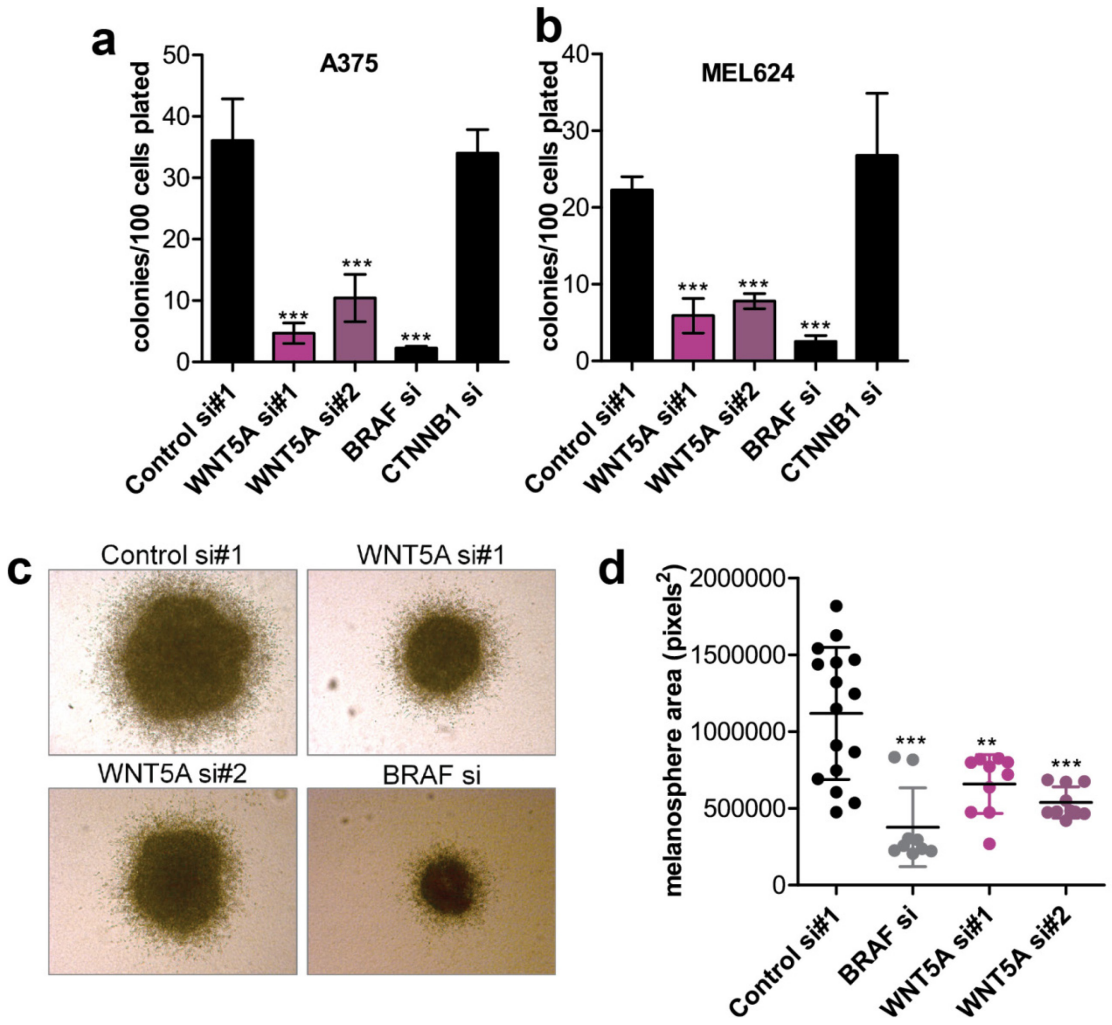


Figure 38: WNT5A loss of function reduces melanoma growth in three-dimensional culture

(**a**-**b**) Average number of colonies per 100 A375, (a) or MEL624, (b) cells plated in soft agar. Colonies were scored 6-7 days following siRNA transfection and embedding in soft agar. Error bars indicate standard deviation calculated from three independent experiments with duplicate wells for each siRNA. (**c**) Representative photographs of A375 cells grown in three-dimensional collagen I culture as described in Materials and Methods following 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 bar indicates the mean melanosphere area and error bars show the standard deviation. See methods for further details on measurement procedure. Statistically significant differences in colony formation in soft agar and in melanosphere size were calculated using One-Way ANOVA. (** $p<0.01$, ***$p<0.001$).

WNT5A loss of function decreases the viability of BRAFi-resistant melanoma cells

We then asked if WNT5A-dependent signaling also contributes to melanoma cell viability in the presence of PLX. Two days after transfecting A375 or MEL624 cells with either control siRNA or WNT5A siRNAs we treated these transfected cells with either DMSO or 2 μM PLX4720 for two days and then quantified the proportion of apoptotic cells. As expected, PLX4720 increases the percentage of apoptotic cells detected by terminal deoxynucleotidyl dUTP nick end labeling (TUNEL+) (Figure 39a) and also increases the abundance of cleaved PARP, an additional marker of apoptosis (Figure 40). Knockdown of WNT5A is not sufficient to induce apoptosis in vehicle-treated cells (Figure 39b and Figure 40). However, WNT5A knockdown enhances apoptosis induced by PLX4720 treatment in both A375 and MEL624 cells (Fig. 3a and Figure 40). Since a relatively small percentage (~10-25%) of melanoma cells undergo apoptosis under these conditions, we also tested whether combining WNT5A siRNAs with BRAFi can reduce the overall viability of melanoma cells. Indeed, combining WNT5A siRNA transfection with PLX4720 treatment further reduces the viability of these cells (Figure 39b,c).

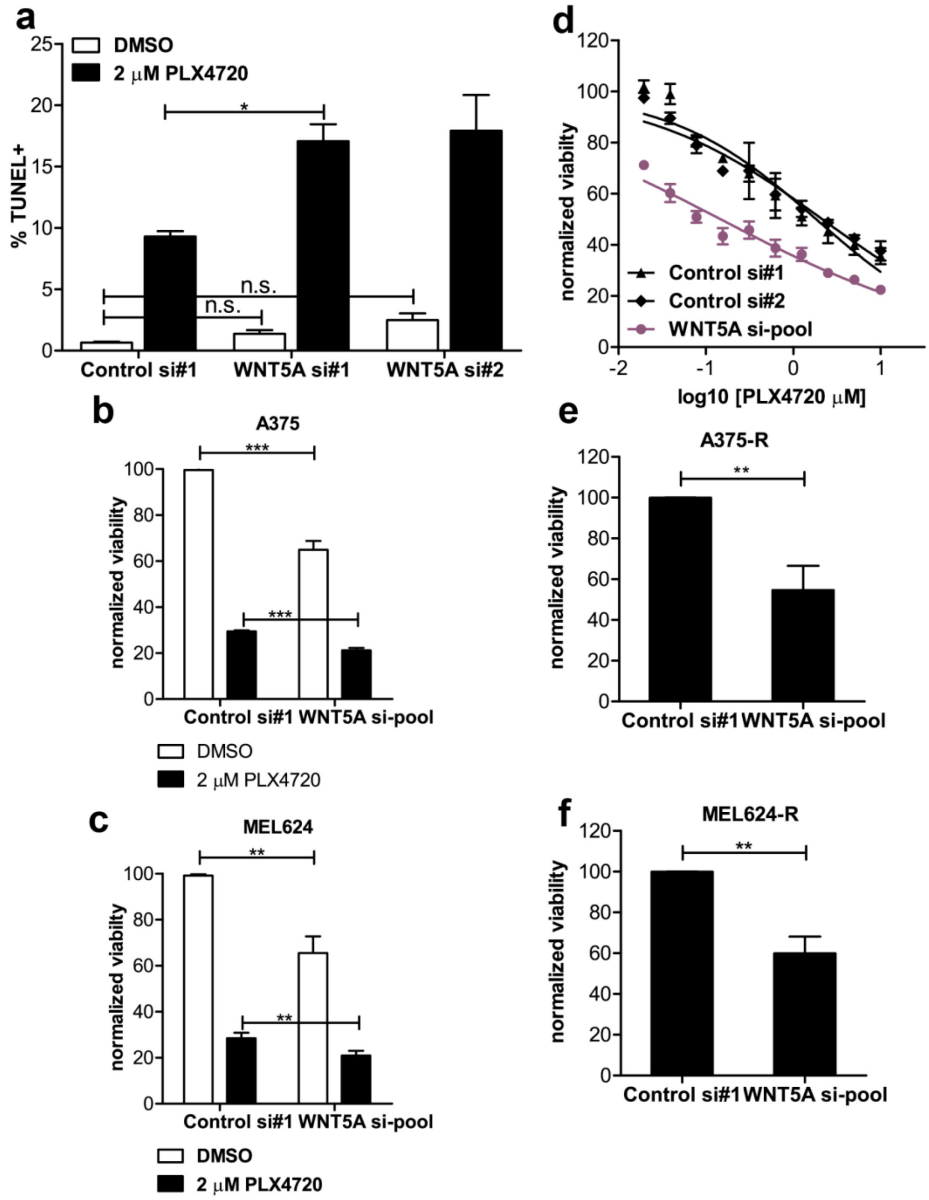


Figure 39: WNT5A loss of function decreases the viability of PLX-resistant melanoma cells.

(**a**) Quantification of the proportion of A375 cells which were apoptotic (TUNEL+) after transfecting these cells with siRNAs and then treating two days later with either DMSO or 2 μM PLX4720 for a total of 48 hours. Error bars indicate the STDEV. (**p*<0.05, Two-tailed T-Test). (**b**-**c**) Normalized viability of (**b**), A375 and (**c**), MEL624 cells following transfection with the indicated siRNAs and then treatment two days later with either DMSO or 2 μM PLX4720 for three days. Data were normalized to Control si#1+DMSO condition set to 100%, and error bars indicate standard deviation of four independent experiments. (**P<0.01 and ***P<0.001). (d) Normalized viability of A375 cells following transfection with either Control si#1, Control si#2, or pooled WNT5A siRNA followed by treatment with increasing doses of PLX4720 for two days. Normalized viability was determined by setting the Mock+DMSO condition at 100% and error bars show standard deviation calculated from three independent experiments. Non-linear best fit curves were obtained using Graphpad software (Control si#1; R^2=0.9472, Control si#^{2}; R^2=0.9144, and WNT5A siRNA pool; R^2=0.9016). (**e-f**) Normalized viability of (**e**), A375-R and (**f**),MEL624-R cells which were plated in media containing 2 μM PLX4720 48 hours following siRNA transfection and allowed to grow for three days. Data were normalized to the Control si#1 condition set to 100%, and error bars indicate standard deviation of three independent experiments. (***p*<0.01, two-tailed T-Test).

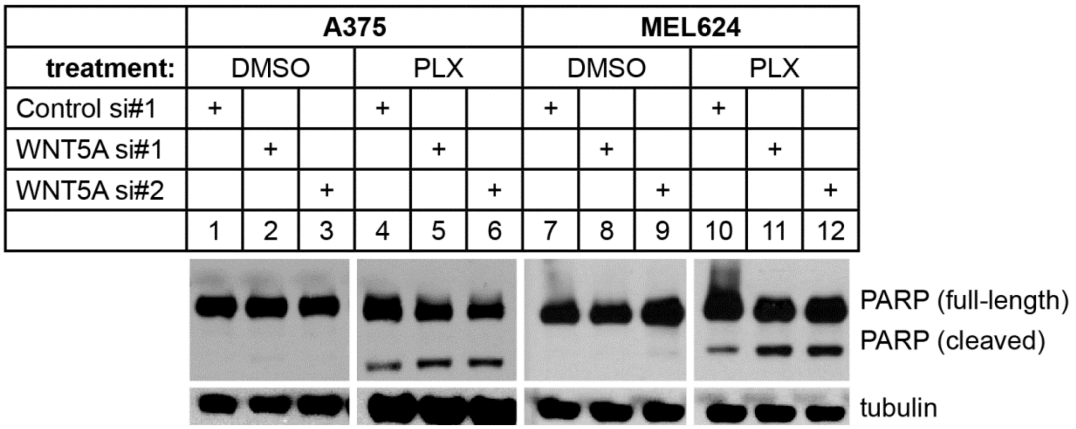


Figure 40: Depletion of WNT5A increases apoptosis in response to PLX4720 treatment.

Western blot analysis of lysates from A375 (lanes 1-6) or MEL624 cells (lanes 7-12) transfected with the indicated siRNAs and then treated two 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.

We also tested the response of WNT5A-depleted melanoma cells to increasing does of BRAFi. Pooled WNT5A siRNA reduces A375 cell viability at all doses of PLX4720 by 40-50% in comparison to mock transfection or Control siRNAs (#1 and #2) (Figure 39d). We calculated the IC_{50} for PLX4720 following transfection with these siRNAs normalizing the vehicle controls to 100% for all conditions. The IC_{50} for PLX4720 calculated in cells treated with WNT5A siRNAs (IC_{50}= 0.1153), differs by less than a factor of ten compared to Control si#1 (IC_{50}= 0.2136) or Control si#2 (IC_{50}= 0.2314), indicating that knockdown of WNT5A does not significantly alter the efficacy of PLX4720. These data raise the possibility that WNT5A regulates a different mitogenic pathway, which acts in parallel to RAS-RAF-MAPK signaling to promote melanoma cell growth. Finally, we determined whether WNT5A promotes the viability of A375-R and MEL624-R cells. Similar to the naïve cell lines, transfection with pooled WNT5A siRNA decreases both MEL624-R and A375-R viability when grown in PLX4720-containing media (Figure 39e,f). Together, these data indicate that loss of endogenous WNT5A expressed in both subsets of naïve melanoma cells and in BRAFi resistant cells results in reduced cell growth and viability.

Overexpression of WNT5A enhances melanoma cell growth in vitro and in vivo

Since loss of endogenous WNT5A reduces the growth and viability of both naïve and

BRAFi resistant melanoma cells, we next asked if WNT5A overexpression is sufficient to promote melanoma growth. We infected melanoma cells expressing low levels of endogenous WNT5A (Figure 33) with lentiviruses to overexpress either GFP as a control or WNT5A and validated the expression of exogenous WNT5A by western blotting (Figure 41a). As we predicted, WNT5A overexpression enhances the anchorage-independent growth of both A2058 and A375 cells as the WNT5A-transduced cells form greater numbers of colonies and form larger colonies in soft agar assays compared to the GFP-transduced control cells (Figure 41b-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 and single melanoma cells. To test this hypothesis, we first plated UACC1273 cells transduced with either GFP control or WNT5A leniviruses in ultra-low attachment plates in order to grow these cells in suspension. After one week we found that the WNT5A expressing cells grew to form large and highly pigmented aggregates, whereas the GFP expressing cells grew in small clusters showing little pigmentation (Figure 42a). We also monitored the clonal growth of UACC1273 cells by plating single cells in 96-well ultra-low attachment round bottom plates and then monitoring spheroid formation 10 days later. Single WNT5A-transduced cells survived and grew to form colonies at a greater frequency than the GFP-transduced control UACC1273 cells (55% compared to 30%).

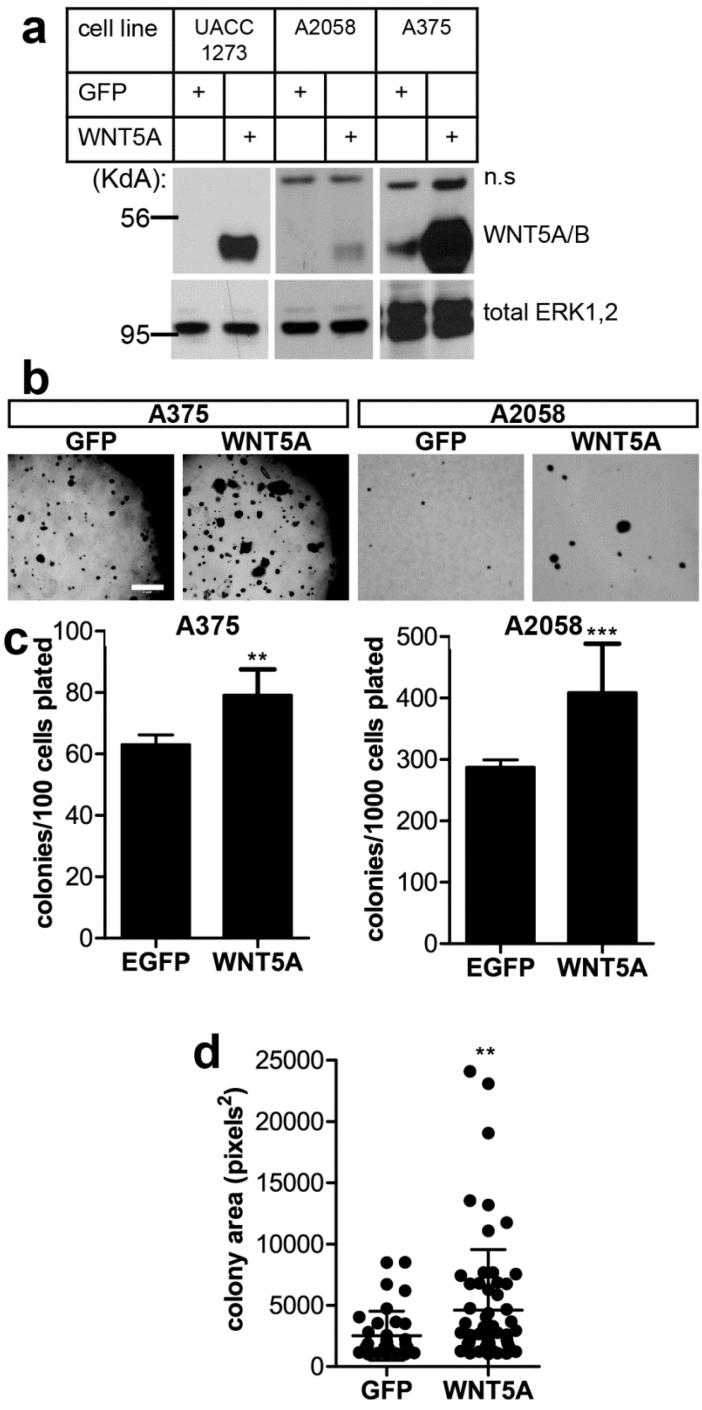
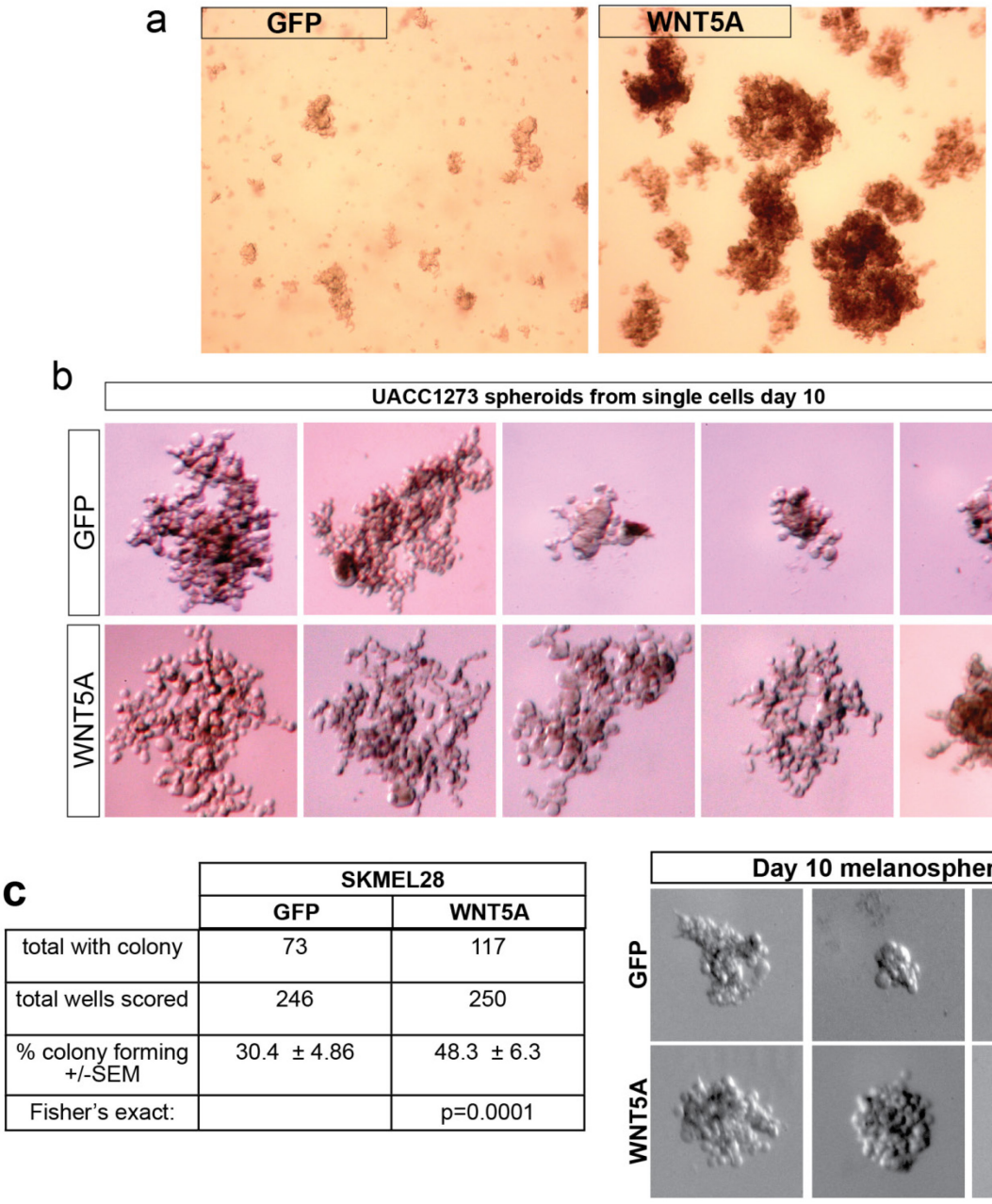


Figure 41: WNT5A overexpression enhances melanoma cell growth

(a) Western blots of lysates collected from A2058, A375, and UACC1273 cells infected with either WNT5A-IRES-GFP or IRES-GFP control lentiviruses, and from SK-MEL-28 cells infected with control GFP-hygromycin or WNT5A-hygromycin lentiviruses. Blots were probed with antibodies to detect the expression of WNT5A, total ERK1/2, and GFP. (b) Representative photographs of colonies formed in soft agar three weeks after embedding 100 A375 cells or 1000 A2058 cells in soft agar. (c) Average number of colonies formed in anchorage-independent growth conditions per 100 A375 cells (left) or 1000 A2058 cells (right) plated in soft agar. Error bars show standard deviation calculated from three independent experiments with duplicate wells counted for each condition. (d) Quantification of A2058 soft agar colony area. Each point represents the area of a single colony determined by image analysis. Center bar indicates the mean colony area and error bars show the standard deviation. Statistical significance of differences in colony number and size were calculated using a two-tailed Student's T-test (*p<0.05, **p<0.01, ***p<0.001)



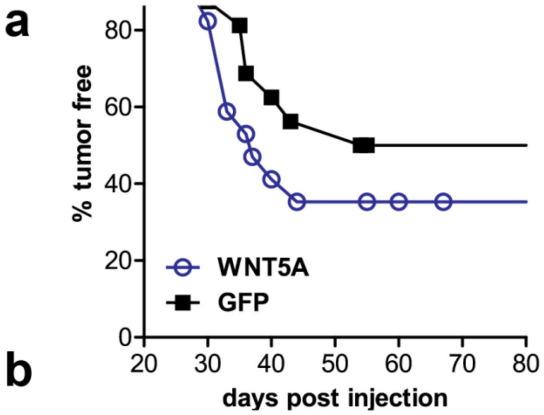
	SKMEL28	
	GFP	WNT5A
total with colony	73	117
total wells scored	246	250
% colony forming +/-SEM	30.4 ± 4.86	48.3 ± 6.3
Fisher's exact:		p=0.0001

Figure 42: WNT5A overexpression enhances melanosphere formation from single cells.

(a) Representative photographs of UACC1273 cell aggregates growing in ultra-low attachment plates taken one week after plating equal numbers of GFP-control or WNT5A-IRES-GFP infected cells. (b) Representative photographs of melanospheres taken 10 days after plating single UACC1273 cells in low attachment plates. Note the increase in size of spheroids derived from WNT5A-transduced cells. (c) Summary of the spheroid-forming potential of SK-MEL-28 cells infected with either WNT5A or GFP control lentiviruses. Clonogenic growth of these cells was determined by plating single melanoma cells in low attachment plates and counting the number of spheroids that grew 6 days later. Representative photographs of melanospheres taken 10 days after plating single cells are included to the right of the table summarizing the proportion of sphere forming cells compiled from three independent experiments.

WNT5A-transduced cells also tended to produce larger melanospheres from single cells compared to GFP control cells (representative photographs in Figure 42b). Next, we plated single SK-MEL-28 melanoma cells in 96-well low attachment plates in order to determine the effects of WNT5A expression on the clonogenic expansion of an additional human melanoma cell line. We find that approximately 30% of the single GFP control SK-MEL-28 cells survive and proliferate to form colonies, while a significantly higher percentage of the WNT5A-expressing cells (48%) form colonies in this assay ($p<0.001$, Fisher's Exact Test) (Figure 42b, statistics summarized in table on right).In contrast to UACC1273 cells we did observe any obvious differences in the size of melanospheres derived formed by either GFP- or WNT5A-expressing SK-MEL-28 cells (Figure 42b, representative photographs in right panel).

Given that WNT5A overexpression enhances the growth of melanoma cells in vitro we then asked whether WNT5A can also promote tumorigenesis in vivo using a xenograft model of tumorigenesis. We transplanted 50,000 GFP- or WNT5A-transduced A2058 cells subcutaneously in the flanks of immunocompromised mice and monitored tumor formation. Overexpression of WNT5A decreases the time to palpable tumor formation following cell transplantation (Figure 43a,b). 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.



	no tumor	tumor in 35 days	total
A2058-GFP	15	2	17
A2058-WNT5A	10	7	17
Total	25	9	34
		Fisher's Exact:	p=0.0285

Figure 43: WNT5A overexpression enhances tumorigenesis in xenografts

(**a**) Kaplan-Meier survival curve indicating the tumor free survival of mice injected with 50,000 A2058 melanoma cells expressing either WNT5A or GFP controls. The proportion of tumor-free mice is indicated on the Y-axis and the number of days following xenotransplantation of melanoma cells is indicated on the X-axis. (b) Summary table indicating the proportion of mice from each group developing tumors by 35 days post cell injections is included below the tumor-free survival curve.

Crosstalk with WNT/β-catenin signaling dose not account for reduced cell viability observed following WNT5A depletion

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 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[41,85]. Since numerous studies indicate that WNT5A can act as a negative regulator of WNT/β-catenin-dependent transcription[384,385],these data suggest that WNT5A might promote melanoma cell growth by inhibiting WNT/β-catenin signaling. We monitored WNT/β-catenin signaling using a luciferase reporter of β-catenin-dependent transcription and find that WNT3A but not WNT5A can enhance β-catenin-dependent transcription in both A375 and A2058 melanoma cells (Figure 44a,b). Transfecting reporter cells with β-catenin siRNAs two days prior to stimulation inhibits WNT3A induced reporter activity, which confirms that activation of luciferase transcription is β-catenin-dependent (Figure 44c).

Given that A375 cells endogenously express WNT5A we then asked if reducing WNT5A can alter their response to WNT3A. We find that knockdown of endogenous WNT5A increases the sensitivity of A375 cells to stimulation with WNT3A as indicated by a leftward curve shift (Figure 44c).

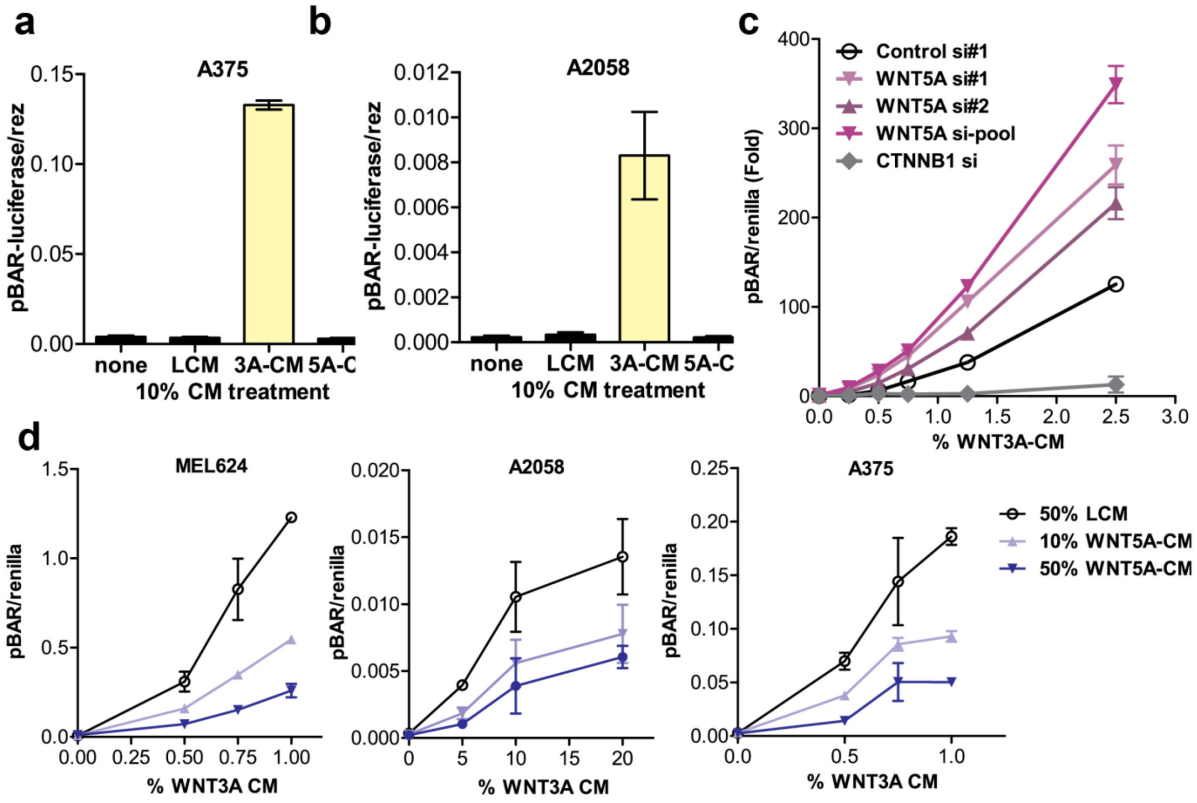


Figure 44: WNT5A negatively regulates WNT/β-catenin signaling in melanoma.

(a-b) Average normalized activity of a WNT/β-catenin-activated luciferase reporter (pBAR) following treatment of (a), A375 cells or (b), A2058 cells, with either control LCM, WNT5A-CM, or WNT3A-CM. Error bars indicate standard deviation from biological triplicates. (c) Average fold increase in pBAR-luciferase reporter activity induced in response to increasing doses of WNT3A-CM relative to LCM treatment following transfection with either negative Control si#1,β-catenin siRNA (positive control), or WNT5A siRNAs. (d) Average normalized activity of WNT/β-catenin -activated luciferase reporter (pBAR) following treatment with increasing doses of WNT3A conditioned media (WNT3A-CM) as indicated on the X-axis, combined with either L-cell control conditioned media (LCM) or two concentrations of WNT5A conditioned media (WNT5A-CM). Data were obtained from MEL624 (left), A2058 (center), and A375 (right) cells. Transcriptional reporter data in panels, (a-d) are representative of at least three independent experiments conducted using each cell line, with error bars indicating the standard deviation calculated from triplicate wells.

Consistent with previous studies involving other cell and tissue types[384,385], these data suggest that endogenous WNT5A also negatively regulates the WNT/β-catenin pathway in melanoma. We then asked whether exogenous WNT5A can similarly negatively regulate β-catenin-dependent transcription. Adding WNT5A conditioned media inhibits β-catenin-dependent transcription induced by WNT3A in multiple cell lines. These results further support our conclusion that WNT5A can inhibit the WNT/β-catenin pathway in melanoma.

Since WNT5A promotes melanoma growth and survival (Figures 35-43), whereas WNT/β-catenin signaling inhibits melanoma growth and survival, we then determined the consequences of simultaneously activating WNT/β-catenin signaling and inactivating WNT5A by siRNA knockdown. Consistent with previous results, either knockdown of WNT5A or treatment with WNT3A conditioned media results in reduced A375 cell number (Figure 45a). Co-treatment with WNT3A and WNT5A siRNAs results in a further reduction in A375 cell proliferation compared to either treatment alone (Figure 45a, compare yellow bars). These data indicate that a switch from WNT/β-catenin signaling to WNT5A-dependent signaling can result in increased melanoma growth and survival.

We next tested our hypothesis that WNT5A loss of function might inhibit melanoma cell growth by activating the WNT/β-catenin signaling pathway. To this end, 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[386].

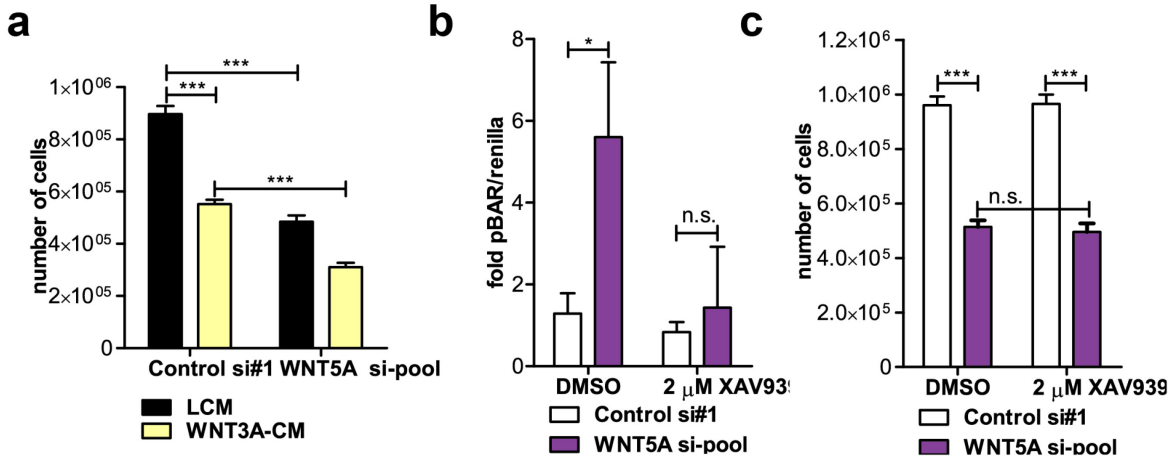


Figure 45: WNT5A inhibition of WNT/β-catenin signaling does not account for growth defects observed due to WNT5A depletion.

(**a**) Average number of cells counted following transfection with either Control siRNA #1 or pooled WNT5A siRNAs (2 days) and subsequent treatment with either LCM or WNT3A-CM for three days. (**b**) Average normalized activity of pBAR luficerase following transfection with the indicated siRNAs followed by treatment with 2 μM XAV939 for three days (*p<0.05). (**c**) Average number of cells following transfection with the indicated siRNAs and treatment with 2 uM XAV939 for three days (***p<0.001).

XAV939 treatment efficiently blocks β-catenin -dependent transcription induced by WNT5A siRNA (Figure 45b). However, XAV939 does not rescue WNT5A siRNA-mediated reduction in melanoma cell number (Figure 45c). In summary, these studies indicate that WNT5A acts as a negative regulator of WNT/β-catenin-dependent transcription in melanoma, yet crosstalk between WNT5A-dependent and β-catenin-dependent signaling does not account for the growth defects observed following WNT5A depletion.

WNT5A enhances AKT pathway activity in melanoma

We next asked whether WNT5A enhances melanoma cell growth via kinase-dependent signaling pathways. For these experiments, we monitored the activation of different kinase-dependent signaling pathways in A375 (lanes 1-12) and MEL624 (lanes 13-20) cells following transfection with either control or WNT5A siRNAs and a short time course of serum stimulation (Figure 46a). As a positive control, we monitored the phosphorylation of Protein Kinase C (PKC), which has been previously shown to be induced by WNT5A[43,387]. As expected, transfection of A375 cells with both pooled WNT5A siRNAs, and individual WNT5A siRNAs #1 and #2, reduces the phosphorylation of PKC in comparison to control (Figure 46a). Since A375 and MEL624 cells lines have activating BRAF leading to constitutively high levels of ERK phosphorylation, we were not surprised to find that WNT5A siRNAs do not enhance or reduce the phosphorylation of ERK1 and 2 in either A375 or MEL624 cells (Figure 46a).

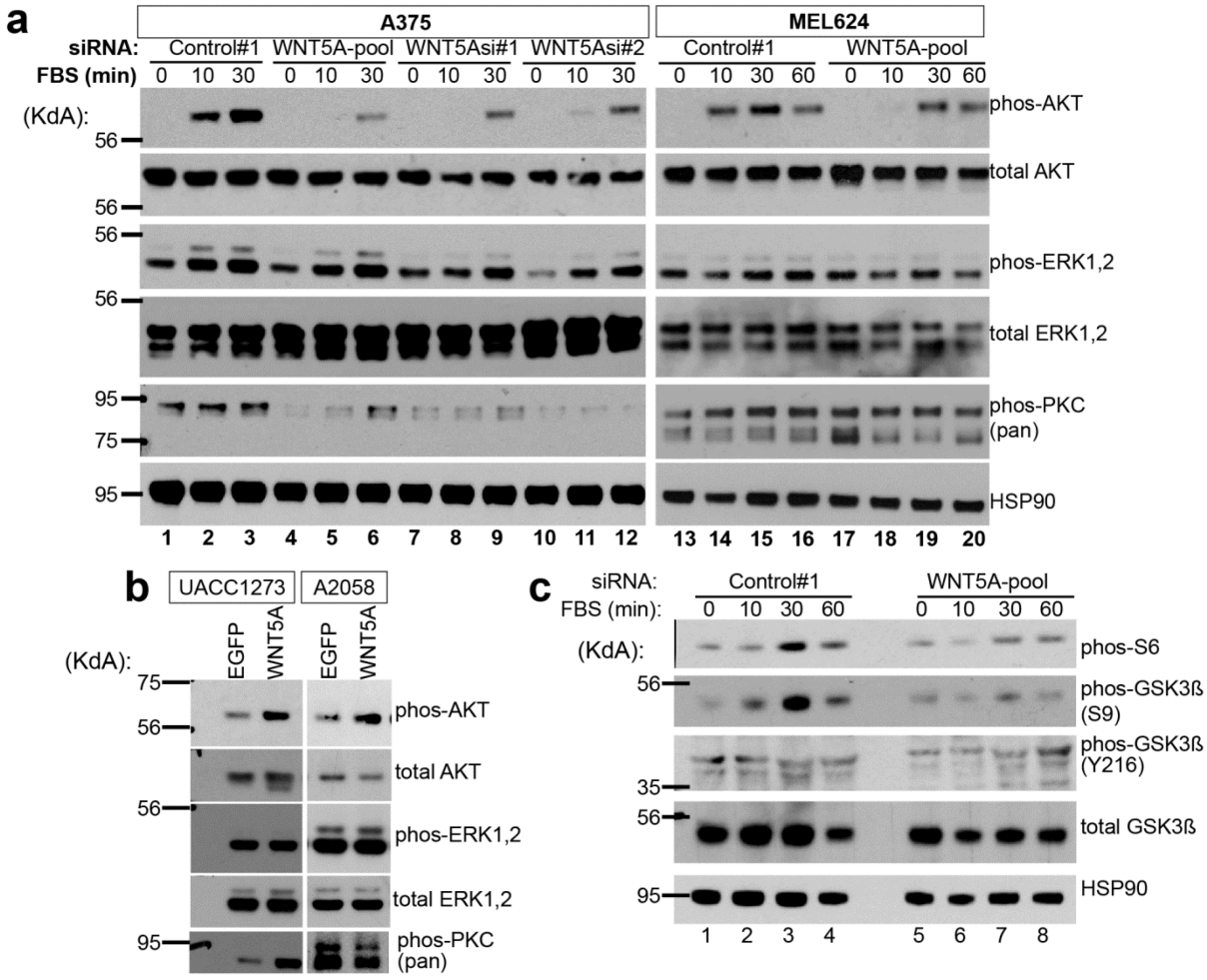


Figure 46: WNT5A enhances AKT pathway activity in melanoma.

(**a**) Western blots of lysates obtained following transfection of A375 cells with control siRNA (lanes 1-3), pooled WNT5A siRNAs (lanes 4-6), WNT5A siRNA #1 (lanes 7-9), or WNT5A siRNA#2 (lanes 10-12) followed by Serum stimulation. These blots were probed with antibodies to detect the abundance of total ERK, AKT, and HSP90 proteins as well as the phosphorylated forms of ERK1/2 (Thr202, Tyr204), AKT (Ser473), and PKC (pan phospho βII Ser660). (**b**-**c**) Western blots analyzing lysates collected following transfection of (**b**), MEL624 cells or (**c**), A375 cells with Control si#1 (lanes 1-4), or pooled WNT5A siRNAs (lanes 5-8) followed by Serum stimulation. In (**b**), blots are 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 βII Ser660). In (**c**), blots were probed with antibodies to detect the phosphorylation of the AKT targets, GSK3β (Ser9), and S6 (Ser253/Ser236), the active site phosphorylation of GSK3α/β (Y279/Y216), as well as total GSK3β and HSP90 as loading controls. (**d**) Western blots detecting total protein and phosphorylated protein in UACC1273 (lanes 1-2), or A2058 cells (lanes 3-4) that were transduced with either GFP control or WNT5A lentiviruses. These blots were probed with antibodies to detect to detect total ERK and total AKT, as well as the phosphorylated forms of ERK1/2 (Thr202, Tyr204), AKT (Ser473), and pan phospho-PKC (βII Ser660). All western blots are representative of at least three experiments.

In addition to PKC phosphorylation, we find that WNT5A knockdown similarly reduces AKT phosphorylation at Ser473 in response to serum (Figure 46a). WNT5A knockdown also reduces the phosphorylation of downstream AKT targets including, S6 and GSK3β (Figure 46c, compare lanes 5-8 to lanes 1-4).

Since loss of endogenous WNT5A reduced AKT phosphorylation, we then determined whether overexpression of WNT5A can promote the phosphorylation of AKT. As a positive control we monitored the phosphorylation of PKC by western blotting and find that WNT5A overexpression enhances the phosphorylation of PKC in UACC1273 cells (Figure 46b, left), as previously reported [43,166], but does not regulate PKC phosphorylation in A2058 cells (Figure 46b, right). Consistent with our loss of function studies, exogenous WNT5A enhances AKT phosphorylation at Ser473 in both UACC1273 and A2058 melanoma cells (Figure 46b).

Our results indicating that both exogenous and endogenous WNT5A enhances the phosphorylation of AKT predict that the high levels of WNT5A might similarly promote the activity of PI3K/AKT signaling in BRAFi-resistant melanomas. We then analyzed the phosphorylation status of various kinases in our BRAFi-resistant cell lines.

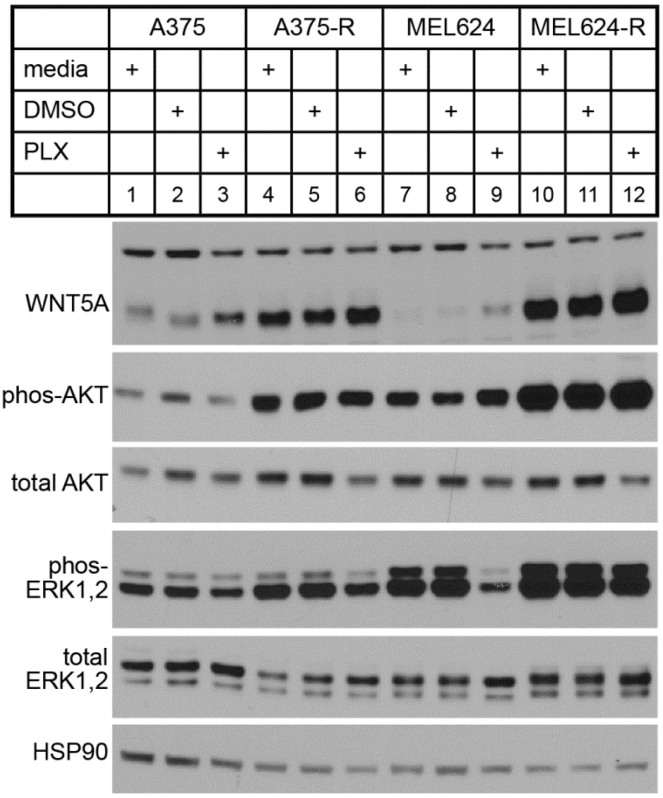


Figure 47: Analysis of protein phosphorylation in naïve and BRAFi-resistant cells.

We treated either naïve A375 cells (lanes 1-3), naïve MEL624 cells (lanes 10-23), A375-R cells (lanes 4-6) ,or MEL624-R cells (lanes 7-9) with either media, DMSO, or 2 uM PLX4720 for 24 hours prior to collecting lysates and analyzing the abundance of various proteins and phospho-proteins.

As we predicted, the phosphorylation of ERK1,2, was decreased by PLX4720 treatment in naïve A375 (Figure 47, compare lanes 1,2 to lane 3) and in naïve MEL624 cells (Fig. 5d, compare lanes 7,8 to lane 9). We observe an overall increase in the ratio of phosphorylated ERK1,2 to total ERK1,2 in both A375-R (Figure 47, compare lanes 1-3 to lanes 4-6), and MEL624-R cells (Figure 47, compare lanes 7-9 to lanes 10-12) compared to naïve cells. We also observe an increase in the ratio of AKT phosphorylated at Ser473 compared to total AKT in both A375-R and MEL624-R cell lines in comparison to naïve cell lines (Figure 47, compare lanes 1-3 to lanes 4-6 and compare lanes 7-9 to lanes 10-11).

Previous studies find that co-treating melanoma cells with BRAF/MAPK and PI3K/AKT inhibitors synergistically reduces melanoma cell growth[218,388–392], suggesting that the BRAF/MAPK and PI3K/AKT signaling pathways cooperate to promote the growth and viability of melanoma cells. In line with these studies, we also find that co-treating naïve melanoma cells with an IC_{50} dose of PLX4720 and with increasing doses of either the PI3K inhibitor GDC-0941 or the dual PI3K/mTOR inhibitor BEZ235 results in a further reduction in cell viability compared to BRAFi alone (Figure 48a,b). We then asked if our BRAFi resistant cells lines also require PI3K/AKT pathway activation for their growth and survival. We find that GDC-0941 and BEZ235 similarly reduce the viability of A375-R and MEL624-R cells (Figure 48c,d). In summary, these results reveal that WNT5A can activate PI3K/AKT signaling which is required for optimum viability of both naïve and BRAFi-resistant cells. These data support the hypothesis that WNT5A can promote melanoma growth by activating PI3K/AKT signaling.

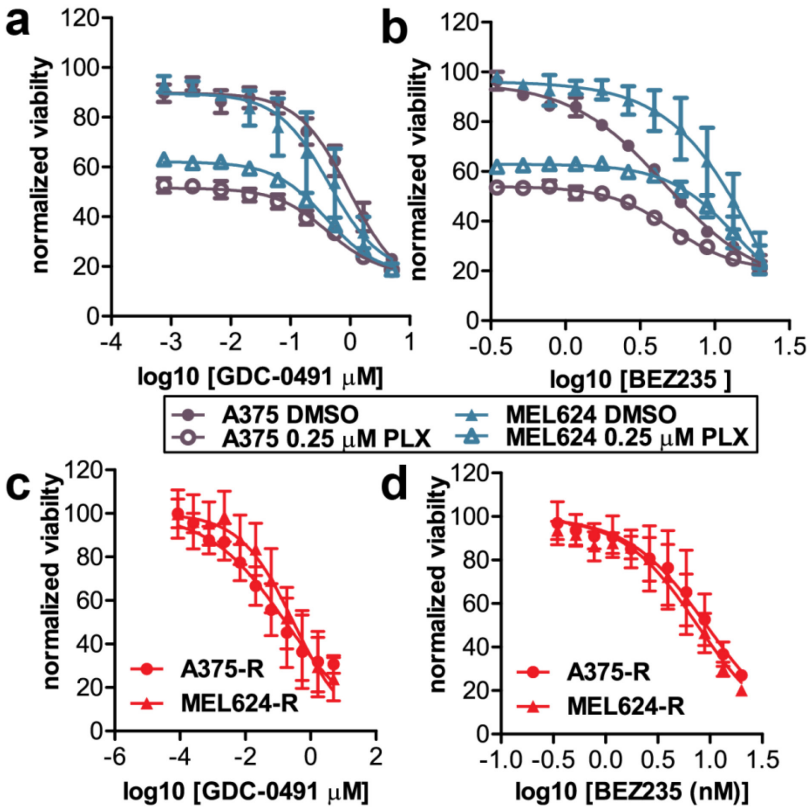


Figure 48: PI3K/AKT pathway activity is required for the growth of both naïve and BRAFi-resistant melanoma cells.

(a-b) Normalized viability of A375 cells (blue lines) and MEL624 cells (purple lines) treated with either DMSO control, or 0.25 μM PLX4720 (open circles and triangles). In (a), cells were treated with increasing concentrations of GDC-0941 in combination with PLX472 or DMSO. In (b), cells were treated with increasing concentrations of BEZ235 in addition to PLX472 or DMSO. (c-d) Normalized viability of A375-R (circles) and MEL624 (triangles) grown in the presence of 2μM PLX4720 and increasing concentrations of (c), GDC-0941 or increasing concentrations of (d), BEZ235. Error barns indicate the standard deviation calculated from four experiments. Best fit curves were determined using GraphPad software and a nonlinear regression model.

The WNT5A receptors FZD7 and RYK are highly expressed in melanoma

To learn more about potential WNT5A-dependent signaling pathways in melanoma we next focused our efforts on identifying transmembrane receptors for WNT5A, a secreted signaling protein. Previous studies have identified multiple putative WNT5A receptors that act in a cell and tissue type-dependent manner[10,393]. Since previous studies indicate that WNT5A is highly expressed in metastatic melanomas with poor clinical outcomes[42–44,380], we reasoned that functional WNT5A receptors would also be highly expressed in melanomas. We analyzed a large microarray dataset profiling transcript expression in 950 human tissue samples including multiple normal and cancerous tissues (Array express, E-MTAB-37).We find that FZD6 ($p=4.93x10^{-5}$), FZD7 (probe 1, $p= 3.84x10^{-9}$, and probe 2, $p= 4.94x10^{-10}$) and RYK (probe 1, $p= 4.76x10^{-4}$, probe 2, p= 2.80x10 and probe 3 $p= 1.45x10^{-6}$) are significantly up-regulated in melanoma while other WNT receptors were not (Figure 49a). We analyzed additional microarray datasets and found that FZD7 is highly expressed in both normal skin and melanoma (Figure 49c), while RYK is overexpressed in melanoma (Figure 49d). These data reveal that the WNT5A receptors, FZD7 and RYK are highly expressed in melanomas.

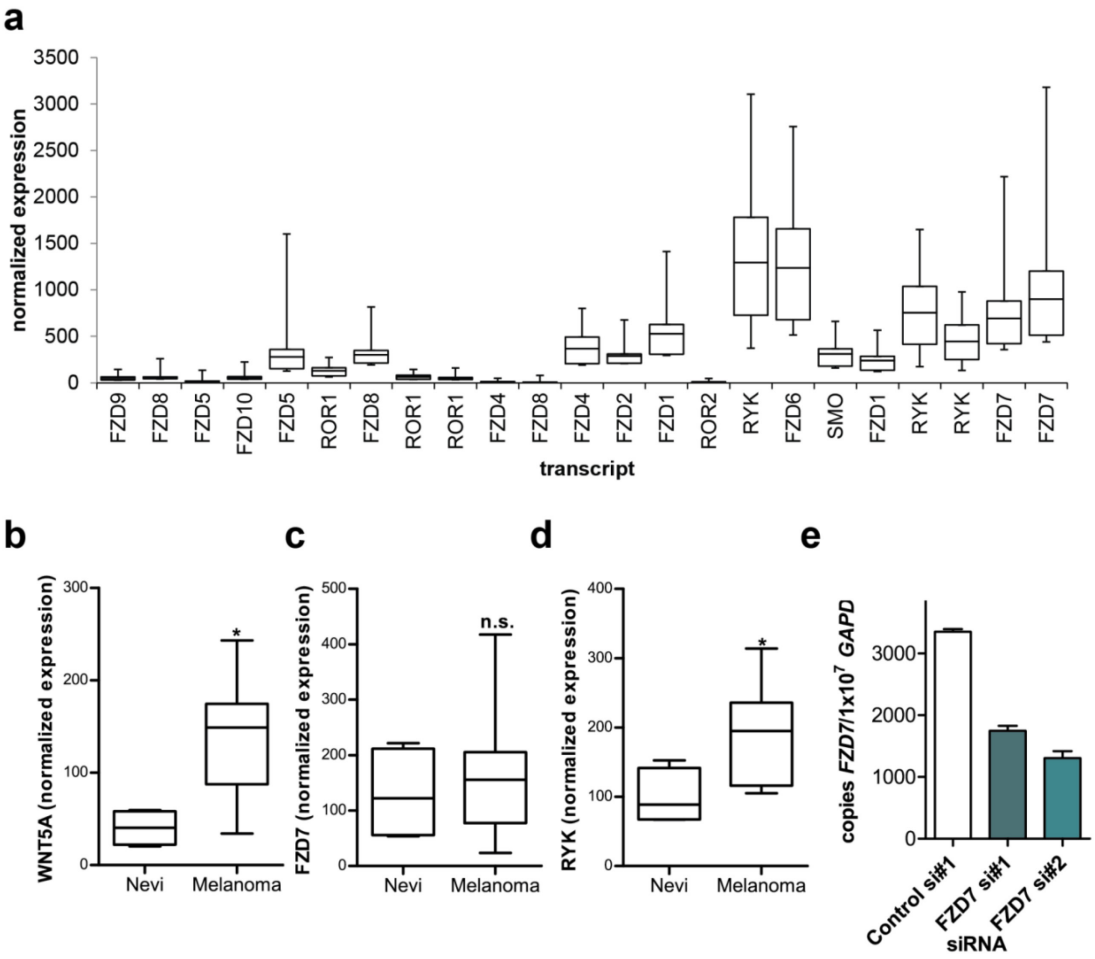


Figure 49: Expression of WNT receptors in melanoma and normal human skin.

(a) Normalized expression of WNT receptors in melanoma relative to other normal and cancerous tissues from a meta-analysis of gene expression patterns in 950 samples (Array expresss, E-MTAB-37) *FZD6* ($p=4.93x10^{-5}$), *FZD7* (probe 1, $p= 3.84x10^{-9}$, and probe 2, $p= 4.94x10^{-10}$) and RYK (probe 1, $p= 4.76x10^{-4}$, probe 2, $p= 2.80x10^{-6}$ and probe 3, $p= 1.45x10^{-6}$) were significantly up-regulated in melanoma. (b-d) Normalized expression of (b), *WNT5A*, (c) *FZD7*, and (d), *RYK* in skin and in melanoma as determined by analysis of publically available microarray dataset (Smith et al 2007). (e) Expression of *FZD7* normalized to *GAPDH* as determined by RT-PCR two days following transfection of A375 cells with siRNAs.

The WNT5A receptors FZD7 and RYK enhance melanoma cell viability and growth

In parallel to our analysis of gene expression profiles, we also carried out functional tests to determine if any of these WNT receptors contributed to melanoma cell viability in a manner similar to WNT5A. As expected from previous results, we find that BRAF and WNT5A siRNAs reduce A375 cell viability in this assay (Figure 50a). In contrast, control siRNAs and siRNAs targeting LRP6 and β-catenin, which inhibit WNT/β-catenin signaling, do not affect viability in this assya(Figure 50a). Although previous reports suggest that the receptor tyrosine kinases ROR1 and ROR2 can act as WNT5A receptors and also enhance the proliferation of cancer cells[100,101], only 1 of 3 ROR1 siRNAs and only 1 of 3 ROR2 siRNAs reduce melanoma cell viability (Figure 50a). However, multiple siRNAs targeting the WNT5A receptors FZD7 and RYK reduce cell viability (Figure 50a). Since loss of WNT5A inhibits melanoma proliferation in three dimensional culture (Figure 38), we asked whether FZD7 and RYK receptors also regulate melanoma cell growth in three dimensions. Like WNT5A, depletion of either FZD7, or RYK inhibits the growth of melanoma cells when grown in soft agar (Figure 50b,c) and when grown as melanospheres (Figure 50d,e).

RYK and FZD7 regulate the viability of naïve and BRAFi-resistant cells in the presence of PLX4720

We next asked if RYK and FZD7 also regulate melanoma cell viability in the presence of BRAFi. We treated melanoma cells with increasing concentrations of PLX4720 following

transfection with pooled RYK and pooled FZD7 siRNAs. Similar to depletion of WNT5A (Fig. 2) we find that loss of RYK or FZD7 further reduces melanoma cell viability at all doses of PLX4720 (Figure 50a). Finally, we asked whether FZD7 and RYK also enahance the growth and viability of our BRAFi-resistant cells. Similar to the naïve cell lines, siRNA knockdown of both RYK and FZD7 reduces the viability of both A375-R and MEL624-R cells (Figure 51b,c). These results indicate that a WNT5A/FZD7/RYK pathway regulates the growth and viability of both naïve and BRAFi-resistant melanoma cells.

FZD7 and RYK enhance AKT signaling in melanoma

As WNT5A enhances AKT signaling in melanoma, we predicted that receptors for WNT5A, such as FZD7 and RYK, would also enhance to AKT phosphorylation in melanoma. Transfection of A375 cells with ssFLAG-FZD7 to overexpress this receptor enhances AKT phosphorylation, but not ERK phosphorylation in comparison to FLAG-GFP (Figure 52a, compare lanes 1 and 3), whereas overexpression of RYK has little effect (Figure 52 compare lanes 1 and 2). We then asked whether endogenous FZD7 and RYK can regulate the phosphorylation of AKT in melanoma cells. We transfected A375 cells with either Control si#1 (lanes 1-3), pooled FZD7 siRNA (lanes 4-6), or pooled RYK siRNAs (lanes 7-9), Serum starved these cells and then treated them with Serum for a short time course (Figure 52b).

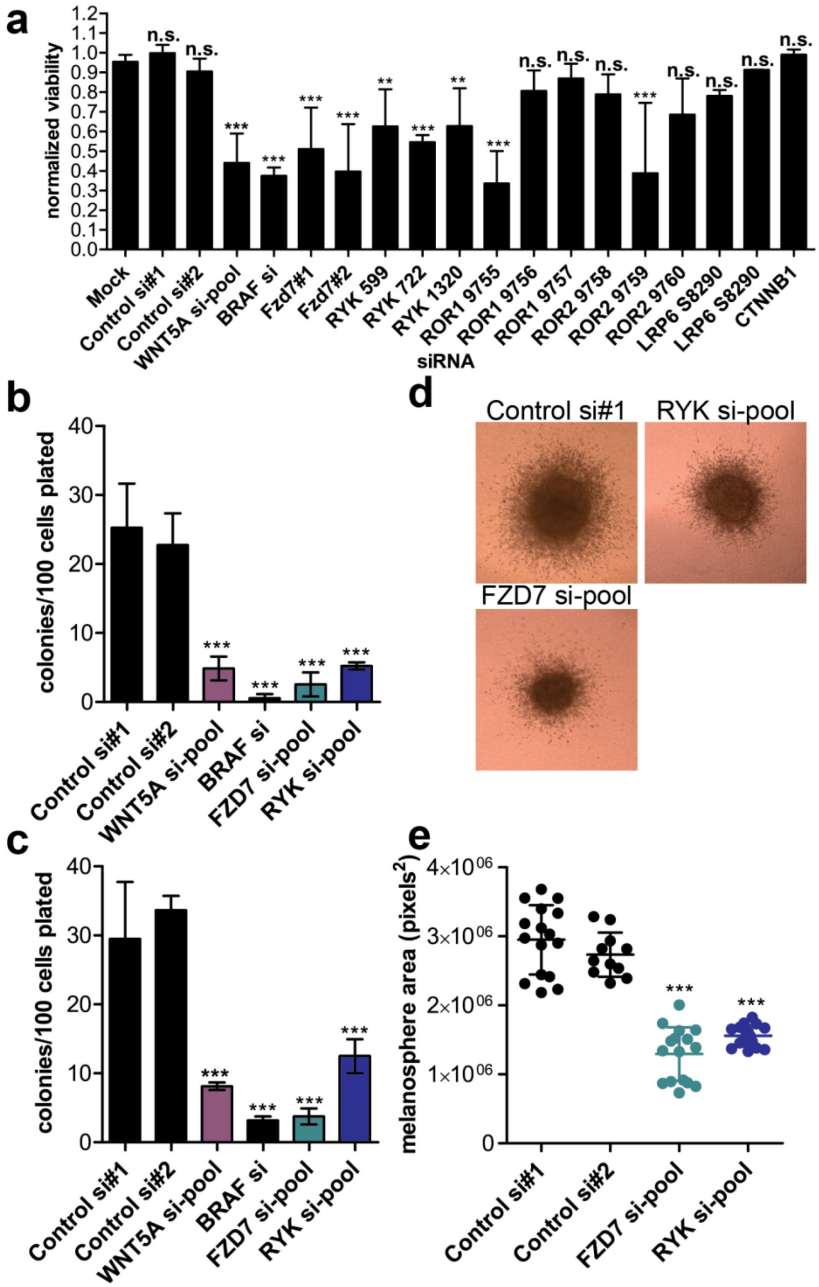


Figure 50: The WNT5A receptors FZD7 and RYK enhance melanoma cell viability and growth.

(a) Average cell viability of A375 cells that were plated at a density of 5000 cells per well in 96-well plates two days following transfection and then allowed to grow for three days. Error bars show standard deviation of at least three experiments with quadruplicate wells for each siRNA. siRNAs resulting in a reduction in cell viability were determined by One-Way ANOVA. (** p<0.01, ***p<0.001). (b-c) Average number of colonies formed in soft agar following transfection of (b),A375 and(c) MEL624 cells with pooled siRNAs followed by growth in soft agar for 6-7 days. (d) Representative photos of A375 cells transfected with control, FZD7 or RYK siRNAs grown as three dimensional melanospheres and embedded in collagen I. (e) Quantification of A375 spheroid area at day 4 following siRNA transfection as described in Fig. 1 and in the Methods section. Statistical significance of differences in colony number and size and in melanosphere size were calculated by ANOVA (** p<0.01, ***p<0.001).

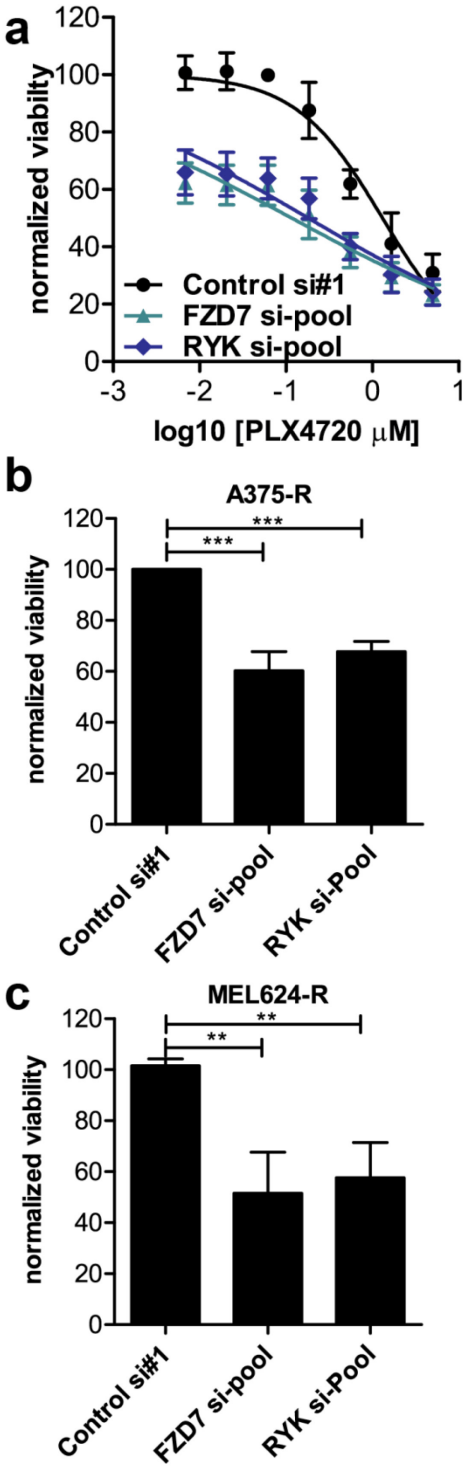


Figure 51: Endogenous RYK and FZD7 regulate both naïve and BRAFi-resistant cell viability in the presence of PLX4720.

(a) Normalized viability of A375 cells that were transfected with the indicated siRNAs, then subsequently treated with increasing concentrations of PLX4720 48 hours after transfection. Transfected cells were grown in the presence of PLX4720 for a total of three days before viability was determine d. All values were normalized to control siRNA with DMSO set at 100% and nonlinear, best fit regression curves were generated using GraphPad software. (b-c) Normalized viability of A374-R cells, (b) and MEL624-R cells, (c) that had been transfected with either control, FZD7, or RYK siRNAs. Data were normalized to the Control si#1 condition set to 100%, and error bars indicate standard deviation of three independent experiments. (**p<0.01, two-tailed T-Test).

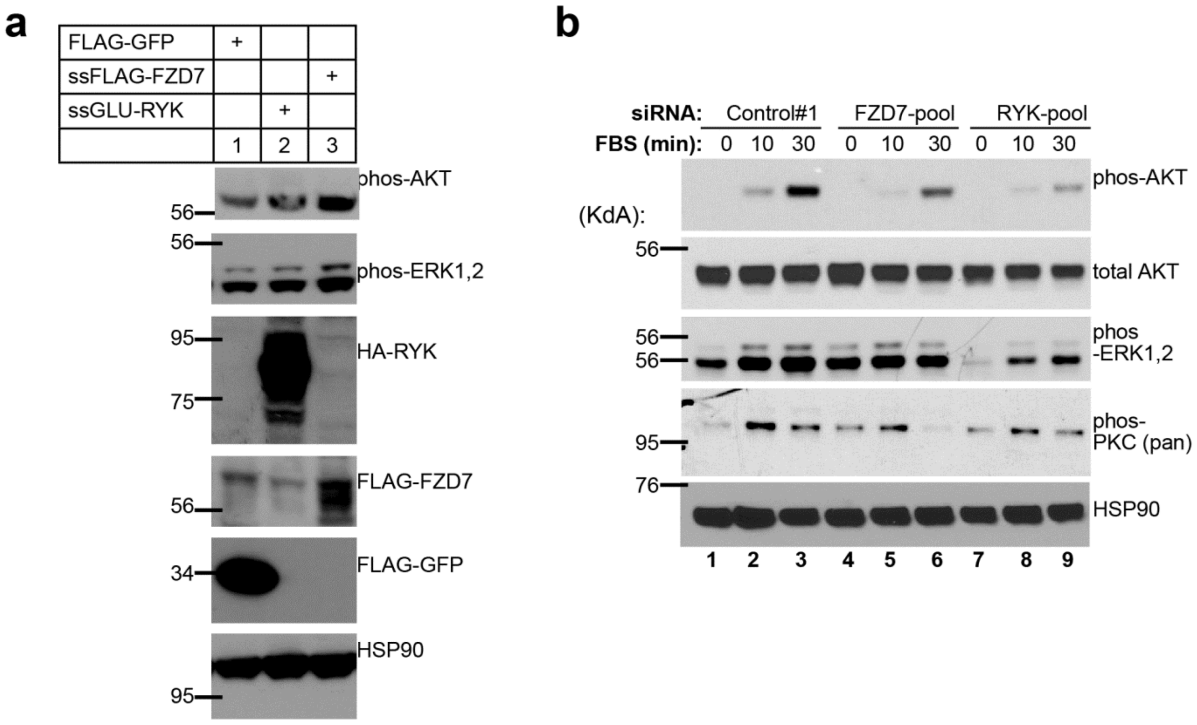


Figure 52: FZD7 and RYK enhance AKT signaling in melanoma.

(a)Western blots of lysates from A375 cells transfected with either (1) FLAG-GFP, (2) ssGLU-RYK, (3) ssFLAG-FZD7. Blots were probed with antibodies to detect HA-tagged RYK, FLAG, the phosphorylated forms of ERK1/2 (Thr202, Tyr204), AKT (Ser473), and total AKT. (b) Western blots of lysates from A375 cells transfected with either Control si#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.

Similar to WNT5A siRNAs (Figure 46a,c), transfecting A375 cells with either pooled RYK or FZD7 siRNAs reduces AKT phosphorylation induced by Serum stimulation, but does not significantly affect either ERK or PKC phosphorylation (Figure 52b). Together, these data reveal that endogenous RYK and FZD7 are necessary for robust phosphorylation of AKT and that overexpression of FZD7, but not RYK is sufficient to increase the abundance of phospho-AKT.

FZD7 and RYK form a protein complex in melanoma cells

We were further incentivized to investigate potential functions of RYK and FZD7 in melanoma based on our proteomic analysis of affinity purified FZD7 and RYK complexes[394]. These datasets indicate a high degree of overlap between FZD7 and RYK proteomes (Fig. 7c). Specifically, we identify RYK peptides in FZD7 pulldowns and FZD7 peptides in the reverse, RYK pulldowns. We also identify several overlapping proteins in the RYK and FZD7 proteomes including genes genetically linked to WNT/PCP signaling including, CELSR1, CELSR2, SCRIB, VANGL1, and PTK7, as well as the predicted G-protein coupled receptors GPR125 and GPR124 (Figure 53a). Due to the high degree of overlap between FZD7 and RYK proteomes, we surmised that RYK and FZD7 function together in an associated protein complex or complexes.

We first validated whether RYK and FZD7 can interact in melanoma cells. We transfected A375 cells with ssGLU-RYK along with various FLAG-tagged proteins and then isolated RYK-associated protein complexes by streptavidin affinity purification (Figure 53b).

ssGLU-RYK co-purifies with ssFLAG-FZD7 but not with FLAG-GFP (compare lanes 8 and 12), nor does RYK associate with ssFLAG-ROR2 (Fig. S6 compare lanes 11 and 12). Next, we asked whether the association between RYK and FZD7 requires endogenous WNT5A by first transfecting cells with either Control si#1 (lanes 1-4), or pooled WNT5A siRNAs (lanes 5-8) and then transfecting these cells with either ssGLU-RYK and FLAG-GFP (lanes 1,2,5, and 6) or ssGLU-RYK and ssFLAG-FZD7 (lanes 3,4,7,and 8) two days later (Figure 53c). Again, we find that ssGLU-RYK associates with ssFLAG-FZD7, but not with GFP (Figure 53c, compare lanes 7-8 with lanes 5-6). Transfection with WNT5A siRNA effectively reduces WNT5A protein (Figure 53b, second panel from bottom) but neither enhances nor inhibits the association between ssGLU-RYK and ssFLAG-FZD7 (Figure 53b, compare lanes 7 and 8).

Closer analysis of the whole cell lysates from our co-affinity purification experiments reveals that co-transfection of ssFLAG-FZD7 and ssGLU-RYK reduces the amount of ssGLU-RYK protein compared to co-transfection with FLAG-GFP (Figure 53b, compare lanes 2 and 6, and Figure 53c, compare lanes 1,2 to lanes 3,4). Co-expression of ssGLU-RYK with ssFLAG-ROR2 also has no effect on RYK levels (Figure 53b, compare lanes 2 and 5), suggesting that the ssGLU-RYK protein abundance is not likely reduced due to some side effect of overexpressing two transmembrane proteins simultaneously. Furthermore, we observe that the ssGLU-RYK protein abundance is further reduced by co-transfection of ssFLAG-FZD7 and ssGLU-RYK following siRNA-depletion of WNT5A (Fig. 6C, compare lane 3 to lane 4).

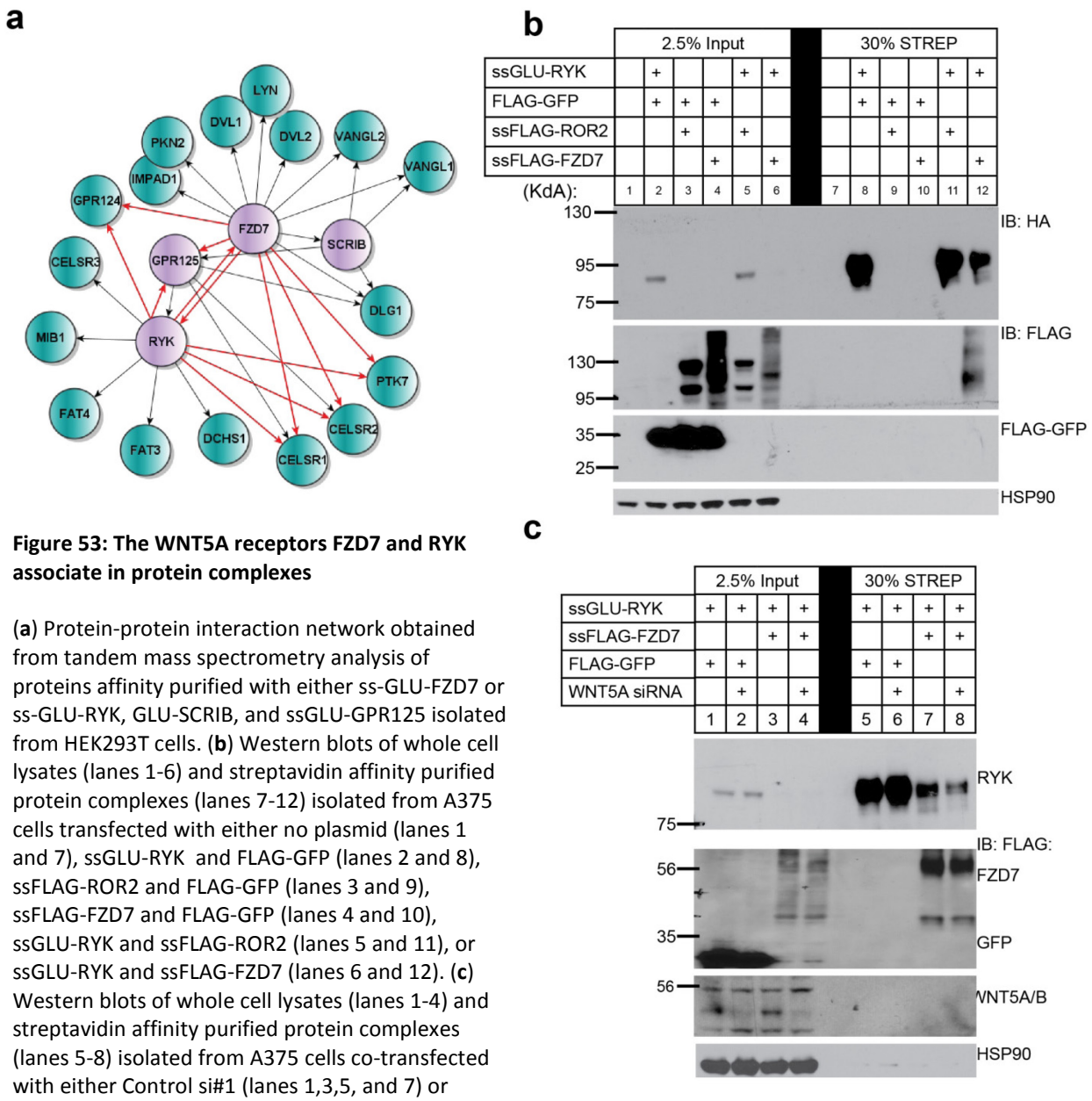


Figure 53: The WNT5A receptors FZD7 and RYK associate in protein complexes

(**a**) Protein-protein interaction network obtained from tandem mass spectrometry analysis of proteins affinity purified with either ss-GLU-FZD7 or ss-GLU-RYK, GLU-SCRIB, and ssGLU-GPR125 isolated from HEK293T cells. (**b**) Western blots of whole cell lysates (lanes 1-6) and streptavidin affinity purified protein complexes (lanes 7-12) isolated from A375 cells transfected with either no plasmid (lanes 1 and 7), ssGLU-RYK and FLAG-GFP (lanes 2 and 8), ssFLAG-ROR2 and FLAG-GFP (lanes 3 and 9), ssFLAG-FZD7 and FLAG-GFP (lanes 4 and 10), ssGLU-RYK and ssFLAG-ROR2 (lanes 5 and 11), or ssGLU-RYK and ssFLAG-FZD7 (lanes 6 and 12). (**c**) Western blots of whole cell lysates (lanes 1-4) and streptavidin affinity purified protein complexes (lanes 5-8) isolated from A375 cells co-transfected with either Control si#1 (lanes 1,3,5, and 7) or pooled WNT5A siRNAs (lanes 2,4,6, and 8) along with either ssGLU-RYK and FLAG-GFP (lanes 1,2,5, and 6) or ssGLU-RYK and ssFLAG-FZD7. Western blots shown in panel, (**b**) and panel, (**c**) were probed using either HA-specific antibodies to detect RYK (top panel), FLAG-specific antibodies (middle panels), or HSP90 as a loading control (bottom panel). Additionally, protein samples in panel, (**c**) were probed with WNT5A antibody (second panel from bottom) to validate siRNA knockdown of WNT5A.

Taken together, these data suggest that RYK and FZD7 form a protein complex independently of WNT5A, and that tightly controlled expression of both FZD7 and WNT5A are required for the maintenance of RYK stability.

High expression of WNT5A has been previously correlated with patient death and metastasis in melanoma[8–10]. We next asked whether the expression of either FZD7 or RYK correlate with patient outcomes in melanoma. We analyzed RYK and FZD7 in a microarray dataset of multiple human melanoma tumors with known clinical outcomes and find that patients with high expression of RYK experience significantly shorter melanoma-related survival in comparison to patients expressing only low levels of RYK ($p<0.006$) (Figure 54a). In contrast, patients expressing high levels of or low levels of FZD7 do not differ in their time to melanoma related deaths (Figure 54b).

Since WNT5A is overexpressed in BRAFi-resistant melanomas (Figure 31-3) and since our results indicated that RYK and FZD7 mediate WNT5A-dependent phenotypes in melanoma (Figure 52-23), we then asked if RYK, FZD7, or other WNT/PCP pathway components were also differentially expressed in BRAFi-resistant melanomas. We analyzed the expression of a variety of genes associated with PCP signaling as well as genes identified in our RYK and FZD7 proteomics. RYK and FZD7 are not consistently up- or down-regulated in the resistant cells. However, we find that other genes associated with β-catenin-independent WNT signaling such as, VANGL2, CELSR1, and FZD5 are reduced in a majority of BRAFi-resistant melanomas whereas FAT3 and DCHS1 expression are increased (Figure 55).

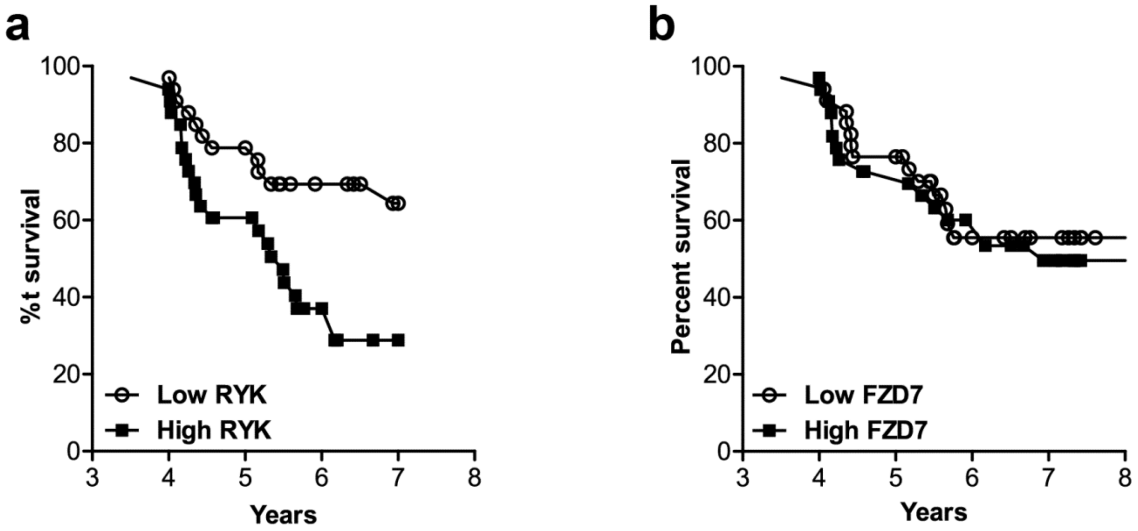


Figure 54: High expression of *RYK* is associated with reduced time to death amongst metastatic melanoma patients

(a-b) Kaplan-Meier survival curves comparing the survival of patients expressing either high or low RYK transcript in (a) and comparing the survival of patients with expressing either high or low FZD7 transcript in (b). High expression of RYK was associated with decreased survival probability in this patent cohort (Log Rank Test, *$p<0.05$), while FZD7 expression did not predict survival. Expression data was obtained by analysis of a previously published microarrary dataset (Xu *et al* 2008).

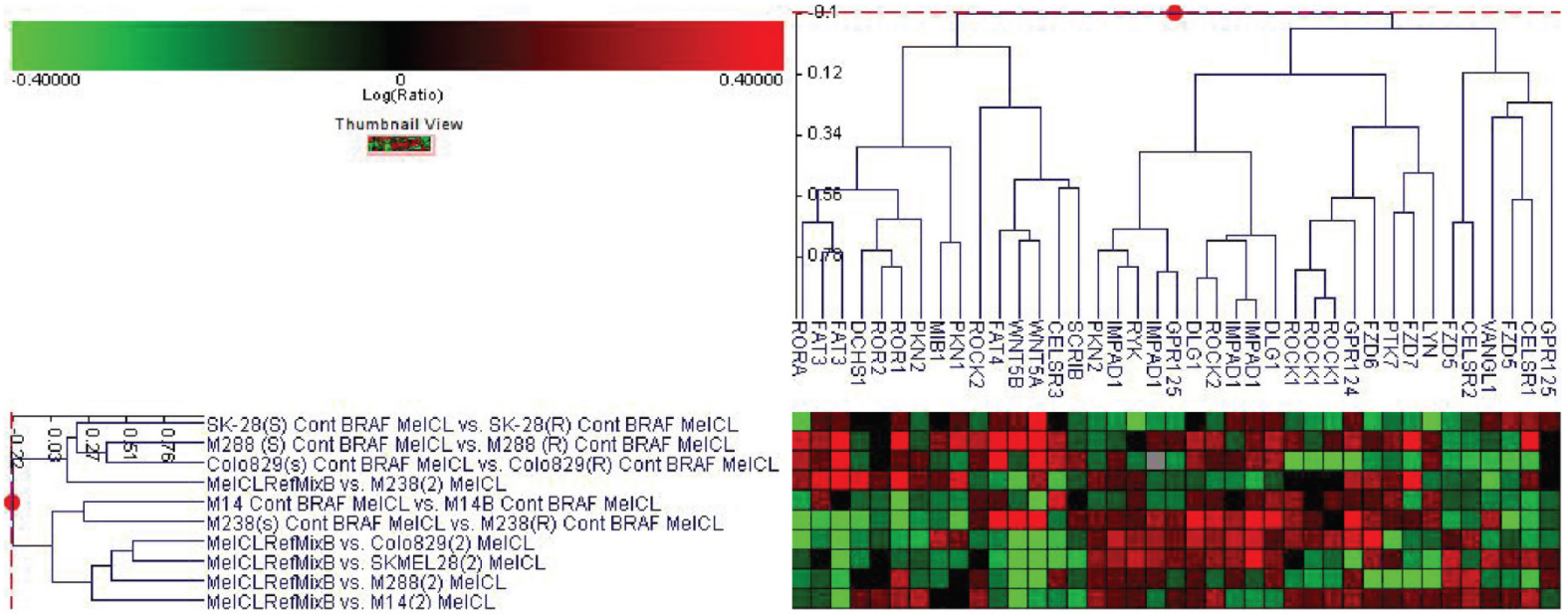


Figure 55: Planar Cell Polarity genes and proteins in the FZD7 and RYK proteomes and differentially expressed in PLX-resistant melanoma cells.

Heat map summarizing of the expression of WNT5A/PCP signaling genes in PLX-resistant melanoma cells arranged by hierarchical clustering. Differences in transcript expression between PLX sensitive (S) and resistant lines (R) were at least two fold for each of the included genes. $p<0.01$.

Although the functional relevance of these gene expression changes is not yet clear, these data further support our hypothesis that β-catenin-independent WNT signaling networks involving WNT5A and numerous other factors may be differentially regulated in therapy resistant melanoma.

Discussion

So far only the $BRAF^{V600E}$ inhibitor Vemurafinib and the immunotherapeutic, Ipilimumab, have resulted in any increase in disease-related survival in melanoma patients, yet these interventions are ultimately ineffective as patients eventually develop drug-resistant disease and only subsets of patients respond to immunotherapy[346,369–371,395]. A more complete understanding of the molecular underpinnings of both melanoma progression and response to targeted therapies will be crucial for the generation of lasting therapies. In this study, we find that WNT5A is dramatically up-regulated in resistant melanoma cells derived by chronically inhibiting $BRAF^{V600E}$ with PLX4720 (PLX). We go on to find that WNT5A promotes the viability of both naïve parental cells and PLX-resistant melanoma cells.

PLX-resistant melanoma cells often exhibit cross-resistance to other BRAF/MAPK pathway inhibitors such as drugs targeting MEK[388,396,397], suggesting that aberrant activity of other signaling pathways and cellular processes also contribute to drug resistance. Indeed, PLX-resistant melanoma cells inactivate apoptotic pathways through expression changes such as

down-regulation of BIM[377], and up-regulation of MDM4, a negative regulator of TP53[379]. PLX-resistant melanoma cells also up-regulate key mitogenic signaling proteins such as RTKs and AKT[372,375,376,391]. The results of this present study add WNT5A to this growing list of signaling proteins that promote both intrinsic and acquired resistance to BRAF/MEK inhibition. Taken together, these studies suggest that combinatorial therapies targeting acquired or intrinsic activation or inactivation of multiple signaling pathways should be explored as possible therapeutic options. Consistent with this hypothesis, co-treatment with BRAF/MAPK inhibitors and PI3K/AKT inhibitors synergistically reduces melanoma cell viability[218,388–392], while inhibition of HSP90 with XL888 can similarly inhibit the growth of PLX-resistant melanoma cells[398].

Previous studies find that WNT5A can act through at ROR2/PKC pathway in melanoma to enhance cell motility[43,166,387]. Our results suggest that WNT5A can activate multiple signaling pathways in melanoma cells since we find that both WNT5A and the WNT5A receptors FZD7 and RYK promote AKT activation. Although we cannot rule out the possibility that other WNT receptors such as, other FZD family members 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. During the course of our studies another group similarly observed that FZD7 can activate AKT in muscle progenitor cells[399], suggesting that WNT/FZD7/AKT signaling is active in multiple cell types. FZD7 has also recently garnered attention for its potential connection to cancer development and progression. Blocking FZD7 activity with siRNAs or peptides can induce

apoptosis and inhibit proliferation and in vitro and in vivo in colon, breast, pancreatic, lung, and hepatocellular cancer cells[95,96,139,140,400]. Potential roles for RYK in cancer are presently less clear. Here, we find that like WNT5A protein expression[44], high RYK transcript in patient tumors correlate with decreased survival. Collectively, these studies identify a WNT5A/FZD7/RYK pathway as a potential therapeutic target for combinatorial therapy in melanoma.

Materials and Methods

Cell culture

Melanoma cell lines used in this study were cultured in various growth medias purchased from Life Technologies supplemented with penicillin/streptomycin and fetal bovine serum (FBS). Briefly A375 cells were cultured in DMEM+5% FBA, A2058 cells were cultured in DMEM+10%FBS, SK-MEL-28 cells were grown in MEM+10%FBS, and COLO829, MEL624m M288, M233, and M14 cells were all cultured in RPMI+10% FBS. A375 and MEL625 PLX resistant cell lines were generated by growth the cells in 2 uM PLX4720 for at least eight weeks. During this process cells were split once they reached confluence and PLX4720 containing media was replenished every 3-4 days. In order to generate additional resitant cell lines, COLO829, SK-MEL-28, M288, and M233 and M14 were treated at their growth-adjusted inhibitory concentration of 50% (IC50g), the other with DMSO. Concentrations of PLX4032 were increased

as resistance developed.. Growth inhibitory IC50 of M288R, M238R, M14R, M288R, SKMEL28R and the parental cell line was determined as follows, cells were plated in duplicate in 12-well plates (10,000 cells/2 ml/well). PLX4032 was added to start concentration 10 μM making 1/10 for a total of 6 dilutions. Media was used as a control of proliferation. Cell counts were performed on 6 after treatment. (Z1 Particle Counter; Beckman Coulter, Brea, CA).

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 fluorescence activated cell sorting to isolate GFP+ cells[41]. SKMEL28 cells expressing either GFP or WNT5A were generated by infecting these cells with additional lentivirus constructs to express either GFP or WNT5A followed by selection with hyromycin. Melanoma reporter cells were generated by infection with lentiviruses harboring a β-catenin responsive firefly luciferase reporter driven by 12x TCF/LEF binding sites and a minimal TK promoter. Polyclonal cell lines were generated following infection by selection with puromycin at 2ug/mL. Reporter cells were also infected with additional lentiviruses harboring a constitutively active Renilla luciferase reporter driven by the EF1α promoter sequence.

Cell proliferation and viability assays

24-48 hours following transfection of the indicated siRNAs melanoma cells were trypsinized and counted and then re-plated for various growth assays. To assess two-dimensional growth 50,000 A375 or MEL624 cells were plated in multiple wells of a 24-well plate 24 hours after siRNA transfection in either normal growth medium or 25% control L-cell control, 25% WNT3A, or 25% WNT5A conditioned media prepared as previously described previously. At various time points following initial platting, cells were trypsinized and counted using a hemactyometer. All growth curves summarize three independent experiments performed with triplicate technical replicates for each time point and condition. For resasurine based viability assays, 2500-5000 cells were plated in 96-well plates 48 hours post siRNA transfection in normal growth media and allowed to grow for 48-72 hours. At this time the media was replaced with a rezasurine and cells were incubated for 2.5-3 hours in the tissue culture incubator before assessing rezasurine change on an Envision plate reader.

Three-dimensional cell culture

Soft agar assays were performed by embedding 100-1000 siRNA-transfected cells in 0.3% agarose over a 0.6% base agarose layer in 6-well plates. For siRNA experiments, 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 modifcations[14]. Briefly, 24 hours after transfecting melanoma cells with various siRNAs 5000 cells were plated in multiple wells of ultra-low attachment round bottom 96-well plates where they grew into melanospheres. Two days later, spheres were embedded in Collagen I and imaged 1-2 hours and 24 hours post-embedding. Melanosphere areas were determined using ImageJ to measure the diameter of each sphere from micrographs. All measurements were done blinded to avoid any biases.

In vivo tumorigenesis

50,000 A2058 cells expressing either GFP or WNT5A-IRES-GFP were injected subcutaneously into the flanks of Nude FoxN1$^{-/-}$ mice. Mice were checked three times a week and the presence of palpable tumors was determined. Tumor volumes were calculated using caliper measurements as previously described[15]. All animal procedures and care were approved by IACUC.

Co-immunoprecipitation and affinity purification

18-20 hours prior to immunoprecipitation 1.5x10^6-1.5X10^6 melanoma cells were transfected with 6 µg of each plasmid encoding FLAG and GLU-tagged proteins of interest using Lipofectamine LTX and Plus Reagent (Life Technologies Carlsbad, CA) according to the manufacturer's instructions. Cells were lysed in either 150 µl of IGEPAL -based

immunoprecipitation buffer (150 nM NaCl, 50 mM TrisHCl (pH 7.5), 1% IGEPAL CA-630, 0.1% Na-Deoxycholate) or with protease and phosphotase inhibitors added (Roche) and then cleared by centrifugation at 14,000 RPM. Cleared protein lysates were then combined with 10 µl of Steptavidin sepharose (Amersham) for one hour at 4C to pulldown GLU-tagged proteins. Streptavidin resin was washed with 5X1mL lysis buffer to remove non-specific proteins. Purified protein complexes were eluted using 1XNUPAGE loading buffer (Life Technologies) with β-mercaptoethanol added to 2.5% v/v final concentration. All immuno- and affinity-purification steps were performed on ice or at 4°C.

Western blotting

Cells were washed with ice cold PBS (pH 7.5), and then lysed prior to quantification of protein by BCA 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), anti-WNT5A/B (Cell Signaling #9562), anti-phospho-AKT (S473) (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-phopspho-PKCalpha/β (Cell Signaling), anti-PARP1 (Cell Signaling), anti-total GSK3β (Cell Signaling), anti-phospho-GSK3β (Millipore).

Analysis of gene expression

For microarray analysis of gene expression the RNA was isolated, using the Qiagen RNEasy protocol and quantitated using a Nanodrop Spectrophotomer (Agilent Technologies). About 750 ng of high-quality total RNA with RIN (RNA Integrated Numbers) greater than 8.0 was labeled with cyanine 5-CTP or cyanine 3-CTP, using the low RNA Input Fuorescent Linear Amplification Kit (Agilent Technologies) and purified on RNeasy Mini columns (Qiagen). Labeled RNA was hybridized to Agilent Human 44K expression arrays that includes 44,000 probes and compared to a labeled mixed reference. The mixed reference contained equal amounts of RNA from each of the4 7 melanoma cell lines were used for the mixed reference pool. The analysis of the microarray was done using Rosetta Biosoftware's Resolver system.. ROAST analysis was used to perform similarity searchers to determine the correlation between WNT5A expression and transcripts with similar expression patterns. All RNA isolation and expression microarray experiments were performed in triplicate

Chapter 6: Conclusions

Jamie N. Anastas

WNTs are a family of secreted glycoproteins, which play crucial roles in the regulation of diverse cell behaviors including, cell fate, proliferation, survival, differentiation, migration, and polarity. WNT signaling evolved early in eumetazoans as it is present in species as distantly related as hydra and humans. Disruption of the activity of the WNT ligands themselves, or of the proteins necessary for WNT signal transduction can lead to various diseases ranging from Alzheimer's disease to cancer. My graduate work has focused on trying to better understand the many roles of WNT signaling pathways in cancer. Despite intensive investigation, the WNT signaling field has not yet fully delineated the multifaceted roles for various WNT pathways in specific cancer subtypes and at different stages of cancer progression. Many fundamental questions central to determining if and how WNT signaling pathways regulate cancer development and progression remain:

1. What are the components of the β-catenin-dependent and -independent WNT signaling networks functioning in different cancer contexts?

2. What are the nodes of crosstalk between these major arms of WNT signaling and what are the nodes of crosstalk functioning at the interface between WNT signaling networks and other signal transduction pathways and molecular processes?

3. Does this extended signaling network differ between different cancer cell types and, if so, what endogenous and exogenous factors modulate WNT signaling in specific cancer subtypes and cancer stages?

The work presented in this thesis begins to address several of these important questions by identifying and characterizing the functional roles of several modulators of WNT signaling functioning in different contexts.

AGGF1: a novel regulator of β-catenin-dependent transcription in colorectal cancer

One of the main challenges facing researchers working to develop strategies to therapeutically target WNT signaling pathways in cancer is that the homeostatic maintenance of healthy adult tissues also requires the activity of WNT signaling pathways. Increasing our knowledge of cancer-specific modulators of various WNT signaling pathways will be instrumental in the development of more targeted therapeutics. The protein AGGF1, which we identified as a novel activator of β-catenin-dependent transcription in colorectal cancer in our near genomewide siRNA screen, provides one example of a context-specific modulator of WNT

signaling. Specifically, we first determined that AGGF1 regulates endogenous WNT target genes by analyzing the expression of AXIN2 and LEF1 in colon cancer cells with constitutively activated β-catenin signaling due to inactivating mutations in APC. We find that knockdown of either β-catenin (a positive control) or AGGF1 reduced the expression of these target genes by RT-PCR, validating the results of the RNAi screen. Interestingly, we find that AGGF1 enhances β-catenin dependent transcription in colorectal cancer cells, but does not regulate β-catenin target genes in either HEK293T embryonic kidney cells or in HT1080 fibrosarcoma cells (data not shown). These results suggest that AGGF1 can enhance β-catenin dependent transcription on colorectal cells, but not in other cell types.

We then sought to understand the mechanisms mediating AGGF1-dependent transcription of β-catenin target genes. AGGF1 was originally characterized as a pro-angiogenic, secreted protein[255]. However, AGGF1 protein complexes identified by affinity purification and mass spectrometry contain nuclear proteins like BAF57, a component of the SWI/SNF chromatin remodeling complex. To determine if AGGF1functions in the nucleus, we then stained colorectal cells with antibodies detecting AGGF1 and BAF57. We find that AGGF1 co-localizes with BAF57 in colorectal cell nuclei, which suggests that AGGF1 can also function in the nucleus. We then asked if AGGF1 can act as a transcription co-factor for β-catenin. The promoter sequences of the β-catenin target genes AXIN2 and LEF1 contain a number of TCF/LEF and β-catenin responsive cis-acting elements. We optimized chromatin immunoprecipitation protocols and were able to pull down DNA corresponding to these cis-acting elements using

both β-catenin antibodies and two independent antibodies recognizing AGGF1. Together, these data suggest that AGGF1 not only regulates β-catenin-dependent transcription in a context-dependent manner, but also that AGGF1 can function as either a secreted protein or as a nuclear protein in different cellular contexts. Whether a switch from acting as a secreted protein to functioning in the nucleus determines the ability of AGGF1 in modulate β-catenin-dependent transcription in cells derived from other sources, or whether these effects are unique to cells derived from APC mutant colorectal tumors is not yet clear.

SCRIB regulates breast cancer cell behavior in a context-dependent manner

My next project in the Moon lab focused on characterizing novel roles for planar cell polarity (PCP) proteins in breast cancer. This project stemmed from proteomics data identifying novel proteins associating with the PCP protein called SCRIB. We performed a series of co-immunoprecipitation experiments and found that SCRIB associates with at least two unique protein complexes: a SCRIB/ARHGEF7 complex and a SCRIB/NOS1AP/VANGL complex as determined by mass spectrometry. In order to further characterize the functional relevance of SCRIB and NOS1AP in breast cancer we generated stable cell lines expressing shRNAs to knockdown these proteins. Although SCRIB and NOS1AP shRNAs do not regulate breast cancer growth in vitro, knockdown of SCRIB inhibits the growth of xenograft tumors. We also observe that depletion of SCRIB, NOS1AP and VANGL with shRNAs slows migration and prevents the

establishment of leading-trailing polarity in breast cancer cells by performing a series of migration assays and analyzing the localization of polarity proteins by confocal microscopy.

These data were surprising because they are in direct contradiction to other studies finding that SCRIB acts as a suppressor of tissue overgrowth in Drosophila melanogaster models of tissue hyperplasia and in mouse models of mammary tumorigenesis[2,3]. One possible explanation for these contradictions is that SCRIB acts to promote epithelial integrity and to prevent tumor growth in early stage tumors, but may conversely enhance the growth and metastatic potential of cancer cells that have begun to lose their epithelial character. Interestingly, we were able to stably express NOS1AP in MDA-MB-231 breast cancer cells which exhibit a mesenchymal-like phenotype, but we were not able stably express NOS1AP in MCF10a mammary cells, which exhibit an epithelial phenotype. Although the mechanisms controlling this potential switch from tumor suppressive to pro-tumorigenic function for SCRIB and SCRIB related proteins are not yet fully understood, these further studies raise the possibility that this transition from a pro-tumorigenic to an anti-tumorigenic role for SCRIB may involve the association between SCRIB and unique protein complexes in different contexts.

Unexpectedly, our analysis of breast cancer patient gene expression profiles concludes that low SCRIB transcript levels are predictive of patient survival. This data suggests that SCRIB's role in regulating signaling pathways necessary for cell migration/polarity trumps its tumor suppressor effects when it comes to mammary carcinomas. WNT5A protein levels are also predictive of breast cancer survival, but unlike SCRIB, greater WNT5A protein levels are

associated with better patient survival[401]. Ultimately, controlled levels of multiple proteins involved in PCP signaling including WNT5A, SCRIB, and, potentially, other factors may be necessary for the maintenance of normal epithelial polarity in breast cancer cells, the loss of which leads to metastasis and poor prognosis. Several lines of evidence lead me to hypothesize that SCRIB may be acting as an important downstream effector for WNT5A. Previous studies have characterized PCP-like phenotypes associated with the loss of SCRIB function in vertebrates. Specifically, SCRIB loss of function alleles cause severe defects in neural tube closure (cranioschisis) and a loss of normal polarity in the inner ear in mice[287–289], while loss of Scrib results in convergent extension defects, and impaired motor neuron migration in zebrafish[290]. Very similar developmental defects are also observed in both zebrafish and mice when WNT5 or WNT11 activity is disrupted[332,402,403]. The striking similarity between SCRIB and WNT5/WNT11 phenotypes raises the possibility that SCRIB and SCRIB-associated protein complexes may play an important role in regulating the activity of a WNT/PCP signal transduction network the context of breast cancer.

A WNT5A/FZD7/RYK/AKT pathway and the RAS/RAF/MAPK pathway cooperate to promote melanoma growth and survival

My most recent work involves trying to understand a potential connection between WNT5A and drug resistance in melanoma. Melanomas rely on hyper-activation of the RAS-RAF-MEK-ERK signaling pathway for their growth and survival. In a majority of melanomas this

constitutive signaling is traceable to a highly specific mutation in a single protein kinase, $BRAF^{V600E}$, which can be inhibited by Vemurafinib, also known as PLX4032 (PLX). In clinical trials, PLX treatment initially reduces tumor burden, but patients invariably develop resistance to PLX treatment. Previous expression microarray profiling studies find that melanoma cell lines that are intrinsically insensitive to PLX express high levels of WNT5A. These data led me to ask whether WNT5A expression is also increased in melanoma cells exhibiting de novo resistance to chronic $BRAF^{V600E}$ inhibition by PLX. We generated resistant cell lines by chronically treating melanoma cells with PLX for more than 10 weeks. We then analyzed the expression of WNT5A in these cells and was surprised to observe a >10 fold increase in WNT5A protein in the resistant versus untreated cells. Intrigued by this result, we contacted our collaborators at the University of Sydney who have analyzed gene expression in patient tumors before PLX treatment and in relapsed tumors arising when patients stopped responding to PLX. Indeed, WNT5A expression is increased dramatically in subsets of PLX-resistant tumors.

These results support the hypothesis that WNT5A signaling contributes to unfavorable therapeutic responses in melanoma. We then determined whether WNT5A functionally promotes the viability of melanomas exhibiting either intrinsic or acquired resistance to $BRAF^{V600E}$ inhibition. We used siRNAs to reduce the levels of endogenous WNT5A in melanoma cell lines exhibiting both intrinsic PLX resistance and acquired PLX-resistance due to long-term drug treatment and monitored cell growth and viability. As we predicted, WNT5A siRNAs reduce the viability of all melanoma cell lines tested. To validate the specificity of these siRNAs

we treated melanoma cells with exogenous WNT5A in the media and found that adding back WNT5A could rescue decreased cell numbers seen following WNT5A knockdown. We also find that WNT5A siRNAs reduce colony formation in soft agar and similarly reduce the size of tumor spheroids by performing a series of 3D culture assays. To ask whether WNT5A is sufficient to promote melanoma proliferation, we transduced melanoma cells with lentiviruses and find that exogenous WNT5A promotes melanoma growth in soft agar, increases clonogenic outgrowth from single cells and reduces tumor free survival in mouse xenograft models. We then investigated whether loss of WNT5A might increase the sensitivity of melanoma cells to PLX. WNT5A siRNAs do not induce apoptosis on their own, but approximately double the number apoptotic cells induced by PLX treatment in comparison to control siRNA as measured by western blotting for apoptotic markers and TUNEL assays. Finally, we note that increased WNT5A observed in the PLX-resistant cells also promotes viability, as knocking down WNT5A in resistant cells reduced cell numbers by ~50%.

We next asked whether WNT5A enhances melanoma cell growth via kinase-dependent signaling pathways using phospho-specific antibodies to analyze melanoma cell lysates by western blotting. We observe that knocking down WNT5A delays both PKC phosphorylation (a positive control) and also inhibits AKT phosphorylation in response to serum stimulation, but has no effect on the phosphorylation of ERK. We also observe increased levels of phospho-AKT and phospho-PKC in cell lines overexpressing WNT5A, as well as increased levels of phospho-AKT in PLX-resistant melanoma cells. We performed additional experiments to show that small

molecule inhibitors of the PI3K/AKT pathway reduce the viability of melanoma cells exhibiting both intrinsic and acquired resistance to PLX. Together, these data suggest that WNT5A/AKT signaling promotes the viability of a variety of melanoma cells including drug-resistant subpopulations.

Previous studies have identified multiple putative WNT5A receptors that act in a cell and tissue type-dependent manner. We took a targeted approach to identify potential WNT5A receptors in melanoma by screening siRNAs against more than a dozen different proteins and eventually found that multiple siRNAs targeting the WNT receptors, FZD7 and RYK reduce cell viability. Like WNT5A siRNAs, depletion of either FZD7 or RYK inhibits anchorage-independent growth of melanoma cells and growth in tumor spheroids. We also tested whether loss of RYK or FZD7 can mediate melanoma cell response to PLX. Like WNT5A siRNAs, RYK and FZD7 siRNAs enhance the extent of apoptosis in sensitive melanoma cells responding to short term PLX treatment and also reduce the viability of long-term PLX resistant cells. Our observation that WNT5A enhances AKT signaling predicts that receptors for WNT5A, such as FZD7 and RYK, will also promote AKT phosphorylation. As expected, we find that overexpression of either FZD7 or RYK enhances AKT phosphorylation, while siRNAs targeting RYK and FZD7 reduce AKT phosphorylation.

Together, these studies suggest that a WNT5A/FZD7/RYK/AKT pathway contributes to the viability of melanoma cells exhibiting both intrinsic and acquired resistance to PLX. These results also identify WNT5A signaling as an important new therapeutic target in drug-resistant

melanoma. Although it is still challenging to target WNT5A pharmacologically, these 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. Alternatively, future studies may also determine if WNT5A can serve as a biomarker to predict PI3K/AKT pathway activity in melanoma. Based on 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. In the future, it will be interesting to determine if WNT5A expression can predict patient response to the PI3K and AKT inhibitors that are the subject of investigation as possible therapeutics for metastatic melanoma in several clinical trials.

Final thoughts

In the past decade, we have witnessed an explosion in the development of strategies to targeting these WNT signaling. Many synthetic modulators of WNT signaling including, small molecules, peptides and blocking antibodies show great promise in animal models of several different cancers. Importantly, activating or inhibiting WNT signaling pathways alone is unlikely to result in significant improvement in disease progression due to co-activation of numerous oncogenic pathways in most cancers. Further research is clearly necessary to not only optimize these reagents for applications in animals and eventually in human patients, but also to further

explore the potential value of combinatorial therapies. Studies aimed at identifying genetic factors and biomarkers that can be used to predict response to treatment with WNT pathway modulators either alone or in combination with other therapies will be an important next step in determining the utility of these potential new therapies. Despite these future challenges, many studies including the work presented in this dissertation suggest that evidence-based targeting WNT signaling pathways in cancer patients will be both a possible and potentially effective strategy in the near future.

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