# Resolving mechanisms of apoptosis in response to WNT3A in Melanoma

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Abstract

Resolving mechanisms of apoptosis in response to WNT3A in Melanoma

Chair of the Supervisory Committee: Professor Randall T. Moon

Department of Pharmacology

Wnt/β-catenin and MEK/ERK signaling regulate the balance of differentiation and proliferation in melanoma. Aberrant activation of MEK/ERK signaling results from the BRAFV600E or NRASQ61X mutations in greater than 70% of primary melanoma samples. Previous data have shown that BRAF inhibition in combination with Wnt/β-catenin activation promotes robust melanoma apoptosis in a subset of melanomas. This finding raised the question of what factors predict sensitivity to apoptosis in response to WNT3A and how Wnt/β-catenin signaling is regulated in melanoma. My thesis research addresses this question by testing if Wnt/β-catenin signaling can promote apoptosis in *BRAF* and *NRAS*-mutant backgrounds, and it addresses how Wnt/β-catenin signaling is regulated in melanoma using combinatorial screening techniques. Here, I present evidence that combined MEK/ERK pathway inhibition and Wnt/β-catenin activation leads to apoptosis in *NRAS*-mutant melanomas. Additional experiments demonstrate that the stability AXIN1 protein, a negative regulator of Wnt/β-catenin signaling, is required for resistance to apoptosis. Finally, I use high throughput screening to discover

FAM129B as a novel positive regulator of Wnt/β-catenin signal transduction and the apoptotic response to WNT3A in melanoma. Collectively, these data expand the genetic contexts by which melanomas undergo apoptosis following WNT3A treatment and MEK inhibition, and further resolves Wnt/β-catenin pathway regulation in melanoma. The findings presented here highlight the importance of AXIN1 depletion across multiple mutational backgrounds, and identify numerous putative regulators of Wnt/β-catenin signaling which may ultimately regulate apoptosis in melanoma.

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# Dedication

I dedicate this work to my family.

# Chapter 1: Introduction

## Overview

Melanoma is a devastating disease responsible for 80% of deaths resulting from skin cancer.[1] The 5-year survival rate of 15% illustrates the poor prognosis for melanoma patients with metastatic disease.[2] Making matters worse, melanoma incidence continues to rise at a rate faster than any other cancer.[3] Our growing knowledge of the molecular drivers of melanoma provides perhaps the best hope for identifying novel therapeutics to this disease. Recently, the first mutation-targeted chemotherapeutic agent for any cancer, vemurafenib (trade name Zelboraf), has exhibited promising results in the treatment of patients with metastatic melanoma. Vemurafenib (previously called PLX4032) specifically inhibits a constitutively active, mutant form of BRAF,[4–7] an oncogene that helps drive approximately 50% of melanoma.[8,9] While this revolutionary targeted approach to cancer therapy resulted in tumor reduction in almost all treated patients, half of all patients relapsed within 6 months. Patients treated with vemurafenib exhibited a median duration of progression-free survival of 7 months and an increase in 2-year overall survival to 23.5%.[10–12] While these results are extremely promising compared with previous melanoma therapies, the universal development of resistance indicates that monotherapy is unlikely to produce clinically durable outcomes. The potent effect of targeted BRAF inhibitors on melanoma cells provides a context where additional therapeutic strategies applied in parallel or in series could lead to durable clinical outcomes.

Recent evidence suggests that Wnt/β-catenin pathway activation could be just such an additional therapeutic strategy.[13–16] Wnt/β-catenin pathway activation alone slows melanoma growth *in vitro* and *in vivo.* It promotes the expression of markers of differentiation. In

combination with vemurafenib, it promotes apoptosis *in vitro* and *in vivo* in a subset of melanomas. However, it needs to be determined which melanomas are sensitive to Wnt/β-catenin pathway activation and how Wnt/β-catenin signaling is regulated in melanoma.

My thesis research focuses on identifying under what genetic backgrounds Wnt/β-catenin signaling acts to promote apoptosis and how this pathway is regulated in melanoma in the hopes that identifying new genetic backgrounds where Wnt/β-catenin signaling promotes apoptosis and new regulators of the pathway will ultimately result in new and effective therapies for this intractable disease.

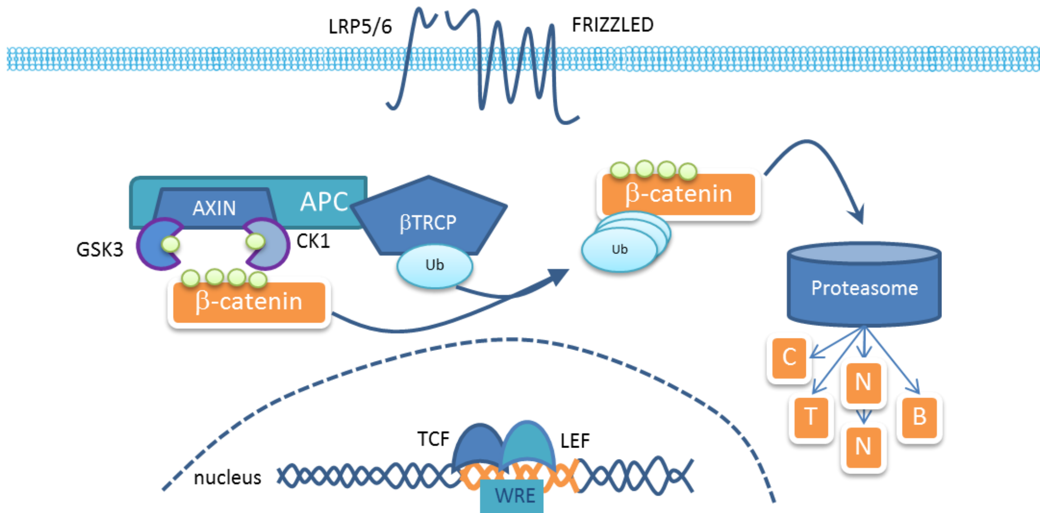
This chapter establishes the background of these two research questions. It describes the Wnt/β-catenin signal transduction pathway and its roles in melanoma. It compares two genotypically and phenotypically distinct melanoma subtypes – *BRAF*- and *NRAS*-mutant melanoma. Finally, it establishes my two research questions. First, does Wnt/β-catenin signaling promote apoptosis in both *BRAF*- and *NRAS*-mutant melanomas? Second, What other proteins regulate Wnt/β-catenin signaling in melanoma?

## Wnt/β-catenin signal transduction pathway and its roles in melanoma pathogenesis

In order to describe the role of Wnt/β-catenin signaling in melanoma, it is first important to describe our understanding of Wnt/β-catenin signal transduction. The human genome encodes 18 Wnts,[17] 10 Frizzled receptors,[18] 2 LRP5/6 coreceptors,[17] and several other Wnt receptors.[18] Through this diverse assortment of ligands and receptors, the signal that a Wnt transduces depends on receptor expression, that Wnt's affinity for a given receptor, and the coreceptors it binds. The overwhelming majority of the evidence suggests that the Wnt/β-catenin pathway is

activated via Wnt interaction with LRP5/6 and Frizzled.[19] Other Wnt receptor interactions have important phenotypes and may even play a role in melanoma pathogenesis.[17,20] However, this thesis focuses on Wnt/β-catenin signal transduction and its role in melanoma pathogenesis.

Absent an activating Wnt ligand, β-catenin is phosphorylated and degraded by the Wnt/β-catenin destruction complex (for review see ref. 21). This complex is comprised of the adaptor proteins AXIN and APC, which bind to β-catenin.[22,23] Axin binds the kinases glycogen synthase kinase 3 alpha or beta (GSK3)[22] and casein kinase I alpha or epsilon (CKI).[24] CKI acts as a priming kinase for GSK3, recognizing a specific phosphorylation site at serine 45 (S45) of β-catenin.[25,26] Once phosphorylated by CKI, GSK3 recognizes β-catenin and phosphorylates it at T41, S37, and S33.[26–28] The ubiquitin ligase βTRCP recognizes phosphorylated β-catenin, ubiquitinating it. Ubiquitinated β-catenin then becomes proteasomally degraded (Figure 1).[29–33]



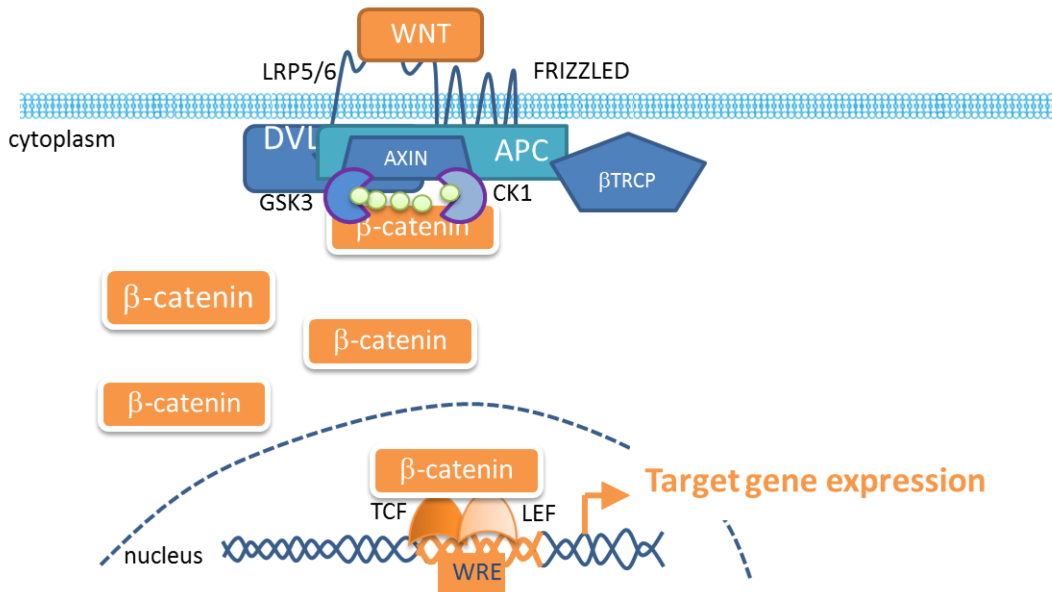




Figure 1 - The Wnt/β-catenin signal transduction pathway. (A) In the absence of a Wnt ligand, cytoplasmic β-catenin is recognized by the β-catenin destruction complex, comprised of the adaptor proteins AXIN and APC. β-catenin is phosphorylated by the kinases CK1 and GSK3, and is ubiquitinated by βTRCP. Polyubiquitinated β-catenin is degraded by the destruction complex. (B) In the presence of a Wnt ligand, the destruction complex becomes inhibited and localizes to the plasma membrane. β-catenin accumulates in the cytoplasm, translocates to the nucleus, and directs target gene expression by binding the transcription factors TCF and LEF and displacing the transcriptional repressor, groucho. TCF and LEF bind a specific sequence, the Wnt responsive element, giving β-catenin signaling specificity for certain target genes, and allowing us to build specific reporters of Wnt/β-catenin signaling based on that WRE.

In the presence of an activating Wnt ligand, Wnt binds the Frizzled and LDL receptor proteins 5 or 6 (LRP5/6) co-receptors.[34–37] The adaptor proteins disheveled becomes phosphorylated and localizes to the membrane along with the destruction complex.[38,39] APC becomes phosphorylated,[40] and AXIN becomes dephosphorylated.[41,42] Importantly, dephosphorylation decreases AXIN1 stability and it degrades. [41,42] AXIN1 is seen as the rate limiting component of the destruction complex.[43,44] With the destruction complex inhibited and localized to the membrane, β-catenin no longer becomes phosphorylated.[45] It is important to note that the destruction complex does not disassemble. Rather it becomes inhibited by localizing to the plasma membrane and interacting with LRP5/6.[45] β-catenin accumulates in the cytoplasm, translocates to the nucleus and binds to the transcription factors T-cell Factor 1 (TCF7) and lymphoid enhancer factor (LEF1). These transcription factors bind to the core consensus DNA sequence (5'-AGATCAAAGGG-3').[46–50] Via this mechanism, β-catenin directs transcription of specific target genes (see Figure 1).

One target gene that has been shown to be broadly expressed in response to nuclear β-catenin accumulation is *AXIN2*.[51–53] As mentioned above, AXIN expression is the rate-limiting step in controlling β-catenin via the destruction complex. While degradation of AXIN1 following Wnt/β-catenin treatment acts as a feedforward mechanism directing transcription,[41,42] transcription of *AXIN2* acts as a common feedback mechanism to inhibit Wnt/β-catenin signaling.[54]

Expression of other Wnt target genes varies by cell type. For instance, in the developing embryo Wnt/β-catenin signaling is required for establishment of the anterior /posterior axis and dorsoventral axis.[55] To establish the vertebrate dorsal organizer Wnt/β-catenin signaling drives expression of target genes *Siamois and Twin.*[56–60] In adult tissue homeostasis the same pathway

drives expression of completely different genes. For instance, Wnt/β-catenin signaling acts as a proliferative signal to replace sloughing tissue in the colon driving expression of target genes like *CMYC.*[61,62]

*A protective role for Wnt/β-catenin signaling in melanoma*

Evidence from development and disease suggest that Wnt/β-catenin signaling plays a protective role in melanoma. During melanocyte development, Wnt/β-catenin signaling is necessary and sufficient to drive neural crest cells toward a melanocyte cell fate, in large part through direct regulation of transcriptional targets, such as microphthalmia transcription factor (encoded by *MITF*).[39–45] In zebrafish, most lateral neural crest cells coinjected with *β-catenin* mRNA and GFP adopt melanocyte and cartilage fates, whereas lateral neural crest cells injected with GFP alone become neurons and glia.[63] Furthermore, medial neural crest cells, which are exposed to endogenous Wnts, normally become pigment cells.[63,70,71] When Wnt/β-catenin signal transduction is inhibited via dominant-negative TCF mRNA, these cells adopt neural fates.[63]

As mentioned above, Wnt/β-catenin signaling directs proliferation or differentiation of cells by regulating expression of specific target genes. In the neural crest Wnt/β-catenin signaling drives expression of the melanocyte lineage specific transcription factor *MITF*. The zebrafish homolog to *MITF* is *nacre.* Microinjection of a DNA construct of the *nacre* promoter driving the *nacre* gene product is sufficient to rescue loss of melanocytogenesis. However, mutation of the TCF/LEF sites present in the *nacre* construct promoter prevents rescue of melanocyte differentiation.[65]

During melanoma pathogenesis, the observed presence of nuclear β-catenin in the majority of benign nevi, along with the loss of nuclear β-catenin seen with melanoma

progression,[72–74] supports the hypothesis that maintenance of homeostatic levels of Wnt/β catenin signaling is important in this particular context. In a histological analysis of 202 nodular malignant melanomas, primary tumors lacking nuclear β-catenin exhibited a significantly deeper invasive depth compared to those bearing nuclear β-catenin (4.2 vs 2.9 mm, respectively, $P$=0.002).[72] In an analysis of 50 benign melanocytic nevi, 91 primary and 50 metastatic melanomas, it was observed that nuclear β-catenin decreases with disease progression. Histological analysis revealed that, 40% of benign melanocytic nevi bore nuclear β-catenin expression, while only 10 and 15% of primary and metastatic melanomas bore nuclear β-catenin. In this study, nuclear β-catenin significantly negatively correlated with lesion thickness, the strongest marker of disease progression ($P<0.0001$).[73] In agreement with the observation that lesion thickness inversely correlates with nuclear β-catenin, a study of 106 superficial melanomas revealed cytoplasmic localization of β-catenin correlates with disease free survival ($P<0.0006$).[74] These data provided the first evidence for a protective role for Wnt/β-catenin signal transduction in melanoma.

Subsequently, additional evidence connected cytoplasmic and/or nuclear β-catenin found in patient tumor biopsies to increased patient survival, decreased metastasis recurrence and decreased expression of proliferation markers.[13,72,75] A histological survey of 118 primary tumors and 225 recurrences / metastases revealed that nuclear β-catenin correlates with survival.[13] Automated immunohistochemical analysis of these primary tumors demonstrated that patients with metastatic tumors bearing high nuclear β-catenin survived significantly longer than patients with low nuclear β-catenin.[13] Strikingly, nuclear β-catenin inversely correlated to Ki67 positivity, a marker for proliferation, indicating that in melanoma, nuclear β-catenin suppresses proliferation.[13] A separate immunohistochemical analysis of over 500 patient samples using 70

biomarkers identified β-catenin as one of only two protective markers that were shown to correlate with recurrence-free survival.[75] Furthermore, a 7 biomarker signature including β-catenin derived from this cohort predicted improved survival in a separate cohort of patient samples.[75]

While the clinical data described above shows a strong correlation between Wnt/β-catenin signaling and survival in melanoma, additional experimentation shows activation of Wnt/β-catenin signaling can affect disease outcomes. High Wnt/β-catenin signaling mediated by overexpression of WNT3A inhibits tumor growth in mouse models of melanoma, accompanied by an increased expression of melanocyte-associated gene targets.[13,15] 4-day Stable overexpression of WNT3A in mouse B16 cells significantly reduces cell number *in vitro*,[13] as does WNT3A conditioned media.[15] Furthermore, allograft of B16 cells overexpressing WNT3A resulted in smaller tumor size and reduced tumor metastases compared to GFP expressing cells *in vivo*.[13] These findings provided early evidence that manipulating Wnt/β-catenin signal transduction affects melanoma growth. These data from development and cancer support a model whereby Wnt/β-catenin pathway activation suppresses proliferation and drives differentiated phenotypes in melanoma (see Figure 2). These findings suggest a conserved role for Wnt/β-catenin signaling in melanocytes and melanoma.

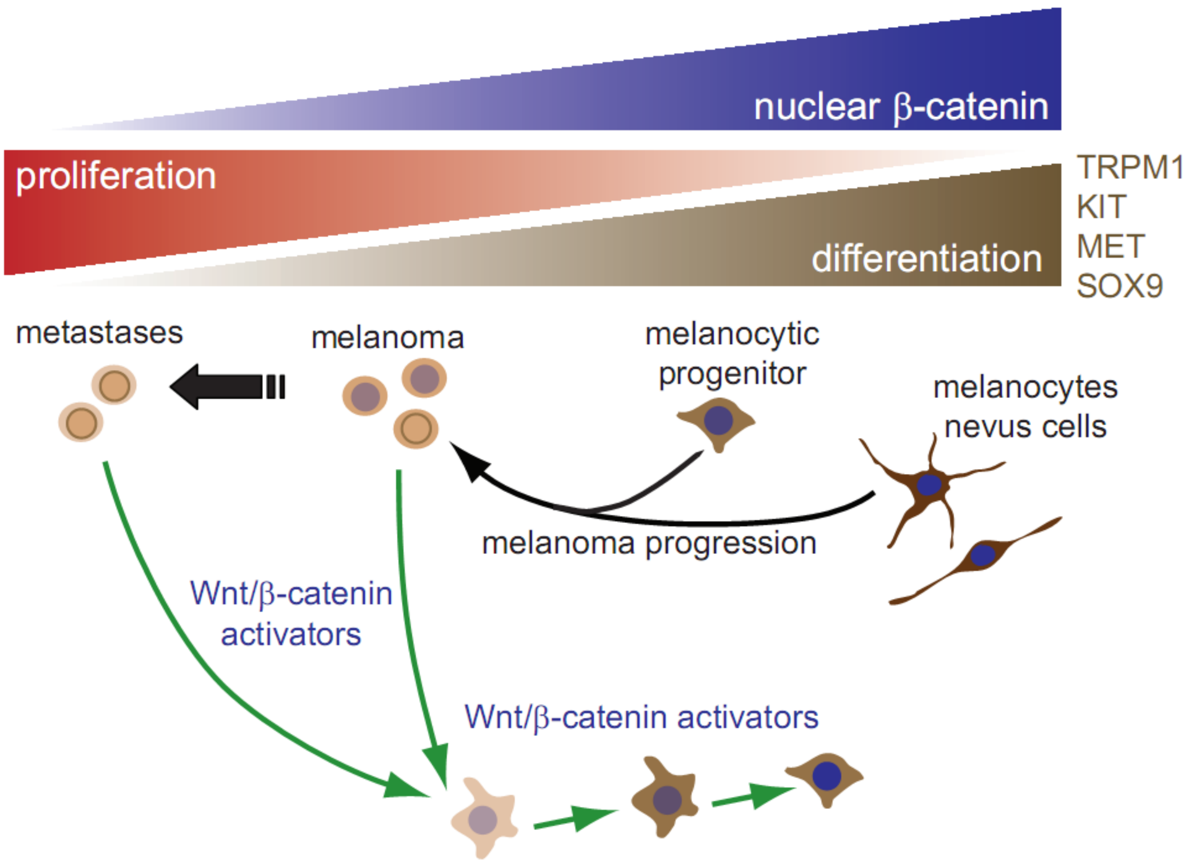



Figure 2 – Wnt/β-catenin signaling increases differentiation and decreases differentiation in melanocyte development and in melanoma. Wnt/β-catenin signaling drives the differentiation of melanocytic progenitors of the lateral neural crest into melanocytes during development.[63,65] Nuclear β-catenin is lost during melanoma progression.[73,74] Activation of Wnt/β-catenin signal transduction decreases proliferation of melanomas and promotes expression of markers of melanocyte differentiation.[13–15]

*An oncogenic role for Wnt/β-catenin signaling in melanoma*

Melanoma is a heterogenous disease. Seemingly contradictory evidence suggests that Wnt/β-catenin signaling plays a rare oncogenic role in melanoma, possibly by promoting tumor initiation. Numerous studies have shown that β-catenin is rarely mutated in melanoma (range 1-5%),[76–78,8,9] with a few exceptions (~25% in one study).[79] Nonetheless, experimental genetic activation of Wnt/β-catenin signaling indicates a capacity for β-catenin mutation to immortalize melanocytes.[80] 95% of melanocyte cell lines derived from stable β-catenin expressing mouse melanocytes proliferated in culture for over 15 weeks, indicating a 95% immortality rate. 31% of wild-type melanocytes proliferated over 15 weeks.[80] None of these melanocytes grew solid tumors when allografted into mice, indicating immortalization, but not complete transformation.[80] Additionally, genetic stabilization of β-catenin in melanocyte precursors cooperates with gain-of-function *NRAS* mutation to promote melanomagenesis.[80] Mice expressing NRAS under the melanocyte-specific tyrosinase promoter experience an incidence of melanoma of less than 30% with a latency of 54+/-21 weeks. Mice expressing NRAS and β-catenin under the tyrosinase promoter experience an incidence of melanoma of 85% with a significantly reduced tumor latency of 27.6 +/- 6.7 weeks.[80]

At least three potential models could explain the discrepancy between evidence linking activating Wnt/β-catenin signaling with improved outcomes in melanoma and evidence indicating an oncogenic role for Wnt/β-catenin signaling in melanoma. The first model is a biphasic role for Wnt/β-catenin signaling where Wnt/β-catenin signaling promotes tumor initiation, and loss of Wnt/β-catenin signaling promotes progression. The high levels of nuclear β-catenin in benign nevi,[72,73] the tumor initiating capacity of β-catenin in conjunction with

NRAS,[80] the reduced proliferation rate of *CTNNB1*-mutant melanoma,[80] and the loss of nuclear β-catenin during tumor progression support a biphasic role for melanoma.[72,73]

The second model predicts that Wnt/β-catenin mutant tumors arise more frequently from hair follicle melanocytes than from dermal stem cell derived melanoctyes. Humans harbor stem cells in both the dermis of the skin and the hair follicle, which can differentiate into melanocytes.[81,82] While the origin of melanoma remains unclear, it is known that follicular melanoma occurs less frequently than cutaneous melanoma.[83] Mice have less skin pigmentation than humans and the majority of their melanocyte-producing stem cells exist in the hair follicle.[81] While mice expressing β-catenin and NRAS under the tyrosinase promoter get melanoma, these melanomas mostly arise from hair follicle melanocytes.[80] It is possible that that β-catenin and NRAS drive melanoma more commonly in follicular melanoma than other melanomas. More epidemiological research is needed to determine if β-catenin mutation correlates with origin of melanoma.

The third model suggests that β-catenin mutation arises preferentially in a subset of melanomas, like those harboring microsatellite instability and that β-catenin promotes apoptosis in a different subset of melanomas. β-catenin or *AXIN2* mutation occurs preferentially in colorectal cancer with high levels of microsatellite instability.[84–91] Similarly, individuals carrying germline mismatch repair mutations who are at risk for microsatellite instability (those with hereditary nonpolyposis colorectal cancer), are at increased risk for both colorectal cancer as well as melanoma.[92] Preliminary evidence indicates that *CTNNB1* mutant and *APC* mutant melanoma concomitantly harbor microsatellite instability via mismatch repair defects.[93] In a panel of melanomas with known mismatch repair status, both melanoma harboring activating

Wnt pathway mutations also harbored mismatch repair mutations driving microsatellite instability.[93]

*Wnt/β-catenin signaling promotes apoptosis in BRAF-mutant melanoma*

Evidence for negative regulation of Wnt/β-catenin signaling by RAF/MEK/ERK signaling in melanoma led to the discovery of a pro-apoptotic role for Wnt/β-catenin signaling in melanoma. A kinome-wide siRNA screen for regulators of Wnt/β-catenin signaling strongly implicated RAF/MEK/ERK signal transduction as a negative regulator of Wnt/β-catenin signal transduction in *BRAF* mutant A375 melanoma cells.[14] Pharmacological inhibition of RAF/MEK/ERK signaling using the targeted BRAF inhibitor PLX4720 (a small molecule closely related to Vemurafenib), or using the MEK inhibitors U0126 or AZD6244, dose-dependently enhances Wnt-dependent transcription as measured by reporter assay and by *AXIN2* transcripts in A375 cells.[14,16] Likewise, silencing *BRAFV600E* by siRNA-mediated knockdown also enhances Wnt-dependent transcription as measured by reporter assay and target gene expression.[14] Following combination MEK/ERK pathway inhibition and WNT3A treatment, A375 cells lose AXIN1 protein in a transcription independent, and proteasome dependent manner, as revealed by Western blot, explaining a mechanism for Wnt/β-catenin pathway hyperactivation.[14,16]

BRAF inhibition promotes apoptosis in melanoma,[94] so it was tested if modulating Wnt/β-catenin signaling affects this phenotype. Combinatorial activation of Wnt/β-catenin signaling and inhibition of MEK/ERK signaling by BRAF inhibitor or MEK inhibitor results in increased apoptosis in A375 melanoma cells. 24 hr co-treatment of A375s with WNT3A and the targeted BRAF inhibitor PLX4720 results in increased apoptosis as measured by TUNEL staining. Similarly, Quantitative flow cytometry analysis of the apoptosis initiation marker

cleaved caspase 3 reveals that WNT3A treatment and PLX4720 treatment alone result in 1.73 and and 7.30% cleaved caspase positivity respectively, while the combination treatment results in around 37% cleaved caspase 3 positivity.[14] WNT3A also increases apoptosis in 3D culture. Co-treatment of spheroid cultures of A375s with PLX4720 and WNT3A results in strong ethidium bromide staining, while individual treatment or control treatment has no effect.[14] Wnt/β-catenin signal transduction proves to be required for PLX4720 mediated apoptosis. At 24 hr, PLX4720 treatment of A375 melanoma cells results in some level of apoptosis, as assayed by cleaved caspase 3 immunoblot. Silencing β-catenin expression by siRNA inhibits apoptosis, as monitored by cleaved caspase 3 immunoblot.[14] Combination of PLX4720 treatment and WNT3A treatment results in increased expression of the pro-apoptoic protein $BIM_s$, providing a possible mechanism by which WNT3A treatment promotes apoptosis in melanoma.[14]

Despite the initial observations that BRAF/MEK/ERK inhibition negatively regulates Wnt/β-catenin signaling and promotes apoptosis in combination with WNT3A, not all *BRAF*-mutant melanomas respond equivalently to combination WNT3A treatment and PLX4720 treatment. A subset of *BRAF*-mutant melanomas does not undergo apoptosis following WNT3A treatment.[14] Both negative regulation of Wnt/β-catenin signaling by MEK/ERK signaling and loss of AXIN1 predict sensitivity of *BRAF*-mutant melanomas to apoptosis.[14,16] Melanomas, which undergo significant apoptosis as monitored by cleaved caspase 3 FACS, also show significantly higher expression of the Wnt-target gene *AXIN2*, when treated with WNT3A and PLX4720.[14] Quantification of AXIN1 levels by immunoblotting also reveals that melanomas that do not lose AXIN1 in response to combination WNT3A and PLX4720 remain insensitive to apoptosis in the presence of WNT3A.[14]

Nonetheless, resistance to the effect of WNT3A on apoptosis can be overcome. While *BRAF*-mutant melanomas respond heterogeneously to WNT3A treatment and PLX4720 treatment, these melanomas respond consistently to PLX4720 treatment and *AXIN1* siRNA.[14,16] siRNA-mediated knockdown of AXIN1 in combination with PLX4720 treatment in resistant SK-MEL-28 cells results in increased cleaved PARP relative to control treatments as assayed by immunoblot.[14] *AXIN1* siRNA increases apoptosis in combination with PLX4720 to a similar degree as WNT3A as measured by cleaved caspase 3 flow cytometry. Addition of WNT3A treatment does not greatly enhance apoptosis in these lines.[14] Taken together, these findings indicate that AXIN1 expression serves as the central regulator of the apoptotic response to WNT3A treatment and MAPK pathway inhibtion in *BRAF*-mutant melanoma.

## Comparing *BRAF*- and *NRAS*-mutant melanoma

The discrepancy between Wnt/β-catenin signaling's positive and negative role in melanoma growth highlights the importance of determining in which cellular contexts Wnt/β-catenin signaling promotes apoptosis and in which cellular contexts it promotes proliferation. Mutational status is one factor that affects melanoma pathogenesis. Oncogenic *BRAF* and *NRAS* mutations are among the most common mutations in melanoma. Approximately 50% of melanomas harbor *BRAF* mutations,[8899] while approximately 20% of melanomas harbor *NRAS* mutations.[95,96] These mutations for the most part occur in different melanomas, and only rarely are found in the same melanoma.[96–98] Second, *BRAF* and *NRAS* mutant melanomas differ phenotypically.[97,99] Patients with *NRAS* mutant melanoma experience shorter survival and more aggressive disease.[97,98] Third, *BRAF* and *NRAS* melanomas respond differently to identical therapies.[99,100] In the subsequent three sections, this introduction will describe *BRAF* and *NRAS*

mutant melanomas and their differences and will establish the hypothesis that these differences explain sensitivity of melanomas to WNT3A treatment.

*Oncogenic BRAF mutation in melanoma*

The BRAF kinase belongs to the RAF family of kinases.[101–105] Humans have three RAF kinases, ARAF, BRAF, and CRAF.[101–105] BRAF is the most frequently mutated oncogene in melanoma.[88,9] 50% of patient samples bear oncogenic BRAF mutations.[88,9] In rare cases, ARAF and CRAF are observed.[88,9] The most common mutations in the BRAF proto-oncogene occur in exon 15.[88,9] The most common mutation translates to V600E, comprising 80% of all known oncogenic BRAF mutations.[9,104,106,107] Another 16% and 3% of melanomas harbor BRAFV600K and V600D/R, respectively.[108] Other more rare activating mutations occur in Exon 11 and also result in BRAF activation, including BRAFG466E, BRAFG466R/V, and BRAFG469R.[8,9,109]. These mutations activate BRAF/MEK/ERK signaling more modestly (2.5 fold vs. >10-fold for V600E).[109] The BRAFV600E mutation occurs in the activation domain of the kinase resulting in a consitutively active mutant kinase.[108]

BRAF signals primarily through the MEK/ERK pathway. BRAF is classified as a mitogen activated protein kinase kinase kinase (MAP3K) in this cascade. BRAF phosphorylates MEK, which in turn, phosphorylates extra-cellular signal related kinase / mitogen activated protein kinase (ERK/MAPK). The kinases ERK1 and ERK2 (ERK1/2) translocate to the nucleus, where the phosphorylate numerous transcription factors including CRAF, c-fos, c-myc, AP2A, MITF, and ELK[110–113] thereby regulating target gene expression. These target genes have multiple effects (described below) including promoting proliferation and inhibiting apoptosis (for review see Dhillon AS *et al.* 2007)[114].

In addition to regulating transcription, hyperactive BRAF exerts its pro-proliferative and anti-apoptotic effects through both transcriptional and post-translational mechanisms.[115] For instance, MEK/ERK signaling affects both gene expression and cell cycle protein phosphorylation to promote proliferation. Activated ERK1/2 phosphorylates transcription factors including CREB, c-fos, c-myc, and ELK.[110–113] These transcription factors drive expression of multiple cell cycle checkpoint regulators including CyclinA and CyclinD1.[110–112] MEK/ERK pathway activation also regulates the cell cycle posttranslationally. Activated RAF phosphorylates and activates the phosphatase Cdc25, which proceeds to dephosphorylate and stabilize cell cycle proteins, including cyclin-dependent kinases.[116]

BRAF signaling suppresses apoptosis through transcriptional and posttranslational mechanisms as well. RAF kinases directly phosphorylate the pro-apoptotic proteins BAD,[117] Bim,[118–121] and caspase 9,[122] which results in their inhibition by sequestration or proteasomal degradation.[117] The inhibitors of apoptosis Bcl-2 and Bcl-xL are both transcriptionally upregulated downstream of oncogenic hyperactivation of BRAF signaling.[123,124] These examples indicate not only how hyperactive BRAF/MEK/ERK/MAPK pathway transduces its signal transcriptionally and posttranslationally, but also that the oncogenic effects of MEK/ERK signaling are activated at many levels of the pathway (Figure 3).

BRAF hyperactivation is often an early event in melanomagenesis.[8,125–127] However, hyperactivation of BRAF alone is not sufficient to transform normal cells into malignant melanoma.[128–132] Around 80% of benign melanocytic nevi contain hyperactivating mutations of BRAF signaling, which never undergo oncogenic transformation (Figure 4).[125,128,133–135] There exist many regulatory mechanisms to prevent melanomagenesis, and to induce senescence in melanocytic cells.[136]

Following prolonged activation, BRAF/MEK/ERK signaling directs expression of negative feedback regulators to prevent overproliferation. Once again, MEK/ERK signaling controls these growth suppressive and anti-apoptotic effects through both transcriptional and posttranslational mechanisms. For instance, oncogene-induced MEK/ERK signaling drives the expression of cell cycle inhibitory proteins CDKN2A, CDKN1A, and TP53.[137–140] These targets counteract the pro-growth and pro-survival effects of MEK/ERK pathway, including cyclinA and cyclinD1 expression, and are critical for suppression of tumor growth.

Malignant melanoma are often found attached or in close proximity to benign nevi.[141] Furthermore, these melanoma bear the same BRAF mutations as the nevi, supporting a progressive model for melanoma whereby initial lesions undergo further mutation to become malignant melanoma.[141,142]

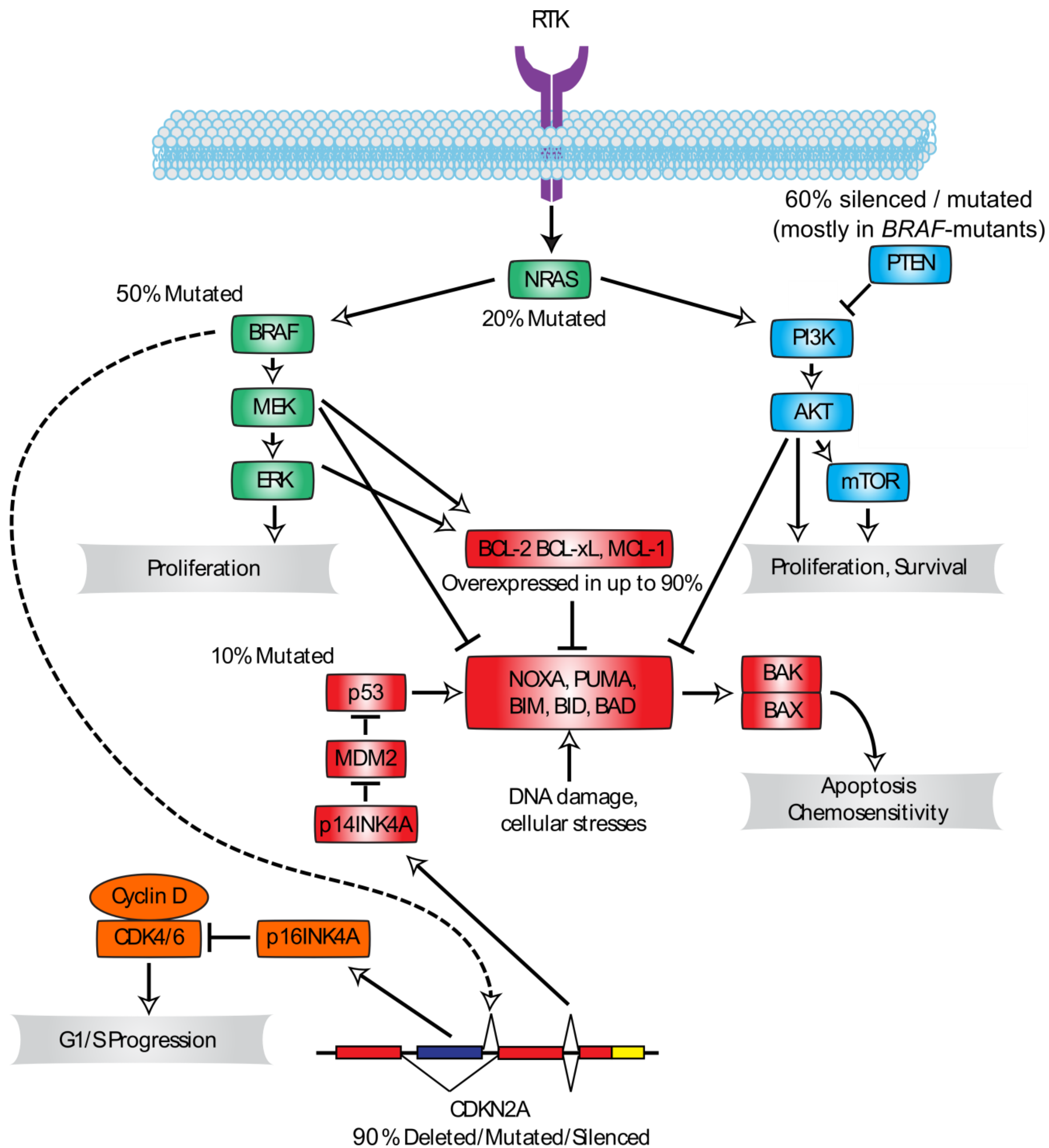



Figure 3 - Genetic factors in melanoma. Approximately 50% of melanomas harbor activating mutations in the *BRAF* proto-oncogene. These melanomas also exhibit hyperactive AKT signaling via PI3K or AKT mutation, or more commonly *PTEN* silencing. Another 20% of of melanomas harbor activating mutations in the *NRAS* proto-oncogene. *NRAS* alone hyperactivates RAF/MEK/ERK signaling and PI3K/AKT signaling. Both *NRAS* and *BRAF* mutant melanomas exhibit a high rate of silencing / deletion at the *CDKN2A* tumor suppressor locus. RAF/MEK ERK and PI3K/AKT signaling promote proliferation by increasing CyclinD expression and CDK4 expression, and inhibiting apoptosis by promoting expression of antiapoptotic proteins while suppressing proapoptotic factors.

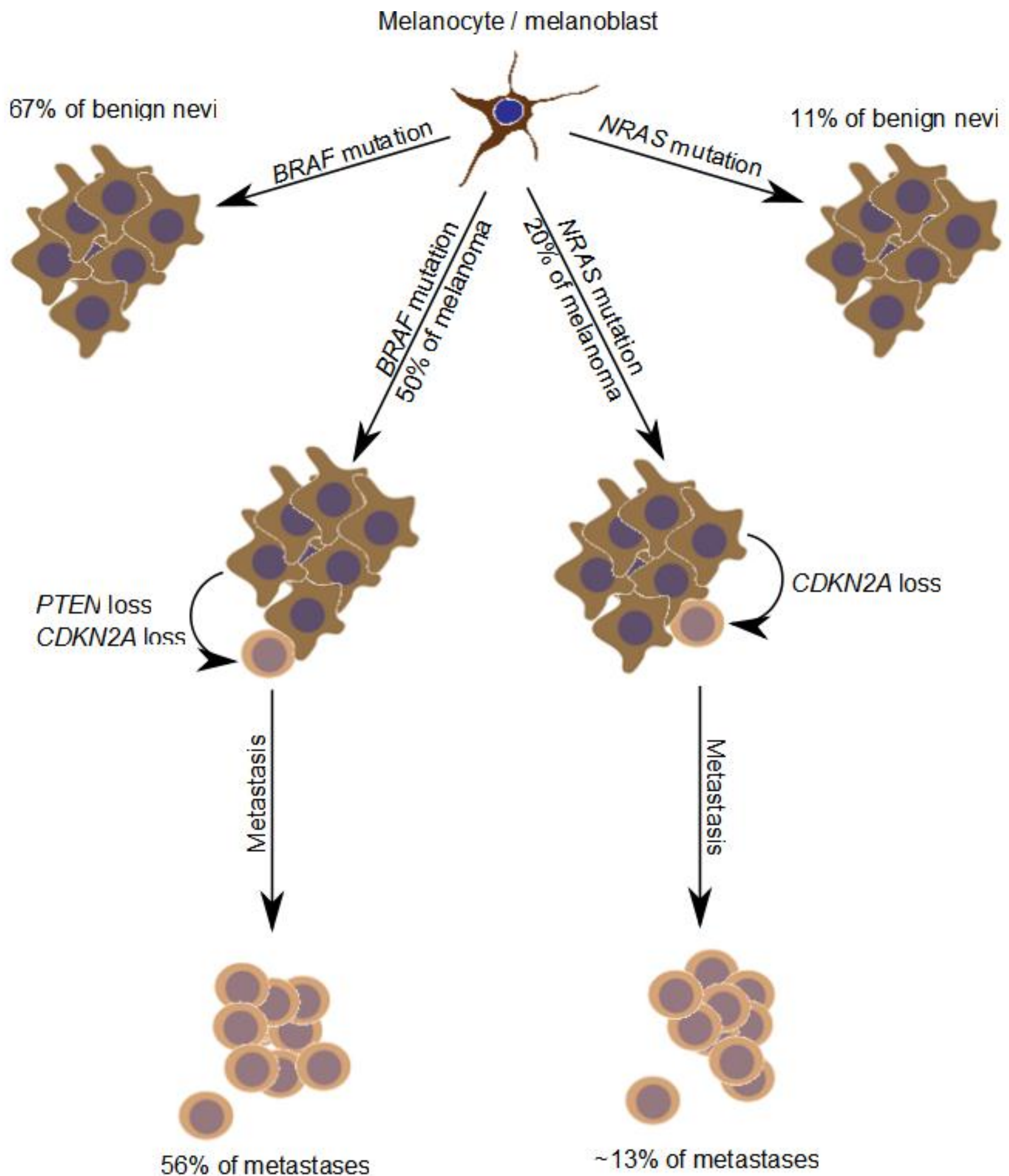



Figure 4 - Schematic of oncogenic events in *NRAS*- and *BRAF*-mutant melanomas. *BRAF* and *NRAS* are often initiating events in melanomagenesis. In the absence of further mutation, these mutations drive oncogene induced senescence (OIS), resulting in benign melanocytic nevi. Most nevi harbor *BRAF* or *NRAS* mutations. A statistically significant number of malignant melanoma are localized on or near benign nevi, indicating progression from nevi. In some cases, further mutations overcome OIS. In *BRAF*-mutant melanomas *PTEN* loss and *CDKN2A* loss overcome OIS. In *NRAS*-mutant melanomas, *CDKN2A* loss is sufficient to overcome OIS. 56% of metastases harbor *BRAF* mutations, whereas 13% of metastases harbor *NRAS* mutations.

Melanomas must overcome oncogene-induced senescence (OIS) brought on by BRAF hyperactivation in order to proceed to malignant melanoma.[136] The pathways both BRAF and NRAS-mutant melanoma modulate to overcome oncogene induced senescence are described in Figure 3and Figure 4. One strategy by which melanoma overcome oncogene induced senescence is by amplification of proproliferative genes, or by deletion or silencing of antiproliferative genes. Overexpression of oncogenic BRAF in normal human melanocytes arrests proliferation via overexpression of the protein P16ink4A (encoded by *CDKN2A*).[128] P16ink4A acts as a specific inhibitor of the pro-proliferative kinase, cyclin dependent kinase 4 (CDK4). To overcome senescence induced by P16ink4A expression, melanoma must shift the balance of CDK4 and P16ink4A. Malignant melanomas often harbor activation of the pro-proliferative cell cycle protein CDK4. Furthermore, the anti-proliferative p16ink4A expression decreases with disease progression, as assayed by immunohistochemistry.[143,144] In one study, P16ink4A was detected in 16% of melanomas, as opposed to 61% of benign nevi.[144] In another study, P16ink4A was detected in 50% of melanomas and 87% of benign nevi.[143] Indeed, well over 90% of melanomas carry deletion of the *CDKN2A* locus encoding P15ink4A (Figure 3).[145] In another set of melanomas, CDK4 amplification occurs, overcoming inhibition of this kinase by P16ink4A.[146] Deletion or silencing of P16ink4A or increasing CDK4 expression disrupts the ratio of P16ink4A:CDK4, overcoming senescence and driving proliferation.

Another common mechanism by which *BRAF* mutant melanoma overcome oncogene-induced senescence is by activation of PI3K/AKT signaling (Figure 3). PI3K is a lipid kinase that recruits the kinase AKT to the plasma membrane via its pleckstrin homology domain, which binds phospholipids. AKT proceeds to phosphorylate numerous substrates to suppress apoptosis, including the proapoptotic protein BAD and the initiator caspase, caspase 9. AKT signaling also

promotes cell cycle progression by promoting phosphorylation of cyclin dependent kinase inhibitors p21cip and p27waf (for review see Davies MA 2012[147]). In *BRAF* mutant melanoma, the PI3K pathway is frequently hyperactivated by silencing of the negative pathway regulator PTEN.[126,148] More rarely, hyperactivating mutations of PI3K or AKT are found.[149–151] Interestingly, *PTEN* silencing and PI3K mutations are rarely found in *NRAS* mutant melanoma.[126,148,152] A genetic interaction between *PTEN* and *BRAF* has been demonstrated using transgenic mouse models.[153] Mice overexpressing oncogenic *BRAF* under the tyrosinase promoter develop numerous melanocytic nevi. Conditional knockout mice lacking *PTEN* under the tyrosinase promoter only rarely develop melanocytic nevi. However, mice expressing oncogenic *BRAF* in combination with conditionally knocked out *PTEN* generate melanoma 7-10 days of *PTEN* silencing.[153] The median survival of mice with activated BRAF and silenced *PTEN* is approximately 60 days compared to >500 days for BRAF activation or *PTEN* silencing alone.[153] So, although the most frequent mutations found in malignant melanoma are oncogenic *BRAF* mutations (Figure 3), they are not sufficient to drive melanoma, and require either loss of tumor suppressors like CDKN2A or gain of oncogenic PI3K/AKT signaling (Figure 4).

Despite accumulation of multiple mutations following an initial *BRAF* mutation, many *BRAF*-mutant melanomas respond to mutant BRAF inhibition or downstream MEK inhibition in preclinical models of melanoma. Furthermore, improvement of preclinical effects of RAF/MEK/ERK pathway inhibitors have resulted in improved clinical outcomes. The nonselective RAF inhibitor sorafenib showed some preclinical effects *in vitro*, inducing caspase-independent apoptosis.[154] However, sorafenib ultimately proved not efficacious in a phase II clinical trial, and its effects were found to be independent of *BRAF* mutational status.[155] Downstream MEK inhibitors show effects consistent with oncogene addiction of melanoma for

RAF/MEK/ERK signaling *in vitro*. Inhibition of MEK using inhibitors like PD98059, PD0325901, CI-1040, and AZD6244 leads to inhibition of cyclin D1 expression, increased p27kip1 expression, and ultimately cell cycle arrest at G1 *in vitro.*[100100,156] MEK inhibition by AZD6244 also results in apoptosis.[157] Nonetheless, clinical trials of AZD6244 resulted in a response rate of only 12%, equivalent to that of the extant chemotherapeutic temozolamide.[158] Most recently, targeted BRAF inhibitors have shown clinical efficacy both *in vitro* and *in vivo*. These inhibitors selectively target oncogenic BRAF over wild-type BRAF or other RAF isoforms.[4,159] One example is vemurafenib, which inhibits BRAFV600E with an $IC_{50}$ of 31 nM, and WT BRAF with an $IC_{50}$ of 100 nM.[4,159] Vemurafenib induces both a G1 arrest and apoptotic response induced by BIM expression in melanoma xenograft models.[7] While this revolutionary targeted approach to cancer therapy resulted in tumor reduction in almost all treated patients, half of all patients relapsed within 6 months. Patients treated with vemurafenib exhibited a median duration of progression-free survival of 7 mo and an increase in 2 year overall survival to 23.5%.[41111]

The progressive development of BRAF and MAPK pathway inhibitors described above has shown the therapeutic promise of inhibiting this pathway. However, the failure of highly selective, highly potent pathway inhibitors to produce lasting clinical responses indicates the need for combination therapy.[160–163] Combination of BRAF inhibitors and PI3K inhibitors show promise in preclinical models of melanoma.[161–163] As described above combination BRAF inhibitors and Wnt/β-catenin pathway activators promote apoptosis in *BRAF*-mutant melanomas.[14] The combination of WNT3A, MEK/ERK pathway inhibition and PI3K/AKT inhibition has yet to be tested in *BRAF*-mutant melanomas.

*Oncogenic NRAS mutation in melanoma*

NRAS is a small GTPase belonging to the RAS family of small GTPases. Humans have three RAS GTPases, HRAS, NRAS, and KRAS.[164] *NRAS* is the second most frequently mutated proto-oncogene in melanoma.[8,9] Approximately 20% of patient samples bear this mutation.[95,96] In rare cases (less than 2%[8,9]), HRAS[165] and KRAS[166] are observed. The most common mutations in the NRAS and HRAS proto-oncogene translates to Q61 (80%), whereas G12 is the most common mutation in KRAS mutant melanoma (70%).[8] These mutations inactivate the GTPase domain of the enzyme resulting in a constitutively active GTP-bound form.

NRAS exerts its oncogenic effects through multiple signal transduction cascades. Like BRAF, NRAS signals through the MEK/ERK pathway. NRAS is directly upstream of the RAF family of MAP3K kinases. Oncogenic NRAS activates ARAF, BRAF or CRAF, which transduce the MEK/ERK signal as described above.[167] Unlike BRAF, NRAS also activates PI3K / AKT signaling, by activating PI3K itself (Figure 3).[168,169] NRAS also exerts its effects on proliferation, survival and migration via activation of proteins including RALGEFs, the RAC activator TIAM1, and phospholipase Cε.[168]

*BRAF and NRAS mutant melanomas differ at the genetic, cellular, and phenotypic levels.*

Although NRAS and BRAF mutant melanoma share some common elements (activation of MEK/ERK pathway and activation of PI3K signaling, for instance), their mutational differences, difference in signal transduction, and differences in gene expression translate to different phenotypes and ultimately different disease severity. *NRAS*-mutant melanomas regulate tyrosine kinase receptor signaling, RAF/MEK/ERK and PI3K signaling to different degrees than *BRAF*-mutant melanomas. *NRAS*- and *BRAF*-mutant melanomas undergo different cellular senescence patterns. *BRAF*- and *NRAS*-mutant melanomas express genes like *FYN* and *MMP14*

differently. In addition they silence different genes to a different extent, including the tumor suppressor CDKN2A. These differences in signaling, genomics and gene expression translate to worse outcome for *NRAS*-mutant melanomas and ultimately different therapeutic strategies.

*NRAS*-mutant melanomas regulate tyrosine kinase receptor signaling, RAF/MEK/ERK and PI3K signaling to different degrees than *BRAF*-mutant melanomas. For instance, even though NRAS activates both BRAF and PI3K signaling, these pathways are activated to different degrees in *NRAS*- and *BRAF*-mutant melanoma. *NRAS*-mutant melanomas exhibit lower AKT signaling than *BRAF*-mutant melanomas.[170] Immunohistochemical analysis of *NRAS* and *BRAF* mutant tumors indicates that *BRAF* mutant tumors lacking *PTEN* show much higher phospho-AKT staining than *NRAS* mutant melanomas.[170] Receptor signaling differs between *BRAF* and *NRAS*-mutant melanoma, as well. Immunohistochemical analysis of phospho c-met in a panel of *BRAF*- and *NRAS*- mutant melanomas reveals that 54% of *NRAS*-mutant and 29% of *BRAF*-mutant tumors stain positive for phospho c-met.[171] These data indicate a variability of activity of signaling between BRAF and NRAS mutant melanomas.

Differences in signal transduction pathways and intensity translate to differences in cellular phenotype between *NRAS* and *BRAF*-mutant melanoma. For instance, melanocytes undergo different forms of cellular senescence following oncogenic *NRAS* or *BRAF* transduction.[172] *NRASQ61R*-expressing melanocytes became SA-β-Gal-positive, and flat and extensively vacuolized within 4–6 days after transduction, whereas, melanocytes overexpressing BRAFV600E became SA-β-Gal-positive and developed a more rounded shape without extensive vacuolization starting 12–15 days after infection.[172] Overexpression of c-MYC reverses oncogene induced senescence of BRAFV600E and NRASQ61R, although it does so to different extents, almost completely ablating OIS in BRAFV600E expressing melanocytes, while

inhibiting OIS less so in NRASQ61R expressing melanocytes.[130] As mentioned above, *NRAS*-mutant melanomas also display higher c-met phosphorylation than *BRAF*-mutant melnomas.[171] Inhibition of c-met with the chemical PHA665752 decreases migration 88% in *NRAS*-mutant melanoma and 20% in *BRAF*-mutant melanoma by Boyden chamber assay.[171] This finding suggests that *NRAS*-mutant melanomas respond more strongly to some therapies, and supports the need to investigate the role of Wnt/β-catenin signaling in *NRAS*-mutant melanomas.

In addition to having phenotypic differences, *BRAF* and *NRAS*-mutant melanoma show differences in their genomes and gene expression as well. The oncogene *FYN* and the matrix metalloproteinase *MMP14* are overexpressed in *BRAF*- mutant melanoma, as validated by QPCR. *NRAS*-mutant melanoma overexpress components of the glutathione S transferase system (*GSTM1* and *GSTM4*), the Ras related protein *RRAGD*, and the dual specificity phosphatase *DUSP4*.[173] While these gene products have not been linked to functional differences between *NRAS* and *BRAF* melanoma, it is enticing to speculate that they could be exploited to different effect between these melanoma subtypes.

In addition to differences in gene expression, genomic differences exist between NRAS and BRAF mutant melanoma as well. DNA microarray-based comparative genomic hybridization reveals that CDKN2A copy number is lost or mutated in both *BRAF*- or *NRAS*-mutant melanomas relative to melanomas wild type. This observation indicates a tumor suppressive role for the gene product of *CDKN2A*, P16ink4A, in both genetic backgrounds.[174] Mutation and deletion of the *PTEN* locus occurs almost exclusively in *BRAF* mutant melanoma, consistent with the observation that NRAS signals through both RAF/MEK/ERK signaling and PI3K/AKT and BRAF signals only RAF/MEK/ERK.[174] *TP53* copy number loss occurs only in cell lines harboring *NRAS* mutation.[174] In another study, loss of the genomic region containing

tumor suppressor *OPCML* correlated with *NRAS* mutation, but loss of *TP53* did not, possibly due to sensitivity in techniques, or possibly reflecting an as yet unknown variability between *NRAS* mutant lines.[175]

The differences in cellular phenotype, genome, and gene expression between *NRAS*- and *BRAF*- melanoma may translate to differences in outcome. In one study of >200 melanomas, *NRAS* mutant melanoma correlated with increased tumor thickness, increased mitotic rate, and increased tumor depth.[97] In this study, *NRAS*-mutation was an adverse prognostic indicator relative to wild type melanoma, with a hazard ratio of 2.96 (P=0.04), and *BRAF*-mutation was not an adverse prognostic indicator with a hazard ratio of 1.73 (*P*=0.23).[97] In another study designed to settle the question of *NRAS* mutation as an independent prognostic indicator of overall survival, multivariate cox regression was performed on a cohort of 313 patients. In this study, known independent predictors of overall survival were confirmed (elevated serum LDH, metastasis category, etc). In this study, *NRAS* and *BRAF* mutations were both independent predictors of overall survival, although to different extents (Hazard Ratio = 2.05 and = 0.45, respectively).[99]

*NRAS*- and *BRAF*-mutant melanomas also respond differently to therapeutic interventions. The targeted *BRAF* inhibitor vemurafenib is now available for *BRAF* mutant melanomas. These treatments show some effect on survival, with a 7 month increase in progression-free survival and a 23.5% increase in progression-free survival, and represent the first new monotherapy for melanoma in decades.[41111] Not surprisingly, the outcome is quite the opposite for *NRAS* mutant melanoma. Treatment of *NRAS* mutant melanoma with the targeted BRAF inhibitor Vemurafenib actually increases MEK/ERK signaling by increased activation of CRAF.[176] Indeed, *NRAS* transduces its signal primarily via CRAF to activate MEK and ERK.[177]

Targeting CRAF would make no sense in *BRAF*-mutant melanoma, however inhibition of CRAF in *NRAS* melanoma results in apoptosis and cell cycle inhibition.[107]

Emerging evidence suggests that *NRAS* mutant melanomas are more susceptible to p21 activated kinase (PAK) inhibitors. Unlike BRAF, mutant NRAS activates the small GTPase Rac, which in turn activates PAK.[178] PAK acts as an anti-apoptotic protein in *NRAS* mutant melanoma.[179] Treating *NRAS*-mutant melanoma lines with an $IC_{50}$ dose of the PAK inhibitor IPA3 induces apoptosis, but has no effect in multiple *BRAF* mutant melanomas. This effect was shown to be specific by rescue of apoptosis via PAK overexpression.[179] This evidence points to the potential Achilles heel of *NRAS*-mutant melanoma, that it activates and manipulates a multitude of downstream effectors, which can potentially be targeted using small molecules.

*NRAS*-mutant melanomas respond more strongly to IL2 mediated immunotherapy than *BRAF* mutant melanomas.[180] IL2 induces cytotoxic effects by increasing natural killer cell levels.[181] In a recent trial of high-bolus IL2, 47% of *NRAS*-mutant melanomas exhibited a complete recovery or partial recovery, while only 23% of *BRAF* mutant melanomas showed a complete or partial recovery.[180] Furthermore, patients with NRAS mutations had a significantly higher response rate than those with BRAF mutations (49% vs. 19%, P=0.04).[180] Unfortunately, this treatment is only effective for about 6% of melanoma patients (Figure 5). [182]

## Research questions

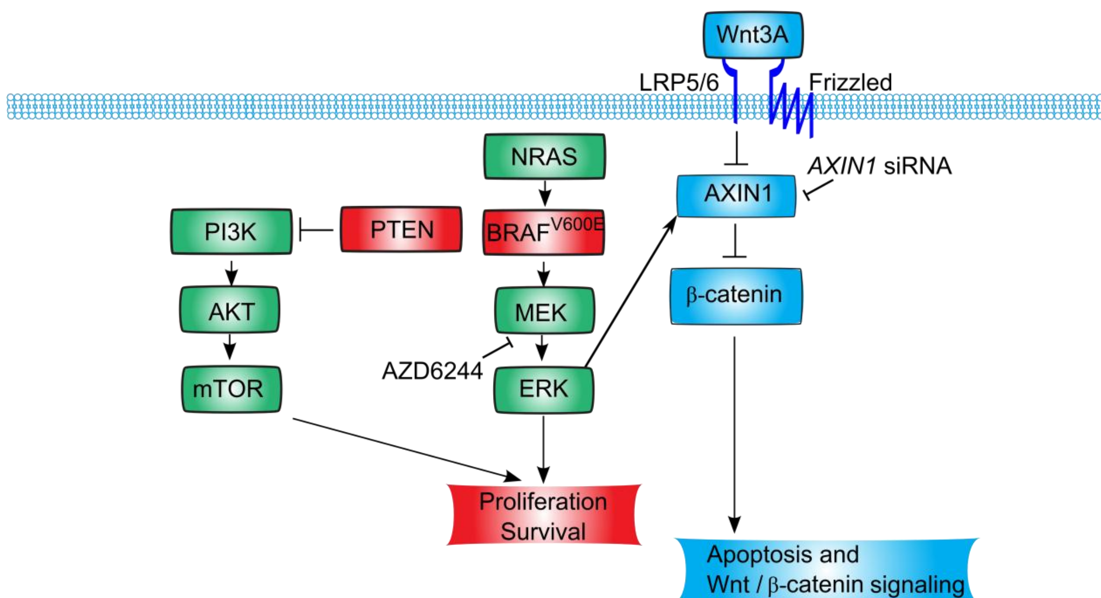
### *Does Wnt/β-catenin signaling regulate apoptosis in both BRAF- and NRAS-mutant melanoma?*

Because *BRAF*-mutant melanoma displayed a diverse set of apoptotic responses to WNT3A treatment, because *NRAS* mutation has been identified as an independent prognostic

factor, because *NRAS*- and *BRAF*-mutant melanoma respond differently to treatment,[179,180] and because fewer viable therapies exist for patients harboring *NRAS*-mutant melanoma, I dedicate the first part of my thesis research to determine if WNT3A promotes apoptosis in *NRAS*-mutant melanomas.

The apoptotic response of some *BRAF*-mutant melanomas to WNT3A treatment provides additional testable hypotheses regarding the role of WNT3A in *NRAS*-mutant melanomas. Research in *BRAF*-mutant melanoma led to the observations that sensitivity to apoptotic response to WNT3A correlates both to negative regulation of Wnt/β-catenin signaling by MEK/ERK signaling and to loss of AXIN1, and also to the observation that *AXIN1* siRNA cooperate with MEK/ERK pathway inhibition to promote apoptosis in cell lines resistant to WNT3A treatment. In addition to asking if WNT3A promotes apoptosis in *NRAS* mutant melanomas, my thesis research also asks if negative regulation of Wnt/β-catenin signaling by MEK/ERK signaling and AXIN1 stabilization by MEK/ERK predicts apoptotic response to WNT3A treatment in *NRAS*-mutant melanoma. It also asks if AXIN1 silencing by siRNA promotes apoptosis in combination with MEK/ERK pathway inhibition (Figure 5).

5A *BRAF*-mutant melanoma




5B *NRAS*-mutant melanoma

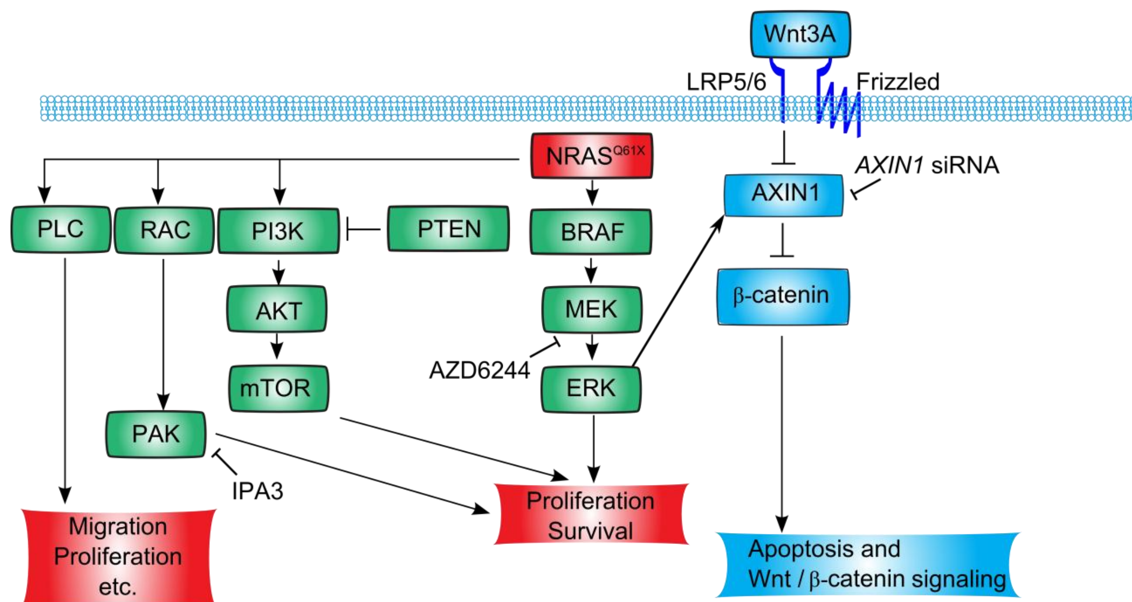



Figure 5 - Proposed models by which *BRAF*- and *NRAS*-mutant melanomas undergo apoptosis in response to WNT3A. (A) *BRAF*-mutant melanoma undergo apoptosis following MEK/ERK pathway inhibition and AXIN1 loss. In all *BRAF*-mutant melanomas tested thus far, AXIN1 siRNA combines with MEK/ERK pathway inhibition to suppress proliferation and drive apoptosis. In a subset of melanomas, WNT3A treatment and MEK/ERK inhibition decrease AXIN1, and drive apoptosis. (B) Does WNT3A promote apoptosis in *NRAS*-mutant melanomas? NRAS activates numerous additional pathways relative to BRAF. My thesis research tests, if this different subset of melanoma responds to MEK/ERK pathway inhibition (via AZD6244) and Wnt/β-catenin pathway activation, or alternatively to *AXIN1* siRNA.

*What other proteins regulate Wnt/β-catenin signaling in melanoma?*

In addition to resolving whether or not Wnt/β-catenin signaling regulates apoptosis in NRAS- and BRAF-mutant melanoma, it is important to further resolve how Wnt/β-catenin signaling is regulated in melanoma to identify additional therapeutic targets. In addition to the core regulatory proteins (described in Figure 1), many additional adaptor proteins, kinases, and signaling pathways regulate Wnt/β-catenin signal transduction from the membrane to the nucleus in different contexts. In the kidney, the adaptor protein WTX negatively regulates Wnt/β-catenin signaling in a manner similar to APC in the colon.183 In B-cell lymphoma tissue, the kinase BTK is often hyperactivated and can serve as a negative regulator of Wnt/β-catenin signal transduction in this context.183 In the colon, hyperactive MEK/ERK signaling positively regulates Wnt/β-catenin signaling, promoting nuclear translocation of β-catenin and increasing expression of Wnt-dependent target genes.184 This role for MEK/ERK signaling is exactly the opposite in melanoma where hyperactive MEK/ERK signaling serves as a negative regulator of Wnt/β-catenin signaling. In this context, inhibition of MEK/ERK signaling promotes Wnt-dependent signal transduction.185 Each cell type regulates Wnt/β-catenin signaling differently, and this difference potentially affords an opportunity to specifically modulate Wnt/β-catenin signaling in melanoma, and highlights the need to focus specifically on melanoma to resolve pathway regulation. In chapter 3, I will describe FAM129B as a novel regulator of Wnt/β-catenin signaling in melanoma.

# Chapter 2: Wnt/β-catenin signaling promotes apoptosis in *NRAS*- and *BRAF*- mutant melanoma. (Adapted from Conrad WH, *et al*. 2012)

## Introduction

As described in more detail in Chapter 1, preliminary evidence from melanocyte development and patient-derived human models implicate the Wnt/β-catenin signaling pathway in melanoma pathogenesis. During melanocyte development, Wnt/β-catenin signaling is necessary and sufficient to drive neural crest cells toward a melanocyte cell fate, in large part through direct regulation of transcriptional targets, such as microphthalmia transcription factor (encoded by MITF).[63–67] During melanoma pathogenesis, the observed presence of nuclear β-catenin in the majority of benign nevi, along with the loss of nuclear β-catenin seen with melanoma progression,[72–74] support the hypothesis that maintenance of homeostatic levels of Wnt/β catenin signaling is important in this particular context. Furthermore, high cytoplasmic and/or nuclear β-catenin staining of patient tumor biopsies correlates with increased patient survival, decreased metastasis recurrence and decreased expression of proliferation markers.[13,72,75] Indeed, high Wnt/β-catenin signaling mediated by overexpression of WNT3A inhibits tumor growth in mouse models of melanoma, accompanied by an increased expression of melanocyte-associated gene targets.[13,14]

In light of the inhibitory role for Wnt/β-catenin signaling in this context as well as existing clinical efforts to target BRAF-mediated signaling in melanoma, we examined the combinatorial effects of Wnt/β-catenin activation with MEK/ERK inhibition. In doing so, we found that simultaneous activation of Wnt/β-catenin signaling pathway and inhibition of BRAFV600E by PLX4720 synergistically induced apoptosis in a selection of melanoma cell

lines. Furthermore, overexpression of WNT3A in human A375 cells in combination with PLX4720 treatment resulted in significant inhibition of tumor growth in relevant mouse models of melanoma.[14] Not only did Wnt/β-catenin signaling enhance PLX4720-mediated apoptosis, it is also unexpectedly required for this apoptosis, which was inhibited in cell lines sensitive to PLX4720 alone by pretreatment with siRNA targeting β-catenin.[14]

Although a subset of cell lines exhibited significant apoptosis with the combination of WNT3A and PLX4720, several cell lines showed no response to this combination treatment. [14] Further analysis revealed that apoptotic sensitivity correlated with dynamic loss of AXIN1, a negative regulator of Wnt/β-catenin signaling.[14] In support of the central role of AXIN1 in mediating this response, the cell lines that did not exhibit significant apoptosis with WNT3A and PLX4720 could be sensitized to this combination by siRNA-mediated knockdown of AXIN1, thus conferring a significant apoptotic response. Taken together, these findings define a preliminary context wherein Wnt/β-catenin signaling and the loss of cellular AXIN1 abundance can promote BRAF-inhibitor-mediated apoptosis.

Our initial work was performed in BRAF-mutant melanoma lines, as approximately 50% of melanomas carry BRAF-activating mutations, which activate the MEK/ERK pathway.[8,9] However, an additional 20% of melanomas harbor activating NRAS mutations, which likewise, activate MEK/ERK signaling.[8,9] Based on the initial finding that Wnt/β-catenin signaling cooperates with targeted BRAFV600E inhibitors to promote apoptosis in BRAF-mutant melanoma, I hypothesized that WNT3A treatment would also cooperate with MEK/ERK inhibitors in a subset of NRAS-mutant melanomas.

## Materials and Methods

### *Reagents*

Transient transfection of siRNA was performed with RNAiMAX (Invitrogen), as directed by the manufacturer. Control siRNA #1 was purchased from Ambion. The sequence used for AXIN1 siRNA was 5'-GAAAGUACAUUCUUGAUAAtt-3' (Ambion) . Protease and phosphatase inhibitor tablets were purchased from Roche. AZD6244 was purchased from Selleck Chemicals and used at a dose of 2 uM. Antibodies recognizing ERK (p42 and p44), phopho-ERK (p42/44), cleaved PARP1, cleaved PARP1 Alexa Fluor 488 conjugate were purchased from Cell Signaling. Antibodies recognizing β-tubulin or β-catenin were purchased from Sigma-Aldrich. The antibody recognizing AXIN1 was purchased from R&D Systems.

### *Cell lines*

The human melanoma cell lines A375 and A2058 from C. Yee (Fred Hutchinson Cancer Research Institute, Seattle, WA). The human melanoma cell line SK-MEL-2 was purchased from the American Type Culture Collection. M202 and M207 cell lines were a generous gift of Antonin Ribas and were derived as described previously.[186]

### *Cell culture*

The human melanoma lines A375 and A2058 were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS) and 1% antibiotic. The human melanoma lines SKMEL5 and SKMEL28 were grown in Eagle's minimum essential medium (EMEM) supplemented with 10% FBS and 1% antibiotic. The human melanoma lines COLO829 and MEL624 were grown in RPMI supplemented with 10% FBS and 1% antibiotic.

Synthetic siRNAs were transfected into cultured cells at a final concentration of 20 nM with RNAiMAX (Invitrogen).

*Low-throughput siRNA transfections*

Cells were reverse-transfected with 20 nM siRNA (final concentration) in six-well plates with RNAiMAX reagent per manufacturer's suggestions (Invitrogen). Cells were incubated for 48 hours after transfection and then treated with the indicated conditions for the indicated amount of time.

*RNA purification and qRT-PCR analysis*

RNA was purified with the RNeasy kit according to the manufacturer's protocol (Qiagen). Complementary DNA (cDNA) was synthesized with RevertAid M-MuLV Reverse Transcriptase (Fermentas). LightCycler FastStart DNA Master SYBR Green 1 (Roche) was used for realtime PCR as previously described.[187] Quantitative PCR results presented in the manuscript are representative of a minimum of three biologic replicates.

*Flow cytometry for cleaved PARP*

Cells were seeded in a six-well dish at a density to achieve 90 to 100% confluence at harvest. Twenty-four hours after seeding, cells were treated with the indicated conditions for the indicated amount of time. At the time of collection, supernatants were collected and pooled with trypsinized cells. Cells were fixed with 4% paraformaldehyde and permeabilized according to the vendor's protocol for cleaved PARP antibody (Alexa Fluor 488 conjugate) (Cell Signaling). The antibody was used at a final dilution of 1:100. Flow was performed on a BD FACSCanto II, and data were analyzed with FlowJo 8.8.6 (Tree Star) software. Experiments were performed in biological triplicate, and data are representative of at least three independent experiments.

*Statistics*

Except where indicated, a student's t test was used to assess the statistical significance of the differences between the different groups; a p value of <0.05 was considered significant.

## Wnt pathway activation promotes apoptosis in *NRAS*- and *BRAF*-mutant melanomas

To test the hypothesis that WNT3A treatment would also cooperate with MEK/ERK inhibitors in a subset of NRAS-mutant melanomas, I treated a panel of *BRAF*- and *NRAS*-mutant melanomas with all possible combinations of L-cell or WNT3A-cell conditioned media and DMSO or the MEK inhibitor AZD6244. I subsequently quantified the level of apoptosis by cleaved PARP immunostaining and flow cytometry. As hypothesized, melanoma cell lines harboring either the *BRAF* or the *NRAS* mutation exhibit variations in sensitivity to the combination of WNT3A and a small molecule MEK inhibitor, with several lines of each genetic class exhibiting relative resistance. As with *BRAF*-mutant melanoma, some *NRAS*-mutant melanomas are susceptible to apoptosis following WNT3A treatment and MEK inhibition (Figure 6A). Other *NRAS*-mutant melanomas exhibit no significant response.

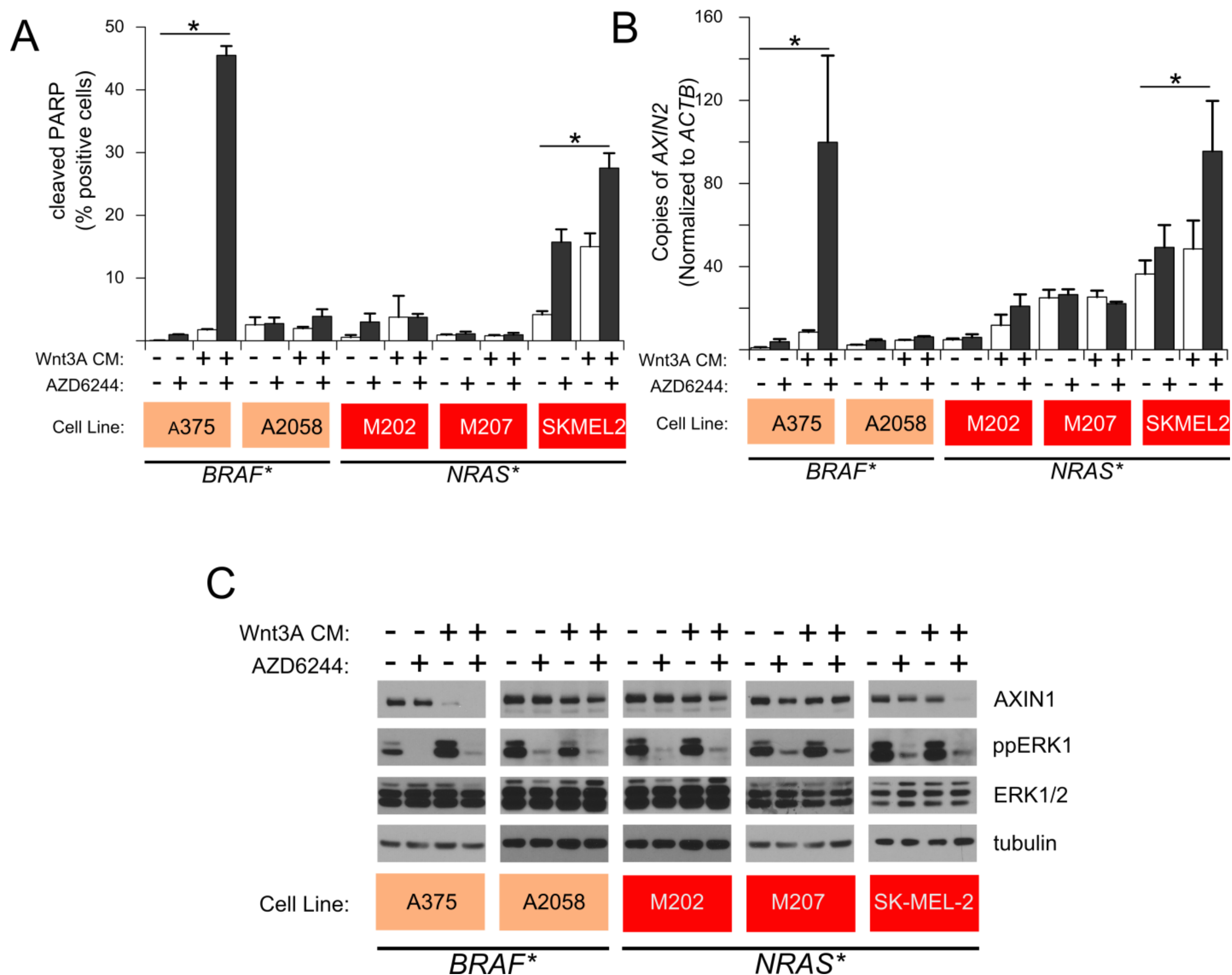



Figure 6 - Regulation of Wnt/β-catenin signaling by MEK/ERK signaling and loss of AXIN1 protein predict apoptotic response to combined Wnt/β-catenin activation and MEK inhibition. (A) Flow cytometry detection of cleaved PARP was used to measure apoptosis in a panel of cell lines harboring activating *BRAF* or *NRAS* mutations as indicated. (B) Enhancement of Wnt/β-catenin signaling by MEK inhibition was measured by quantitative PCR measurements of the endogenous Wnt/β-catenin target gene *AXIN2*. Data are expressed as copies of *AXIN2* per $10^6$ copies of *ACTB*. (C) Immunoblot analysis of same cell lines for loss of AXIN1 protein and for loss of phospho-ERK (ppERK) upon inhibition of MEK by AZD6244. In (A), (B) and (C) cells were treated with the indicated conditions for 24 h. Two uM AZD6244 was used where indicated. For (A) and (B), columns and error bars represent the mean and SEM, respectively, of three biologic replicates. *$p < 0.05$ by one-way ANOVA with Tukey's post-test. (C) is a representative immunoblot of three biologic replicates.

## AXIN1 loss predicts apoptotic response to WNT3A in *NRAS*- and *BRAF*- mutant melanomas

It had been previously observed that WNT3A promotes apoptosis in *BRAF*-mutant melanomas that negatively regulate Wnt/β-catenin signaling via MEK/ERK signaling.[14] If sensitivity to WNT3A is the same between *NRAS*-mutant melanomas and *BRAF*-mutant melanomas, then inhibition of MEK/ERK signaling should increase Wnt-dependent gene expression specifically in apoptosis-sensitive cells. To test this hypothesis, I treated the same panel of *BRAF*- and *NRAS*-mutant melanoma as above with the same conditions as above for 15 hr. I subsequently collected RNA and quantified expression of target genes using QPCR. In both *BRAF*- and *NRAS*-mutant apoptosis-sensitive lines (A375 and SK-MEL-2), MEK/ERK pathway inhibition increased WNT3A-dependent transcription of *AXIN2*, while in apoptosis-resistant lines (A2058, M202 and M207) MEK/ERK pathway inhibition had no effect (Figure 6B).

MEK/ERK signaling negatively regulates Wnt/β-catenin signaling by regulating AXIN1 stability.[14] To test if AXIN1 abundance decreases in parallel to apoptotic response to WNT3A in both *BRAF*- and *NRAS*-mutant melanomas, I once again treated a panel of cell lines with combinations of WNT3A and AZD6244. In all cell lines tested, AZD6244 decreases MEK/ERK signaling, as monitored by phospho-ERK1/2 immunoblot relative to total ERK1/2 (Figure 6C). However, immunoblot analysis detected a decrease in AXIN1 abundance was only observed in A375 and SK-Mel 2 treated with both WNT3A and AZD6244. AXIN1 abundance did not change in either A2058, M202, or M207 cells in response to combination treatment. As seen in *BRAF*-mutant melanoma cell lines, AXIN1 abundance also correlates with susceptibility to apoptosis in NRAS-mutant lines (Figure 6C).

## siRNA mediated knockdown of AXIN1 increases apoptosis in cells that resist apoptosis following WNT3A

It has been previously demonstated in *BRAF*-mutant melanoma, that AXIN1 knockdown is sufficient to enhance MEK/ERK mediated apoptosis in *BRAF*-mutant melanomas that both resist and are sensitive to apoptosis in response to WNT3A.[14] Apoptosis-sensitive A375 and apoptosis insensitive SKMEL28 cells both undergo significantly more PLX4720-mediated apoptosis when pretreated with AXIN1 siRNA.[14] Based on this observation, and based on the evidence that AXIN1 loss correlates with sensitivity to the WNT3A effect on apoptosis in both *BRAF*- and *NRAS*-mutant melanomas, I hypothesized that AXIN1 knockdown would be sufficient to enhance MEK-inhibitor mediated apoptosis in insensitive *NRAS*-mutant melanoma. To test this hypothesis, I treated A2058, M202, and M207 cells with a combination of control or AXIN1 siRNA and DMSO or 2 uM AZD6244 for 24 hr. Immunoblot analysis of all cell lines indicates efficient knockdown of AXIN1 relative to TUBB1 (Figure 7). Immunoblot analysis also indicates that AZD6244 inhibits MEK, as observed previously (Figure 7). Finally, Immunblot analysis shows increased cleaved PARP immunoreactivity in every cell line treated with combination of AZD6244 and AXIN1 siRNA. These data indicate that apoptosis-resistant NRAS-mutant melanoma cell lines can be sensitized to apoptosis by pretreatment with AXIN1 siRNA (Figure 7).

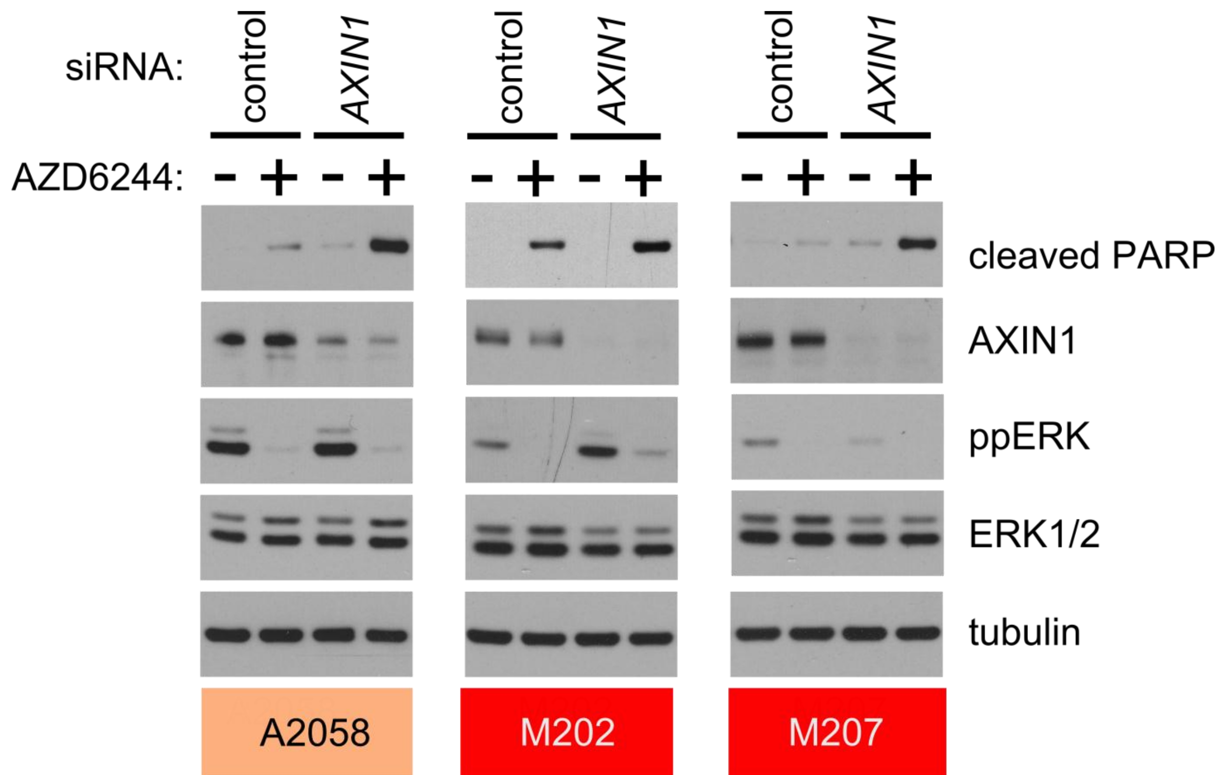



Figure 7 - AXIN1 depletion sensitizes NRAS- and BRAF-mutant melanoma cells to apoptosis mediated by MEK inhibition. Knockdown of *AXIN1* by siRNA sensitizes A2058, M202 and M207 cells to AZD6244 induced apoptosis. Immunoblots show lysates from the indicated cell lines transfected with either control or siRNA targeting *AXIN1* and treated with the indicated conditions for 24 h. Apoptosis, loss of AXIN1, and inhibition of MEK by AZD6244 were monitored by immunoblots for cleaved PARP, AXIN1 and ppERK, respectively.

## Conclusions

These findings point to AXIN1 expression as the central regulator of apoptosis in both *NRAS*- and *BRAF*-mutant melanoma cell lines. MEK/ERK signaling regulates AXIN1 steady-state abundance and Wnt/β-catenin signal transduction in apoptosis-sensitive lines, implicating a common mechanism of sensitivity across multiple mutational backgrounds (Figure 8A). The observation that AXIN1 siRNA enhances apoptosis following MEK inhibitor treatment in both mutant backgrounds suggests that apoptosis-resistant lines can be made sensitive, regardless of differences in NRAS- or BRAF-mutation (Figure 8B)

As with almost all melanoma research, one of the pressing questions is whether these findings have any potential impact on our diagnosis or treatment of patients with the disease. How does enhancement of apoptosis in cultured melanoma cell lines predict the actual response in a patient's tumors? Apoptosis has been widely used as a surrogate benchmark for chemotherapeutic efficacy, and it's inviting to imagine that certain patients with high levels of Wnt/β-catenin signaling (as measured by increased levels of nuclear β-catenin) may be more responsive to treatment with targeted BRAF inhibitors by virtue of enhanced cellular apoptosis. This prediction would also fit with the observation that apoptosis mediated by targeted BRAF inhibitors like PLX4720 requires β-catenin.[14] These studies also raise the question of whether activation of Wnt/β-catenin may have some therapeutic role in enhancing melanoma patient responses to targeted BRAF inhibition. Three large studies using independent tumor microarrays totaling over 1,000 patient tumors have associated increased levels of nuclear or cytosolic β-catenin in patient melanoma tumors to improved patient survival,[13,72,75] so consideration of Wnt/β-catenin activation as a therapeutic approach has already been broached.

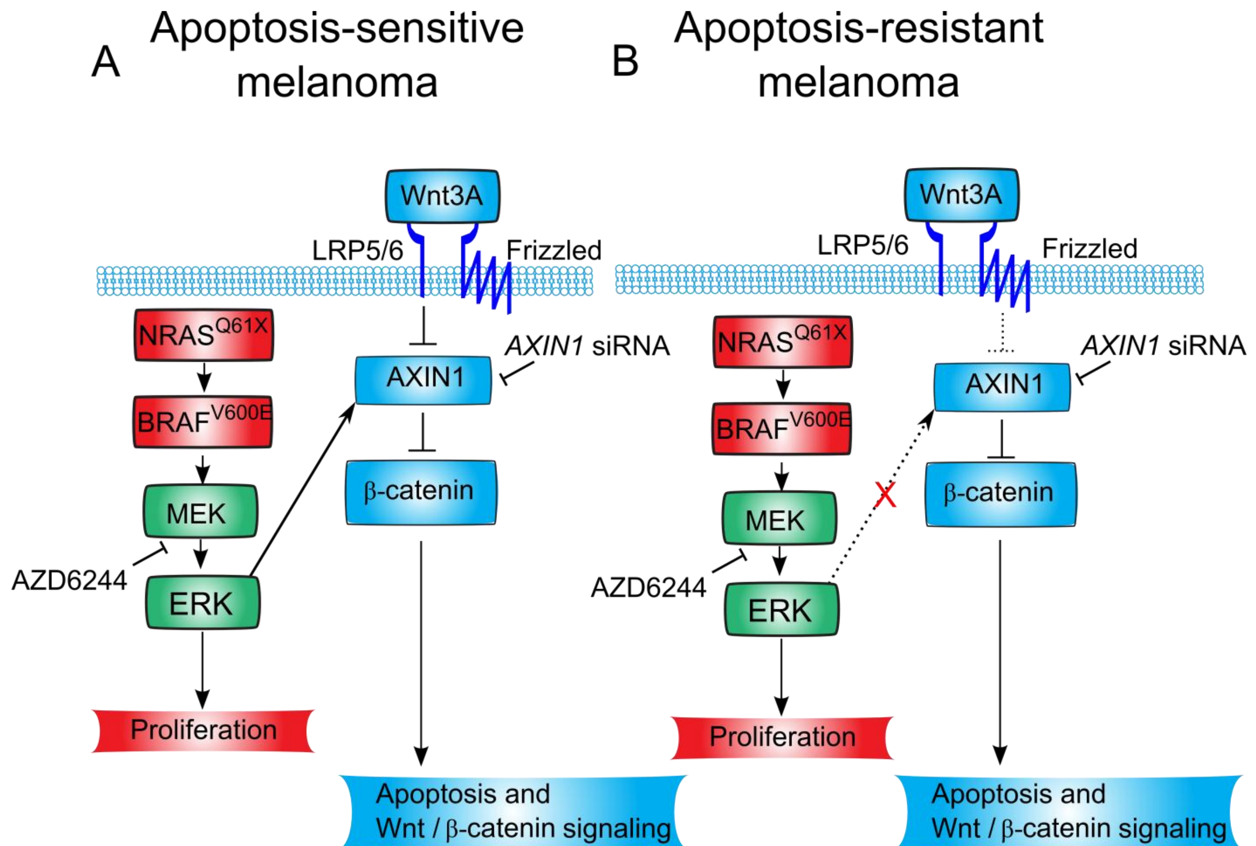



Figure 8 - Melanoma sensitivity and resistance to WNT3A- and AZD6244-mediated apoptosis: a working model. (A) *NRAS*- and *BRAF*-mutant melanoma can both undergo apoptosis in response to WNT3A and AZD6244 treatment. In sensitive lines, WNT3A-treatment and MEK-inhibition cooperate to reduce AXIN1 protein levels. Downstream of AXIN1 loss, apoptosis occurs, and Wnt/β-catenin target gene transcription is synergistically activated. β-catenin is required for both apoptosis and target gene transcription. How exactly MEK/ERK signaling regulates AXIN1 levels remains unresolved. (B) *NRAS*- and *BRAF*-mutant melanoma can both resist apoptosis in response to WNT3A and AZD6244 treatment. In this context, MEK inhibition by AZD6244 does not cooperate with WNT3A treatment to inhibit AXIN1 expression. Additionally, MEK inhibition does not change the cells responsiveness to WNT3A. *AXIN1* siRNA is sufficient to potentiate AZD6244-mediated apoptosis. It remains unknown what factor, or set of factors, uncouples MEK/ERK signaling from AXIN1 degradation, Wnt/β-catenin signal transduction and the coordinate regulation of apoptosis in these cell lines.

Accumulating evidence suggests that any efforts to target Wnt/β-catenin signaling in melanoma (and likely in other cancers as well) will be complicated by the various effects of Wnt/β-catenin activation during different temporal stages of disease development. While patient data indicates that tumors with high levels of Wnt/β-catenin signaling have a more favorable clinical course, the interpretators of these data should keep in mind other studies suggesting a permissive role of Wnt/β-catenin in certain phases of melanoma progression, including melanoma development and metastasis. In transgenic mouse models, the melanocyte-specific expression of a constitutively-active, non-degradable, stabilized β-catenin mutant (β-catSTA, containing mutations at GSK3β phosphorylation target sites that normally target β-catenin for degradation) does not itself lead to increased spontaneous melanomas, but it does increase melanocyte immortalization through suppression of p16Ink4a (encoded in humans by CDKN2A) and also cooperates with melanocyte-specific mutant Nras to promote melanoma at a much higher frequency and rate.[80] In mouse, melanocyte-specific β-catSTA also enhances metastatic spread of melanoma in the presence of combined mutations in Pten (leading to activation of PI3K/AKT signaling) and Braf.[188] How are these results applicable to patient tumors that have already undergone the process of either transformation or metastasis? The answer to this important question will require more studies on how pathway crosstalk between Wnt/β-catenin, MEK/ERK and PI3K/AKT signaling evolves throughout melanoma progression. With continued advances in targeted therapeutics and the mutational analysis of patient tumors, understanding the functional consequences of this pathway crosstalk will be essential for developing effective therapeutics that are tailored to individual patients.

This thesis research, as well as those previously published,[14] have led to some surprising insight on how the interaction between different signaling pathways can impact the response of

melanoma cells to emerging clinical therapies targeting BRAF and MEK signaling. Studies suggest that activation of Wnt/β-catenin signaling in melanoma through mutations in known canonical pathway members is probably rare.[76,189,190] Nevertheless, clinical data has supported an important role for the association of this pathway's activation state with changes in patient outcomes. Our observation that this pathway is required for melanoma cell apoptosis with targeted inhibition of the MEK/ERK signaling pathway was quite unexpected, and reminds us of the complexity of how signaling networks interact to regulate melanoma cell biology. Also surprising was our finding that AXIN1, a protein previously not implicated as a major regulator of melanoma biology, could play a key role in mediating both the crosstalk between Wnt/β-catenin signaling and act as a determinant of the cellular response to targeted MEK/ERK pathway inhibition.

Accumulating literature has also implicated a wide array of molecular mechanisms downstream of oncogenic BRAF mutations that may also be affected by the crosstalk with Wnt/β-catenin signaling. For example, BRAFV600E mutation in melanoma cells can lead to significant changes in epigenetic profiles,[191] particularly given the established association between β-catenin and chromatin modifying complexes.[192] Furthermore, the demonstrated crosstalk in melanoma cells between BRAF/MAPK signaling and metabolically responsive pathways such as AMPK/mTOR signaling, which regulates cellular proliferation and survival, may also involve Wnt/β-catenin signaling at some level.[193,194] Clearly, more studies are needed to investigate how these findings may impact ongoing clinical efforts to target MEK/ERK signaling in patients with metastatic melanoma.[195]

# Chapter 3: FAM129B regulates Wnt/β-catenin signal transduction and apoptosis in melanoma (manuscript in preparation)

## Introduction

Given that Wnt/β-catenin pathway activation can promote melanoma apoptosis, and that Wnt/β-catenin signal transduction is differentially regulated in melanoma, I sought to identify novel regulators of Wnt/β-catenin signaling in melanoma. One successful strategy for identifying regulating Wnt/β-catenin signal transduction has been large-scale siRNA-based screening for regulators of Wnt/β-catenin signal transduction.[15,183,185,196–204] This approach gives researchers the ability to test thousands of siRNAs against a highly sensitive reporter system. However, it also carries disadvantages. For instance, siRNA knockdown requires days to deplete protein expression, and transcriptional reporters only show responsiveness to Wnt signaling many hours after signaling is initiated. These drawbacks potentially limit the ability to monitor rapid events around the initiation of signal transduction. Indeed, many regulatory events occur within minutes of Wnt treatment (as described). Many of the aforementioned regulatory proteins change phosphorylation status following Wnt stimulation and that phosphorylation serves as a critical regulator of Wnt signal transduction. In the absence of Wnt ligand, AXIN1 is phosphorylated and stabilized,[42,205] while β-catenin is phosphorylated and degraded.[17] In the presence of Wnt ligand, LRP5/6 become phosphorylated, DVL becomes phosphorylated, while AXIN and β-catenin become dephosphorylated.[17,42,205–212] As complementary approaches, our lab performed a global quantitative phosphoproteomic screen to identify early phosphorylation changes in response to Wnt3A treatment and coupled those data to large scale siRNA screening data to identify regulators of Wnt/β-catenin signaling.

I identified FAM129B as a regulator of Wnt/β-catenin signaling through phosphoproteomic screening, large-scale siRNA screening and bioinformatic analysis. FAM129B is a 746 amino acid protein that contains an amino-terminal pleckstrin homology (PH) domain and a differentially phosphorylated carboxy-terminal region.[213] FAM129B has previously been connected to apoptosis in HeLa cells, where it was shown to negatively regulate TNF-alpha dependent apoptosis.[214] FAM129B has also been shown to be biologically active in melanoma, regulating melanoma invasion into collagen matrices.[213] With evidence that FAM129B regulates apoptosis in other cell lines, and that it is biologically active in melanoma, this protein served as a promising potential regulator of Wnt/β-catenin signaling and WNT3A's effect on apoptosis in melanoma. In this report we observe that FAM129B positively regulates Wnt/β-catenin signal transduction in melanoma and positively regulates the apoptotic response to WNT3A.

## Materials and Methods

### *Plasmids*

Detailed information on the β-catenin activated reporter plasmid (pBAR) has been previously described.[215] Briefly, the reporters are generated from lentiviral plasmids that contain 12 TCF/LEF binding sites (5′-AGATCAAAGG-3′), TGFβ/SMAD sites (5'-AGCCAGACA-3') or Nuclear Factor Kappa B (5'-GGGAATTTCC-3') signaling pathways separated by distinct 5-base pair linkers upstream of a minimal promoter and the firefly luciferase open reading frame. The reporters also contain a separate PGK (phosphoglycerate kinase) promoter that constitutively drives the expression of a puromycin resistance gene for mammalian cell selection.

*Cell lines and cell culture*

A375 and A2058 cells were a generous gift from Cassian Yee (Fred Hutchinson Cancer Research Institute, Seattle, WA). RKO, 293T and HT1080 cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Stable reporter lines were generated as previously described.[215] Cell lines were cultured in an humidified incubator at 37° C and 5% $CO_2$. All cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum and 1% Penicillin / Streptomycin (Invitrogen, Grand Island, NY), except A375 cells, which were grown in DMEM containing 5% FBS and 1% P/S.

*WNT3A conditioned media*

Control (L) and WNT3A conditioned media used to activate the Wnt/β-catenin signaling pathway were prepared as previously described in reference.[216] To monitor reporter activity and transcript activity, cells were treated with 10% WNT3A CM or LCM overnight before proceeding to subsequent assays. To monitor effects on apoptosis, cells were treated with 1% LCM or WNT3A and DMSO (Sigma) or 2 uM PLX4720 (Symansis).

*Quantitative phosphoproteomics*

The quantitative phosphoproteomics described in this paper follow the protocols described in references [217] and [183]. To perform Stable Isotope Labeling with Amino acids in Cell culture (SILAC)[218] experiments, 2 groups of A2058 malignant melanoma cells were cultured separately, one with labeled, heavy amino acids, and the other with light, normal amino acids. In the quantitative experiments, the heavy cells were stimulated with experimental conditions, and the light with vehicle. After stimulation of 2.5 X $10^6$ heavy A2058 cells (approximately 1 15CM plate) with recombinant WNT3A (Peprotech, Rocky Hill, NJ), and 2.5 X $10^6$ light A2058 cells with vehicle, the cells were lysed on ice with 3 ml of 8 M urea supplemented with 1

mM $Na_3VO_4$. A 10-µl aliquot was taken from each sample to perform bicinchoninic acid protein concentration assay (Pierce, Rockford, IL) according to the manufacturer's protocol. Cell lysates from the heavy and light cells were combined at a 1:1 protein ratio, reduced with 10 mM DTT for 1 h at 56 °C, alkylated with 55 mM chloroacetamide for 30 min at room temperature, and diluted to 12 ml with 100 mM ammonium acetate, pH 8.9. 40 µg of trypsin (Promega, Madison, WI) was added to each sample (∼100:1 substrate/trypsin ratio), and the lysates were digested overnight at room temperature. The whole-cell digest solutions were acidified to pH 3 with acetic acid (HOAc) and loaded onto C18 Sep-Pak Plus Cartridges (Waters, Milford, MA). The peptides were desalted (10 ml of 0.1% HOAc) and eluted with 10 ml of a solution composed of 25% acetonitrile (MeCN) with 0.1% HOAc. Each sample was divided into 4 aliquots and lyophilized overnight to dryness for storage at −80 °C.

For the phosphopeptide enrichment, we adapted a previously published method from Villen and colleagues[217] that combines strong cation exchange (SCX) chromatography with subsequent Immobilized Metal Affinity Chromatography (IMAC). Peptides were dissolved in 1mL of SCX loading buffer (5mM $KH_2PO_4$ [pH 2.65], 30% Acetonitrile) and bound to a hand-packed chromatography column (SCX resin, BMSE1203, Nest group, Southborough, MA). Using a P1 LKB peristaltic pump (Pharmacia, Uppsala, Sweden), peptides were washed and then subjected to a step gradient between of SCX loading buffer and SCX elution buffer (EB: 5mM $KH_2PO_4$ [pH 2.65], 30% Acetonitrile, 350mM KCl). The flow rate was maintained at 0.5 mL/minute and the following elution steps were used for the 13 eight minute fractions: 0.0% EB, 2.5% EB, 5% EB, 7.5% EB, 10% EB, 12.5% EB, 15% EB, 17.5% EB 20% EB, 22.5% EB, 25% EB, 100% EB, 100% $H_2O$. The Acetonitrile was removed from each fraction by dehydration, and

the peptides were subsequently desalted using C18 Sep-Pak (100mg, Waters, Milford, MA), lyophilized and stored at -80 °C.

IMAC beads (10 μL per fraction, P9740, Sigma, St. Louis, MO) were washed three times with IMAC binding buffer (40% Acetonitrile, 25mM Formic Acid). The peptides from each fraction were dissolved in 120 μL of IMAC binding buffer and were bound to the beads by incubation for 60 minutes at room temperature. Subsequently, the bound phosphopeptides were washed three times with IMAC binding buffer, and then eluted in IMAC elution buffer (50 mM $K_2HPO_4/NH_4OH$ pH 10). The eluted pepties from each fraction were acidified by the addition of 40 μL 10% Formic acid, and then desalted using C18 Sep-Pak (50mg, Waters, Milford, MA), lyophilized and stored at -80 °C.

Phosphopeptides from each fraction were loaded onto a 10-cm self-packed C18 capillary pre-column (POROS RL2 10 μM beads, inner diameter, 100 μm; outer diameter, 360 μm). After a 10-min rinse (0.1% HOAc), the precolumn was connected to a 20-cm self-packed C18 (Moniter 5 μM) analytical capillary column (inner diameter, 50 μm; outer diameter, 360 μm) with an integrated electrospray tip (~1-μm orifice). Online peptides separation followed by mass spectrometric analyses was performed on a nanoLC system (nanoAcquity UPLC system, Waters Corp.). Peptides were eluted using a 240-min gradient with solvent A ($H_2O$/Formic Acid, 99.9:1 (v/v)) and B (Acetonitrile/Formic Acid, 99.9:1 (v/v)): 10 min from 0% to 10% B, 180 min from 10% to 40% B, 15 min from 40% to 80% B, and 35 minutes with 100% A. Eluted peptides were directly electrosprayed into a LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Rockford, IL). The mass spectrometer was operated in a data-dependent mode to automatically switch between MS and MS/MS acquisitions. Each full scan (from *m/z* 300-1500) was acquired

in the Orbitrap analyzer (resolution = 60,000), followed by MS/MS analyses on the top ten most intense precursor ions that had charge states greater than two. For each full scan, we performed 10 low energy collision induced dissociation (CID) MS/MS scans that were scanned in a low-pressure ion trap with a normalized collision energy of 35%.

*Data Analysis pipeline:*

MS/MS data files were first processed and searched using the SEQUEST[219] algorithm to identify peptides. Following database searching, estimates of the false positive rate and peptide probabilities will be generated using the Institute for System's Biology's Trans Proteomic Pipeline.[220] and the output was imported into the 'Trans-Proteomic Pipeline' (Institute for Systems Biology[221]). The following variable (Phosphorylation of Serine, Threonine or Tyrosine; 79.8 Da), (Heavy Arginine; 10.0 Da, Heavy Lysine; 6.0 Da) and static (carbamidomethylation of Cystein; 57.02 Da modifications were used in the search. All identified peptides whose peptide probability score exceeded the peptide probability score associated with a < 2.5% false discovery rate were retained. Finally, the software suite XPRESS[222] was used to determine the relative quantification of heavy and light peptides. In-house Python scripts were then used to normalize the data to the mean express ratio for all peptides identified in the entire experiment and transform it to $\log_2$ for further analysis. For all unique peptides that were sampled multiple times in our analysis, we eliminated further consideration of those that had individual replicates with normalized XPRESS ratios that were greater or less than 2-fold different from the mean value of all the replicates or those whose mean value for a given charge state were greater or less than 2-fold different from the mean value of a different charge state.

*Gene ontology analysis*

The 73 Protein targets identified from the quantitative phosphoproteomic screen were entered into the Database for Annotation, Visualization and Integrated Discovery (DAVID ) v6.7.[223,224] A table of the significantly enriched biological processes was generated from the GOTERM_BP_ALL subset with an EASE score between 0 and 0.1 containing at least 2 protein targets.

*Large-scale siRNA screen*

The large-scale siRNA screen was performed as previously described,[224] with minor modifications. Briefly, HT1080 cells stably transfected with the BAR reporter and *renilla* luciferase were reverse-transfected in 1536-well plates, with a final concentration of pooled siRNA at 25 nM. 48 hours after reverse transfection, cells were treated with WNT3A-conditioned media. Background controls were treated with L-conditioned media. Following overnight incubation, β-catenin dependent transcription was measured by assaying firefly luciferase activity and normalized by monitoring constitutively expressed *Renilla* luciferase activity. All siRNAs were designed with a proprietary algorithm.

*siRNA transfection*

200,000 cells were reverse transfected at a final dose of 20 nM siRNA in 6-well format using 5 ul RNAi max / well (Invitrogen, Grand Island, NY). Medium GC universal stealth control siRNA was used as a negative control (Cat. No. 12935-112, Invitrogen, Grand Island, NY). Invitrogen's stealth siRNA targeting *FAM129B* were designed using the BLOCK-iT RNAi deisnger, are described below. The sequence for "FAM129B A" is UCACGGACAUGAACCUGAACGUCAU. The sequence for "FAM129B B" is ACUGAGGUGCGAGAUGUCUUCUUCA. The sequence for "FAM129B C" is

CAGCAGCGAUUUGAUGUGUCCAGCA. As a positive control for inhibition of Wnt/β-catenin signal transduction by siRNA, we used silencer select siRNA targeting β-catenin with the sequence GGUGGUGGUUAAUAAGGCUTT (Invitrogen).

*Low throughput reporter assays*

24 hr after siRNA transfection, cells were plated in 96-well plates at a density of 20,000 cells / well. Twenty-four hours after plating, cells were treated with the indicated conditions, and luciferase activity was measured 15 hours later with a Dual-Luciferase Reporter Assay kit (Promega) and an Envision multilabel plate reader (PerkinElmer) according to the manufacturer's suggestions.

*QPCR*

24 hr after siRNA transfection, cells were split into a 12-well cluster plate at approximately 50% confluency. 24 hr later, cells were treated with WNT3A or L conditioned media. After overnight treatment, RNA was isolated using Trizol reagent according to the manufacturer's instructions (Invitrogen). 1 ug of RNA was reverse transcribed using Fermentas' RevertAid M-MuLV Reverse Transcriptase (Glen Burnie, MD). QPCR was performed on a Lightcycler 480 using Lightcycler 480 DNA SYBR Green 1 master mix (04707516001 Roche). The following primers were used for QPCR: "*AXIN2* F" CTCCCCACCTTGAATGAAGA and "*AXIN2* R" TGGCTGGTGCAAAGACATAG; and, "*ACTB* F" AGAGCAAGAGAGGCATCCTC and "*ACTB* R" CTCAAACATGATCTGGGTCA.

*Cell lysis and Immunoblotting*

To test for siRNA knockdown, replicate cell lysates from low throughput reporter assays were pooled and treated with 10x RIPA lysis buffer (500 mM Tris, pH 7.5, 1.5 M NaCl, 10 mM

EDTA, 10% Igepal CA-630, 1% SDS, and 2% sodium deoxycholate). For monitoring cleaved caspse 3, 90% confluent 12-well plates were treated for 24-hr with the indicated conditions described in "cell culture". Media were collected and cells were rinsed once (gently) with PBS. Cells were lysed on-plate in 100 ul 1X RIPA buffer containing protease and phosphatase inhibitors (Complete EDTA-free and PhoStop by Roche). Cells were disrupted by scraping of a 1000 ul pipette tip against the plate. Apoptotic cells present in the media and PBS wash were centrifuged at 300 x *g*, rinsed once with PBS, and lysed with the RIPA buffer collected from the plate lysis. Cell lysates were cleared by centrifugation at 20,000 x *g* at 4 C for 10 minutes. Protein lysates were separated by SDS-PAGE using NuPAGE 4%-12% Bis-Tris gels (NP0336BOX, Invitrogen) in MES buffer, and transferred onto a Nitrocellulose membrane (162-0115, Bio-Rad, Hercules, CA) using IDEA scientific GENIE transfer apparatuses (idea scientific, Minneapolis, MN). Blots were probed using rabbit anti FAM129B (HPA023261 Sigma), mouse anti Tubulin (T7816 Sigma), mouse anti β-catenin (C2206 Sigma), Rabbit anti Cleaved-Caspase 3 (9661 cell signaling), Rabbit anti ERK1/2 (9102 cell signaling), Rabbit anti phospho ERK1/2 (9211 cell signaling).

*TUNEL immunofluorescence*

Glass coverslips were coated with poly-L-lysine in a 24-well dish, rinsed with PBS, and dried. Following reverse transfection as described above, cells were seeded at a density to achieve 90 to 100% confluency at harvest. Twenty-four hours after seeding, cells were treated with the indicated conditions and incubated for 24 hours with the indicated conditions as described above in "cell culture". TUNEL staining was performed according to the vendor's protocol (Roche). Briefly, the medium was gently aspirated, to keep apoptotic bodies on the slide, and cells were fixed in 4% paraformaldehyde for 1 hour at room temperature. Cells were

gently rinsed twice with PBS and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice. Cells were rinsed twice with PBS and 40 ml of TUNEL reaction mixture was added directly on top of the slide; cells were incubated for 1 hour at 37°C in a humidified incubator. Slips were rinsed three times with PBS and mounted on Superfrost Plus glass slides with ProLong Gold anti-fade mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen). Images were obtained on a Nikon TiE inverted wide-field high-resolution microscope. DAPI and TUNEL positive nuclei were quantified blinded for 5 fields per slide using NIS elements (Nikon Instruments Inc, Melville, NY)

*Statistics*

Except where indicated, a student's t test was used to assess the statistical significance of the differences between the different groups; a p value of $<0.05$ was considered significant.

## Wnt3A promotes changes in phosphorylation in melanoma cells

To assess early changes in phoshporylation in response to WNT3A treatment in melanoma, A2058 cells were prepared for stable isotope labeling by amino acids in cell culture (SILAC). They were subsequently treated with WNT3A for 30 minutes and phosphoproteins were identified and quantified (Figure 9 and materials and methods). Our experiments identified 2377 unique phosphopeptides, belonging to 721 unique proteins. 42 unique phosphopeptides decreased in abundance, while 59 unique phosphopeptides increased in abundance (Figure 10). These phosphopeptides represent 73 differentially phosphorylated proteins.

Analyzing phosphoprotein hits using the Database for Annotation, Visualization and Integrated Discovery (DAVID, v6.7)[223,224] identified 13 biological processes represented by 47 proteins that were significantly enriched in our dataset. 26 proteins from our dataset did not

belong to any significantly enriched biological process. DAVID analysis revealed that Wnt treatment regulates phosphorylation of proteins belonging to rRNA metabolism, M phase of the mitotic cell cycle, nitrogen compound metabolism, and organelle fission, among others (Figure 11). Notably, Wnt/β-catenin signal transduction was not identified as a significantly enriched biological process in the dataset. However, it is important to observe that while these biological processes may feed back to regulate Wnt/β-catenin signaling, it is also possible that Wnt treatment regulates these processes for other purposes. Indeed, no clear regulators of Wnt/β-catenin signaling emerged from the phosphoproteomic analysis alone, indicating a need to use complementary processes to identify regulators of Wnt/β-catenin signaling.

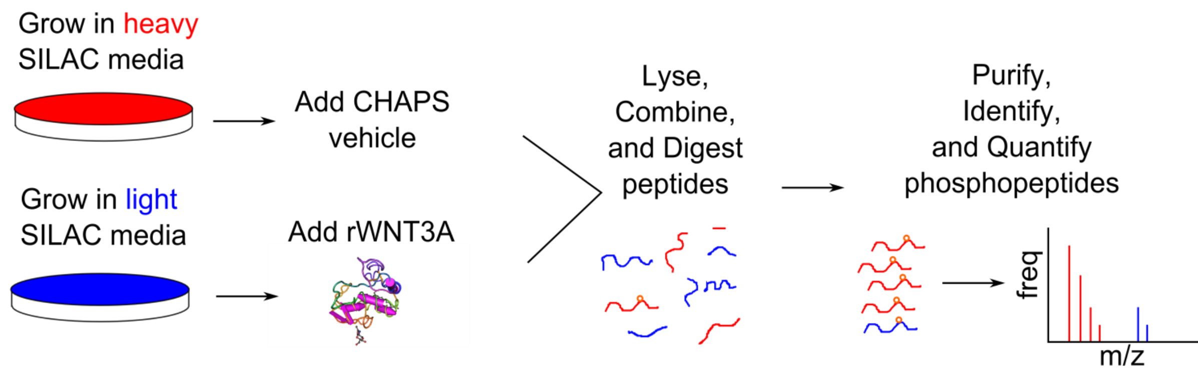



Figure 9 - Schematic of the phoshoproteomic screen. A2058 cells were grown in SILAC media. Cells were then treated for 30 minutes with recombinant WNT3A or vehicle. Following treatment, cells were lysed. Combined lysates were trypsinized, and underwent phosphopeptide enrichment. Enriched phosphopeptides were identified and quantified using mass spectrometry.

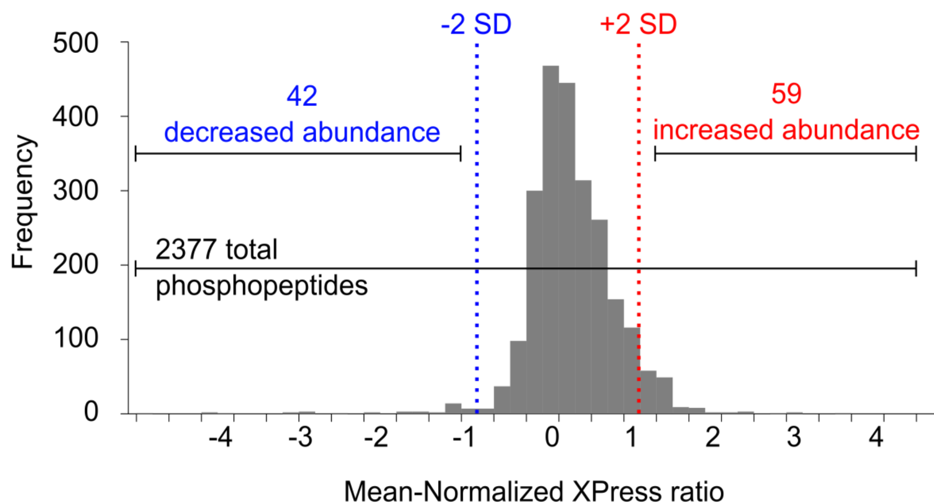



Figure 10 - Distribution of Wnt-dependent phosphopeptides and definition of phosphopeptide hits. The protocol described in Figure 9 identified and quantified 2377 unique phosphopeptides. Data are presented as a histogram with respect to each peptide's Mean-Normalized express ratio. This ratio is defined as the ratio of the area under the curve (AUC) for heavy and light isotopes of an identified peptide. Positive numbers indicate an increase in the phosphopeptide in the WNT3A sample. Data are log[2] transformed. Red and blue dashed lines mark phosphopeptide hits. Phosphopeptide hits were defined as peptides with an XPRess ratio of X where |X|>2 standard deviations from the sample mean. Sample mean = 0.156. S.D. = 0.563.

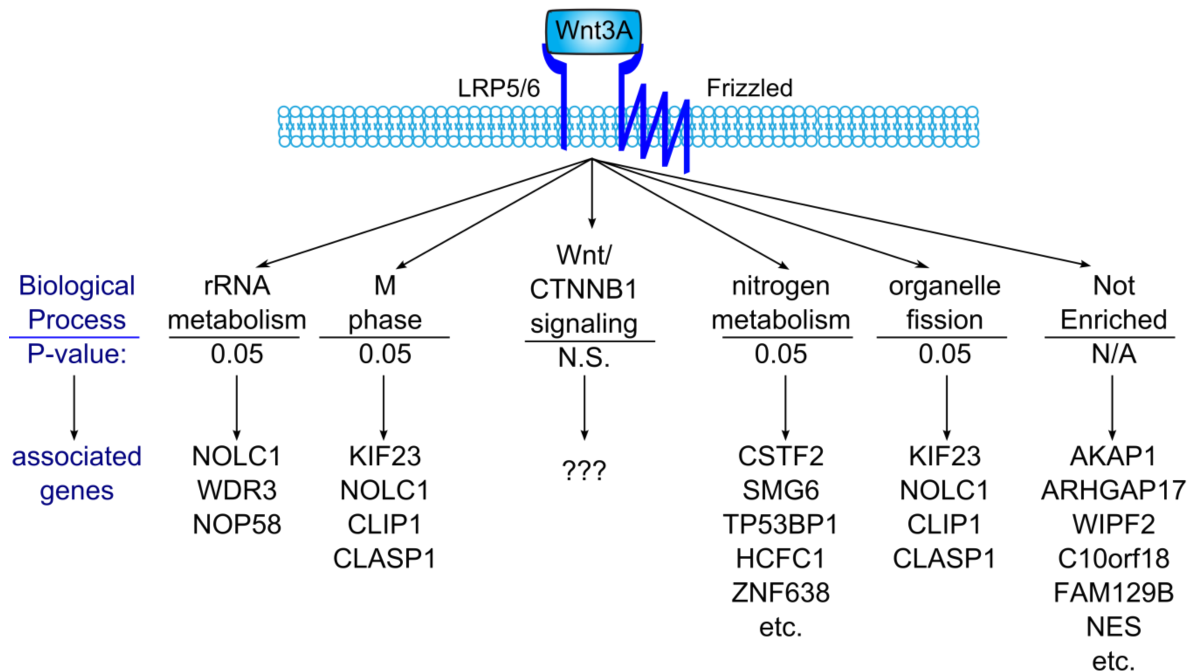



Figure 11 - Depiction of biological processes significantly enriched within Wnt-dependent phosphoprotein dataset. The protein targets of phosphopeptide hits identified in Figure 10 were entered into DAVID. Significantly enriched biological processes were identified, represented by 47 of 73 proteins. A selection of those processes and associated proteins are indicated in this schematic, including their P value and representative proteins. The remaining 26 proteins were not part of any significantly enriched biological process. Wnt/CTNNB1 signaling itself was not identified as a significantly enriched biological process

## siRNA screen identifies FAM129B as a regulator of Wnt/β-catenin signaling

As a complementary approach to our quantitative phosphoproteomic analysis, we designed and performed a large-scale siRNA screen for regulators of Wnt/β-catenin signal transduction (Figure 12). We performed this screen in HT1080 cells stably transduced with the β-catenin activated reporter (BAR reporter) because of the wide dynamic range displayed by this reporter when these cells are treated with WNT3A. We screened HT1080 cells against 28,044 pools of siRNA targeting 19,490 gene products for responsiveness to WNT3A treatment by luciferase assay normalized to constitutive *renilla* luciferase (see materials and methods). siRNA targeting the known Wnt/β-catenin regulator, *AXIN2,* increased BAR reporter activity 2.0 fold with a –log(P-value) of 2.9. Using this positive control as a reference, we examined siRNA pools, which regulated BAR reporter activity greater than twofold with a –log(P-value) > 2. 10,215 siRNA pools met the above criteria. Of the 19,490 gene products tested in our siRNA screen, we identified 5189 gene products for which every siRNA met the above criteria. To focus the results of our siRNA screen, we combined the siRNA screening dataset with our phosphoproteomic dataset. (Figure 13A and B, and Table 1). Because our large-scale siRNA screen was performed in a non-melanoma fibrosarcoma cell line, we generated a list of melanoma-associated genes using a custom Biopython script (supplementary document 1). These 745 melanoma-associated protein targets overlapped with only three phosphoproteomic hits (Figure 13B and Table 1), and with 123 siRNA screen hits (Figure 13A and Figure 13B). FAM129B was the only melanoma-associated protein target present in the siRNA screen and phosphoproteomic screen (Figure 13B and Table 1).

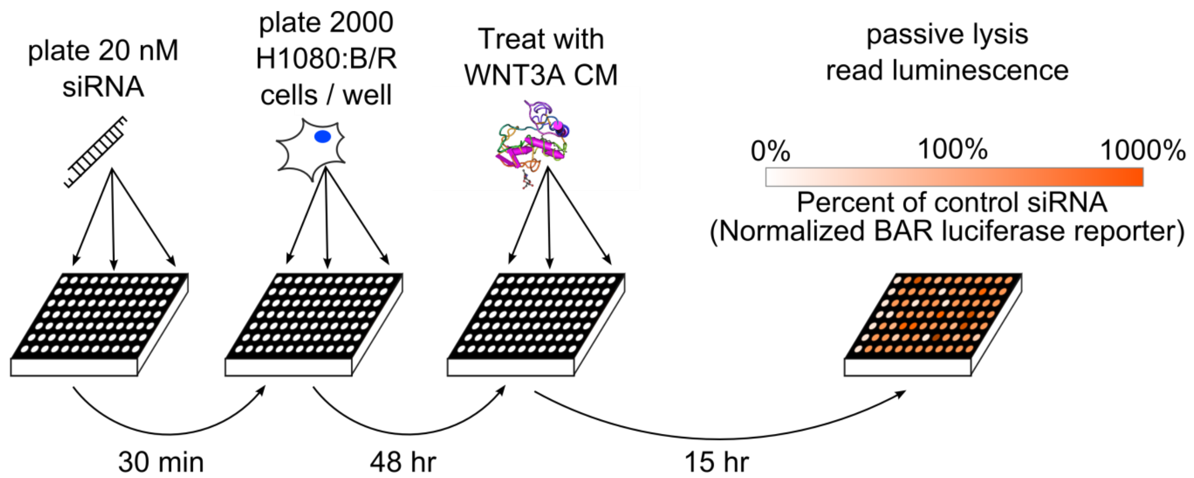



Figure 12 - Schematic of the siRNA screen. HT1080 fibrosarcoma cells stably transduced with the β-catenin activated reporter, were reverse transfected with a library of 28 000 siRNA targeting nearly every protein expressed by the human genome. Following two days of incubation, cells were treated with WNT3A conditioned media, or control. Following overnight incubation, cells were lysed and assayed for Wnt-responsiveness relative to control siRNA treatment *via* luciferase assay. Mean, median and S.D. were generated from n=3 biological replicates.

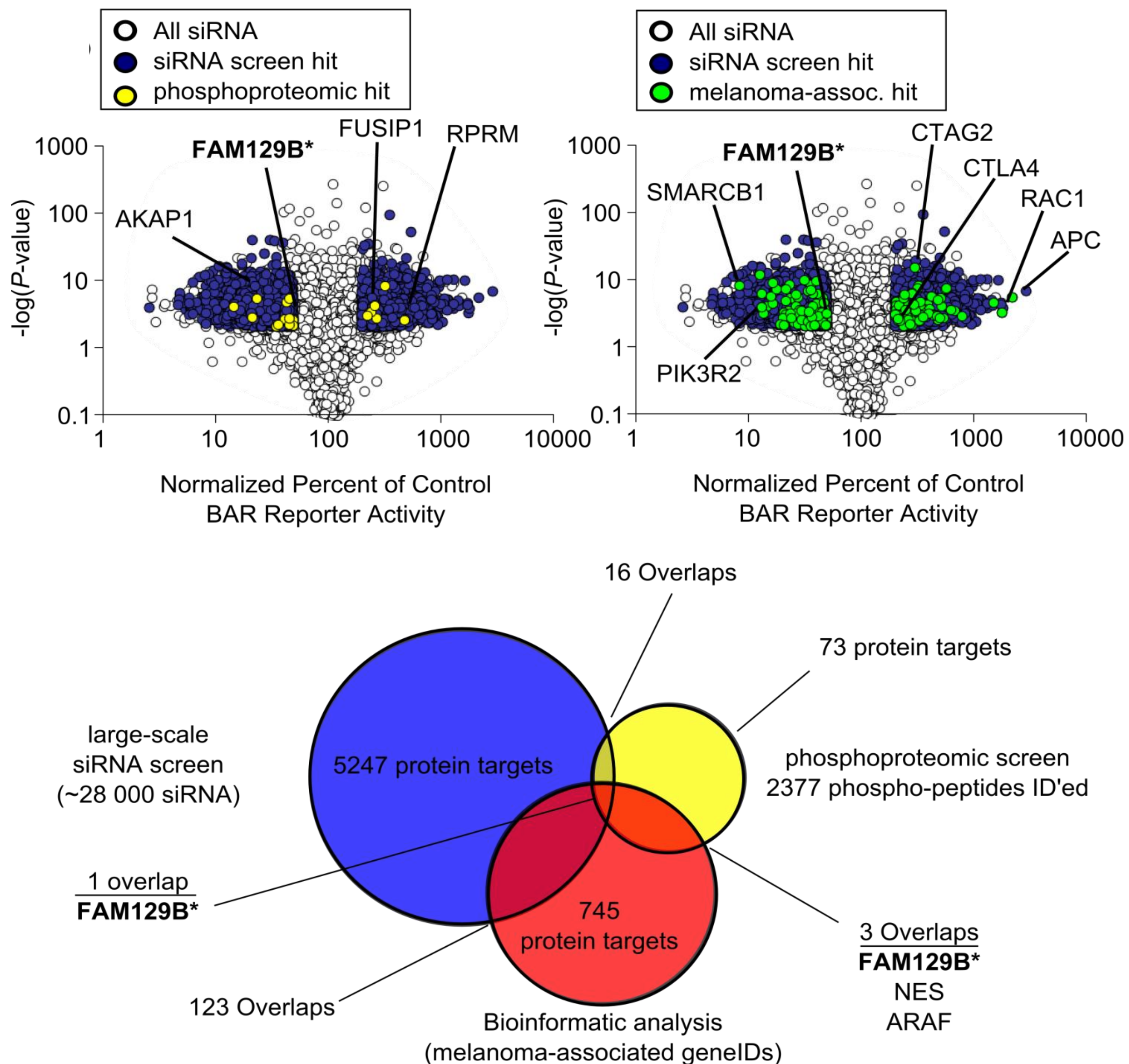



Figure 13 - FAM129B is identified as a putative regulator of Wnt/ β-catenin signaling using phosphoproteomics, large-scale siRNA screens and bioinformatic analyses. (A) Volcano plots depicting siRNA screen hits overlaid with phosphoproteomic data or bioinformatic data. (Left panel) Median effect of each siRNA treatment as a percent of control (med(normFFluc)) siRNA was plotted against the -logP value of that treatment. If, for a given gene, all siRNA showed a twofold change in med(normFFluc) and a -logP >=2, that gene was classified as a hit. siRNA screen hits are depicted in blue. Overlapping phosphoproteomic hits are depicted in yellow. (Right panel). Data plot is the same as the left panel with melanoma-associated hits plotted in green. (B) Venn diagram depicting overlaps between phosphoproteomic dataset, siRNA screen and melanoma-associated genes. 16 protein targets overlap between the phosphoproteomic hits and the siRNA screen, 3 protein targets overlap between the phosphoproteomic hits and melanoma-associated protein targets, and 123 proteins overlap between the siRNA screen hits and melanoma associated protein targets. Only FAM129B overlaps with all three datasets.

Table 1 - protein targets found in both phosphoproteomic dataset and either the siRNA screen or melanoma-associated gene set.

| Gene[a] | Mean-Normalized Xpress[b] | Phospho-Hit (Y/N)[c] | siRNA screen hit (Y/N)[d] | % control siRNA[e] | melanoma-associated gene? |
|---|---|---|---|---|---|
| FAM129B | 1.509 | Y | Y | 49.585 | Y |
| AKAP1 | 1.646 | Y | Y | 23.995 | N |
| ARHGEF7 | -1.059 | Y | Y | 42.195 | N |
| BCAR3 | -1.837 | Y | Y | 237 | N |
| CD3EAP | 1.398 | Y | Y | 272.29 | N |
| CLIP1 | 1.449 | Y | Y | 49.32 | N |
| DCP1A | 1.501 | Y | Y | 45.75 | N |
| EDC4 | -1.322 | Y | Y | 21.63 | N |
| FUSIP1 | -2.737 | Y | Y | 241.82 | N |
| MTDH | 1.291 | Y | Y | 324.28 | N |
| RPRM | -4.059 | Y | Y | 478.75 | N |
| SNTB1 | -3.373 | Y | Y | 226.02 | N |
| SORBS2 | -1.358 | Y | Y | 43.265 | N |
| SRRM1 | 1.321 | Y | Y | 14.9 | N |
| UBAP2L | 2.358 | Y | Y | 45.76 | N |
| ARAF | 1.31 | Y | N | | Y |
| NES | 1.362 | Y | N | | Y |

[a] Official gene symbol for protein target of associated peptide.
[b] Mean-Normalized Xpress ratio is defined as the ratio of the area under the curve(AUC) for heavy and light isotopes of an identified peptide. Positive numbers indicate an increase in the phosphopeptide in the WNT3A sample. Data are log[2] transformed.
[c] Phosphoproteomic Hit defined as peptides with an XPRess ratio of X where |X|>2 Standard deviations from the sample mean.
[d] If, for a given gene, all siRNA shows a twofold change in med(normFFluc) and a -logP <=2, that gene is classified as a hit.
[e] Median percent enhancement or inhibition of BAR reporter relative to control siRNA. All data are normalized to a viability marker.

We next tested whether multiple independent *FAM129B* siRNAs regulate Wnt/β-catenin signal transduction in a panel of cell lines. We tested these siRNA in HT1080s and A2058s to validate our initial screens. We also tested our siRNA in A375 and HEK293T cells to test for cell-type specificity. *FAM129B* siRNA inhibited expression of FAM129B in all lines tested as monitored by Western blot (Figure 14). Validating the results of the siRNA and phosphoproteomic screens, we observed that FAM129B knockdown inhibited both WNT3A-dependent BAR reporter activity and also expression of the Wnt-dependent target gene *AXIN2* (Figure 14). FAM129B does not universally regulate Wnt/β-catenin signal transduction, however. In testing our panel of cell lines from multiple tissues, we observed that while FAM129B knockdown potently inhibits reporter activity and target gene expression in HT1080, A2058, and A375 cells, it does not regulate reporter activity in HEK293T cells (Figure 14). To test if FAM129B specifically regulates Wnt/β-catenin signaling, we tested a panel of transcriptional reporters in A375 cells. We observed that *FAM129B* siRNA inhibit activation of the Wnt-dependent BAR reporter and the TGFβ/SMAD reporter, but not the TNFα/NFκB reporter by their respective ligands (Figure 15).

## FAM129B positively regulates apoptotic response to WNT3A in A375 melanoma cells

A375 cells, unlike A2058 cells, undergo apoptosis in response to combination WNT3A and PLX4720 treatment.[185,16] Knockdown of FAM129B using siRNA inhibits Wnt dependent reporter activity and target gene expression in A375 cells (Figure 14). To test if FAM129B knockdown regulates cellular response to WNT3A, we assayed if FAM129B siRNA inhibits apoptotic response to WNT3A by Western blot and immunofluorescence. Treatment with WNT3A or PLX4720 did not change cleaved caspase 3 immunoreactivity in combination with

either pooled *FAM129B* siRNA or pooled *CTNNB1* siRNA relative to control (Figure 16, lanes 1-9). As expected, cotreatment of A375s with WNT3A and PLX4720 increased apoptosis as monitored by cleaved caspase 3 (Figure 16, lane 10). Furthermore, apoptosis was reduced in A375 cells pretreated with *FAM129B* or *CTNNB1* siRNA (Figure 16, lanes 11 and 12). TUNEL immunofluorescence combined with automated cell counting was used to quantify the level of apoptosis in A375s pretreated with control, *FAM129B*, or *CTNNB1* siRNA. 23.28% of DAPI-positive nuclei were positive for TUNEL staining under the control condition. 17.32% and 0.48% of DAPI-positive nuclei showed TUNEL positivity in *FAM129B* and *CTNNB1* siRNA treated A375s. TUNEL positivity was significantly reduced in *FAM129B* and *CTNNB1* siRNA-treated A375s (Figure 17). *FAM129B* siRNA decreases apoptotic response to WNT3A as measured by both TUNEL immunofluorescence and cleaved caspase 3 Western blot.

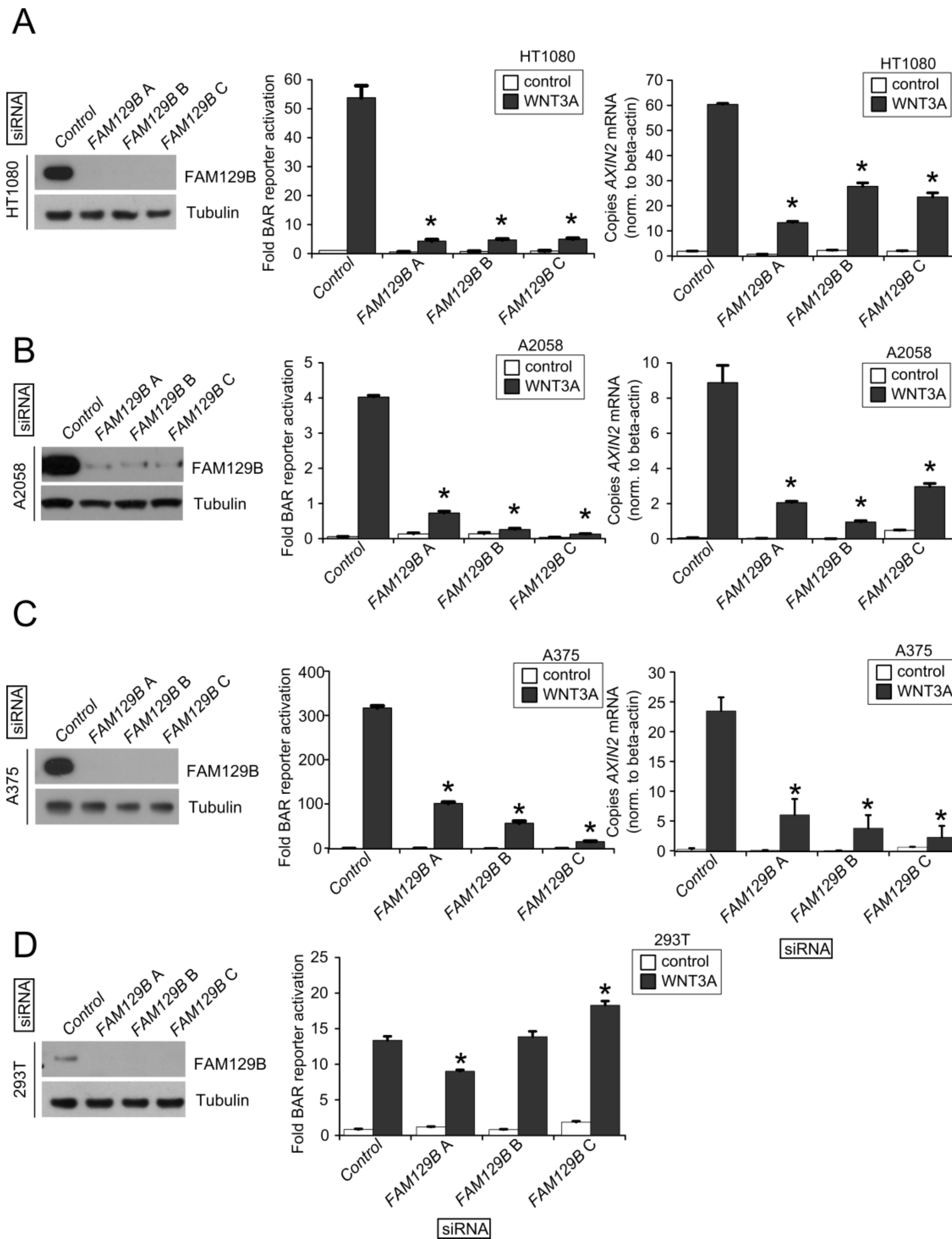



Figure 14 - FAM129B positively regulates Wnt/β-catenin signal transduction in a cell type specific manner. (A) FAM129B positively regulates Wnt/β-catenin in HT1080 fibrosarcoma. (Left panel) Three independent siRNA targeting *FAM129B* inhibit FAM129B expression in HT1080. Immunoblots show three independent siRNA reduce FAM129B steady-state levels following 72 hr of 20 nM siRNA treatment. (Middle panel) *FAM129B* siRNA inhibit Wnt-dependent luciferase reporter activity (BAR reporter) normalized to constitutively expressed *Renilla* luciferase. (Right panel) *FAM129B* siRNA inhibit Wnt-dependent *AXIN2* expression in HT1080 cells relative to β-actin mRNA expression by QPCR. (B and C) *FAM129B* siRNA inhibit FAM129B expression and Wnt dependent transcription in A2058 and A375 melanoma cells as described in (A). (D) FAM129B *siRNA* inhibit expression of FAM129B but not Wnt-dependent reporter activity (as in A). Columns and error bars represent mean and SEM, respectively. *$P$<0.05 by unpaired, two-tailed T-test.

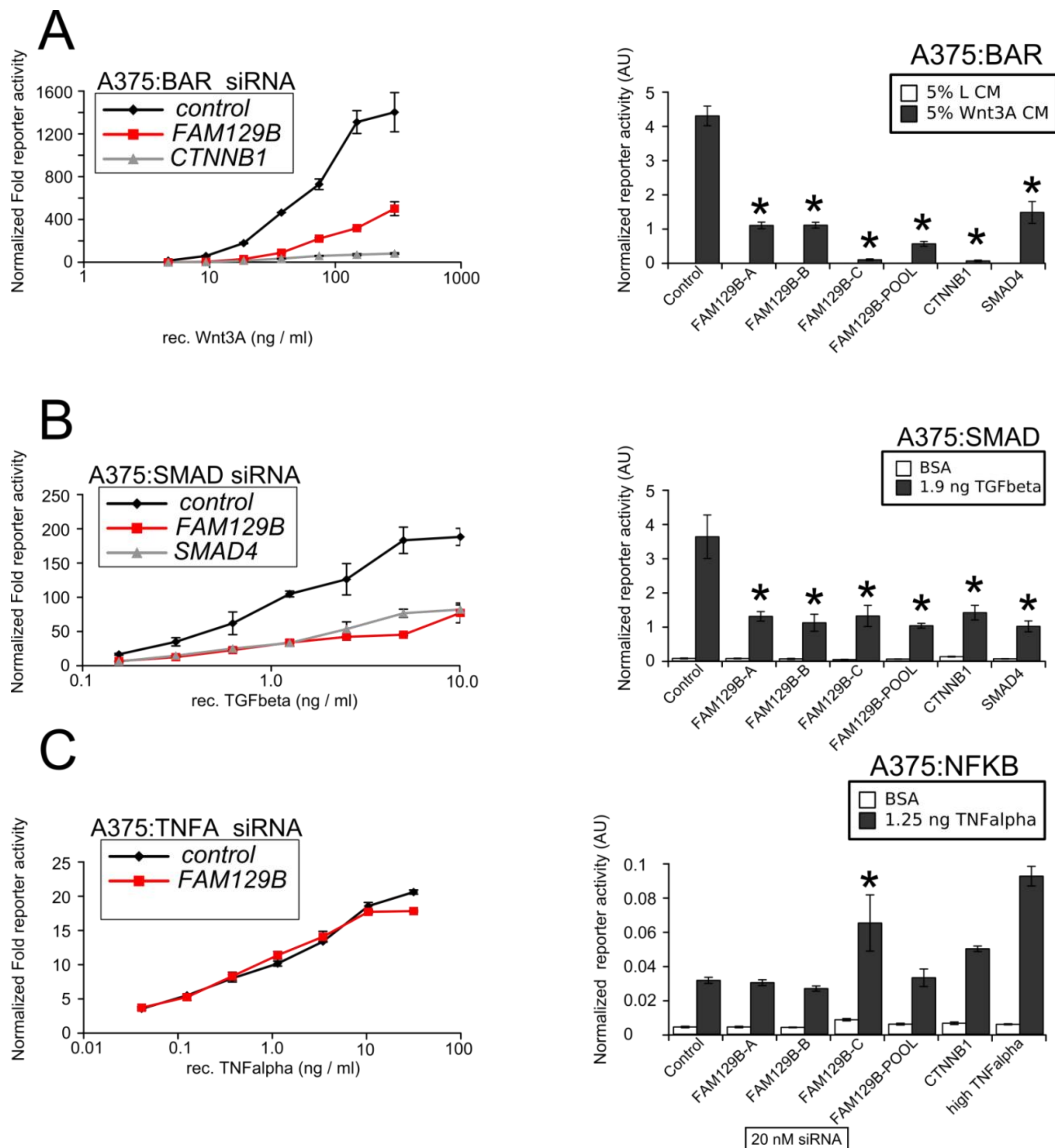



Figure 15 - FAM129B siRNA regulate Wnt-dependent transcriptional reporter and TGFβ/SMAD-dependent reporter, but not TNFα/NFκB dependent reporter. (A, left panel) pooled FAM129B siRNA inhibit Wnt-dependent BAR reporter activity over a wide range of doses. A375 B/R cells were treated for 48 hr with indicated siRNA. They were subsequently treated with a dose curve of WNT3A overnight, and assayed for reporter activity. (Right panel) A375 cells were treated with siRNA as indicated as before and treated with an EC50 dose of WNT3A. All *FAM129B* siRNA and positive control *CTNNB1* siRNA inhibit Wnt-dependent BAR reporter activity. (B and C). The same experiment was carried out as in (A) in A375 lines carrying the TGFβ/SMAD reporter or the TNFα /NFκB reporter. Data in the left panel indicate dose-dependent activation of the reporter by their respective ligands. However, FAM129B data indicate that FAM129B knockdown inhibits the TGFβ/SMAD reporter, but not the TNFα reporter. Data are representative of 3 separate biological replicates.

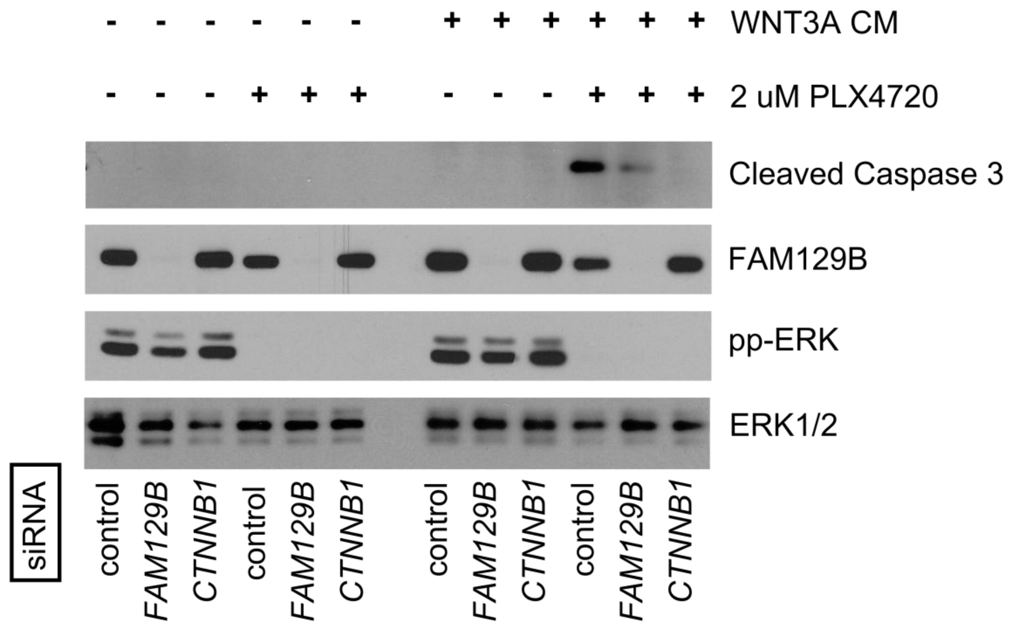



Figure 16 - FAM129B positively regulates apoptotic response to WNT3A in A375 melanoma. *FAM129B* siRNA inhibits apoptotic response to WNT3A as monitored by cleaved caspase 3 immunoblot. A375 cells were treated with pooled control, pooled *FAM129B* siRNA, or *CTNNB1* siRNA as indicated for 48 hr. Cells were subsequently treated with DMSO or 2 uM PLX4720, and L-conditioned or WNT3A-conditioned media for 24 hr as indicated. A375 cells show reduced cleaved caspase 3 expression. Knockdown of FAM129B was monitored by FAM129B immunoblot, inhibition of ERK/MAPK signaling by phospho-ERK immunoblot, and total ERK was used as normalization. Relative levels of cleaved caspase 3 were quantitated by normalizing cleaved caspase 3 pixel density to ERK1/2 for each condition relative to the maximum cleaved caspase 3 level. Data are representative of at least 3 biological replicates. *FAM129B* siRNA inhibit cleaved caspase 3 levels to between 16 and 41% of maximum.

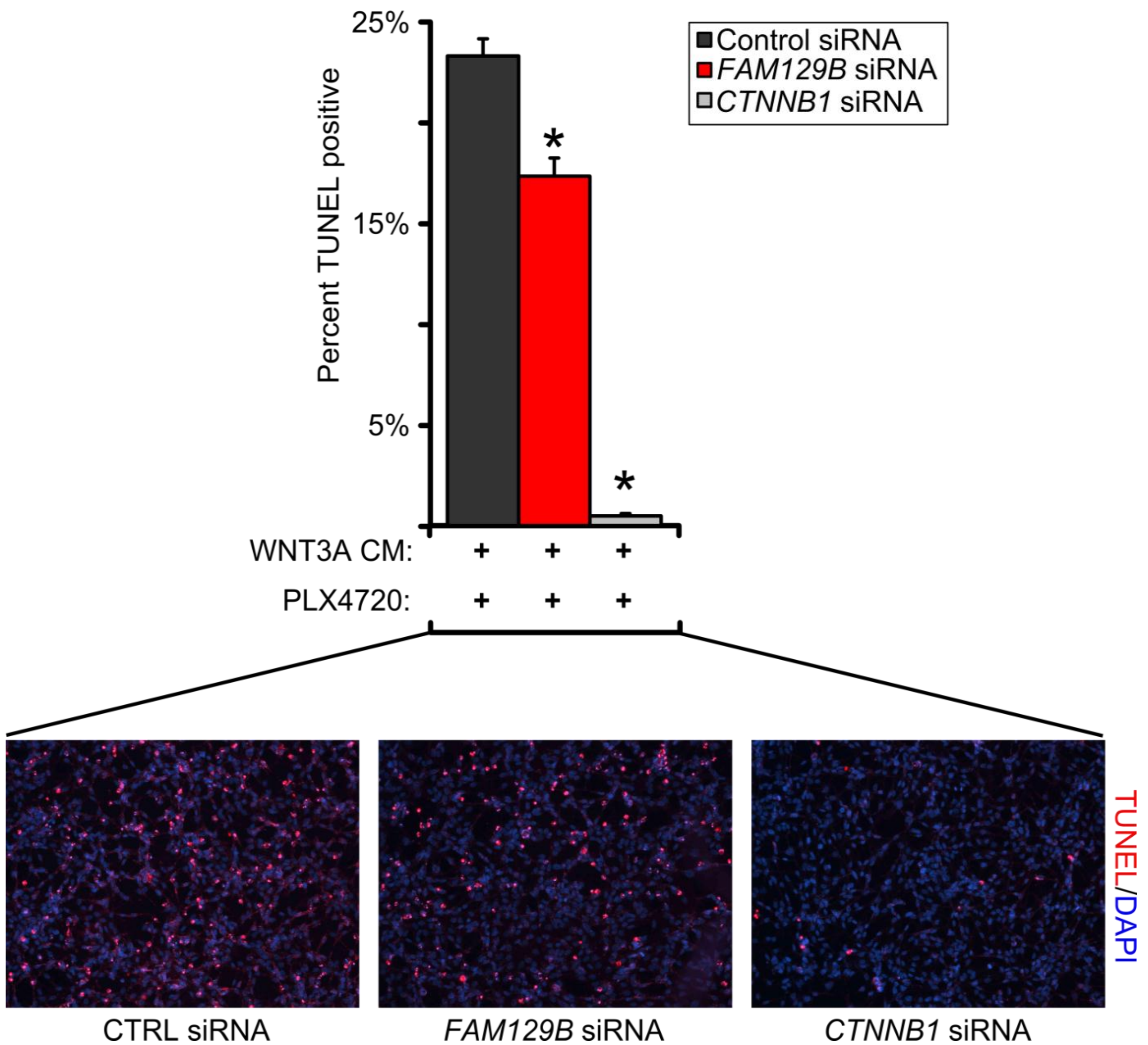



Figure 17 - *FAM129B* siRNA inhibits apoptotic response to WNT3A as quantified by TUNEL IF. A375 melanoma cells were treated as in Figure 16, fixed and stained for apoptosis by TUNEL. Percent TUNEL positive cells calculated as a percent of DAPI positive cells. (Inset) representative immunofluorescence of A375 cells treated with the indicated conditions. TUNEL staining is depicted in red and DAPI staining is depicted in blue. Columns and error bars represent the mean and SEM of three separate biological replicates. **P*<0.05 by student's T-test.

## Discussion

The central finding presented in chapter 3 is that silencing FAM129B expression in melanoma inhibits Wnt-dependent transcription, and apoptotic response to WNT3A. These findings also highlight the cell-type and process-specific role for FAM129B in cellular function. While previous reports show that FAM129B acts as a suppressor of TNFα-dependent apoptosis,[214] our data show that FAM129B promotes apoptosis in melanoma by positively regulating Wnt/β-catenin signal transduction. Furthermore, other studies show FAM129B expression impacts completely orthogonal phenotypes such as invasion.[213] Gaining a more direct understanding of the molecular mechanisms by which FAM129B exerts these effects will be important for explaining these cell-type and biological-process dependent differences in function.

Not only do our studies confirm different cell-type specific roles for FAM129B, our research also found FAM129B to be a cell-type-specific regulator of Wnt/β-catenin signaling. *FAM129B* siRNA regulated Wnt/β-catenin signal transduction in HT1080 fibrosarcoma and A375 and A2058 melanoma cell lines. However, FAM129B knockdown failed to inhibit Wnt-dependent reporter activity in HEK293T cells. Identifying context-specific regulators of Wnt/β-catenin signaling will be important therapeutically as Wnt/β-catenin signaling often serves to promote proliferation rather than suppress proliferation or promote apoptosis. In numerous cancers, including colorectal cancer and hepatocellular carcinoma, activation of Wnt/β-catenin signaling has been shown to promote neoplasia rather than suppress growth (for review see [225,226]). Thus, the identification of context-specific regulators of Wnt/β-catenin signaling will be key for manipulating this pathway specifically in the desired tissue type.

We successfully identified FAM129B as a novel regulator of Wnt/β-catenin signaling by using combinatorial screening methods. Our findings illustrate that interpretation of quantitative phosphoproteomic data can be focused with the combination of siRNA datasets. Analysis of Wnt-regulated phosphopeptides alone was not sufficient to unveil any novel, or even any known regulators of Wnt/β-catenin signal transduction. However, combination of the phosphoproteomic data with large-scale siRNA screening successfully identified a novel regulator of Wnt/β-catenin signal transduction.

Combination of the phosphoproteomic dataset with the siRNA screening dataset also served to focus the results of the large-scale siRNA screen. We were surprised to find that siRNA representing over 5000 gene targets positively or negatively regulated Wnt/β-catenin signal transduction greater than twofold. That population of genes represents almost 20% of the nearly 25000 genes estimated to be present in the human genome.[227] The large number of hits present in our HT1080 large-scale siRNA screen highlights the sensitivity of the BAR reporter over its specificity. It is likely that indirect regulators of Wnt/β-catenin signal transduction can be detected using this reporter, because of its level of sensitivity. For instance, siRNA targeting *COPA*, *COPB2*, *COPG*, and *COPS*, which encode for subunits of the coatomer complex required for transport of proteins between the endoplasmic reticulum and golgi complex, all are identified as positive regulators of Wnt/β-catenin signal transduction. These proteins, however, are likely not direct regulators of Wnt/β-catenin signaling, but rather regulate Wnt/β-catenin signaling very indirectly. For instance, they may act by regulating transportation of receptors and other proteins to the membrane. Identifying very indirect, broad regulators of Wnt/β-catenin signaling may be useful. However, it is more likely informative to enrich hits from this large dataset using

complementary approaches such as phosphoproteomics to highlight novel pathway regulators or for other purposes.

The raw data presented in these screens will serve as a resource for researchers to compare with additional datasets to identify novel regulators of Wnt/β-catenin signal transduction and to identify novel biological processes regulated by Wnt/β-catenin signal transduction. Just as we combined these datasets to identify FAM129B as a regulator of Wnt/β-catenin signaling, it is possible that these datasets could be combined with other data to answer questions as to how Wnt treatment impacts other biological processes and other biological processes impact Wnt/β-catenin signaling. These data will be published as a public resource in the *Journal of Investigative Dermatology*.

Indeed, our dataset still contains a rich set of putative Wnt/β-catenin feedback regulators that require further characterization. 16 protein targets were identified between the phosphoproteomic and large-scale siRNA screen and remain untested as regulators of Wnt/β-catenin signal transduction. These include signaling proteins including the A-kinase Anchoring Protein, AKAP1, and the rho-GEF ARHGEF7, which have never previously been linked to Wnt/β-catenin signal transduction. This list also includes proteins like MTDH, which has been previously shown to regulate Wnt/β-catenin signaling, however not in the context of melanoma. Although these proteins were not identified by our bioinformatic analysis as melanoma-associated, their appearance in the phosphoproteomic dataset indicates their likely expression in melanoma, and leaves open the possibility that they act as regulators of Wnt/β-catenin signaling in that context, or in others.

While our research focused on using phosphoproteomics to identify regulators of Wnt/β-catenin signaling, our phosphoproteomic data may also provide insight into other roles Wnt treatment plays in melanoma beyond changing transcription via β-catenin. Our GO analysis revealed that the phosphopeptides differentially modulated by Wnt/β-catenin signal transduction belong to proteins known to be involved in multiple diverse biological processes ranging from rRNA metabolism to nitrogen compound metabolism to the M phase of the cell cycle. Canonical Wnts, including WNT3A have been shown to regulate cellular phenotypes independent of β-catenin, including dedifferentiation of chondrocytes via Ca++ / CAMKII signaling and Trophoblast migration via PKB/AKT signaling.[228,229] For these reasons, further investigation of the Wnt-regulated phosphoproteome may potetentially identify further β-catenin -independent effects of WNT3A treatment.

Principally, the results presented in this chapter reveal that knockdown of FAM129B inhibits the transduction of Wnt/β-catenin signaling pathway, and inhibits apoptotic response to WNT3A in melanoma. Our study also highlights the importance of cellular context when considering signal transduction. Our model for how signal transduction pathways regulate different cells, and to what extent they do so, remains incomplete, and further understanding of how different molecules act in different contexts will help to refine those models.

# Chapter 4: Future directions

## Identifying the mechanism of AXIN1 degradation in melanoma

Studies by Biechele *et al.*,[14] as well as the data presented in chapter 2, highlight the central role of AXIN1 stability in regulating apoptosis in both *BRAF*- and *NRAS*-mutant melanomas. Previous in vitro studies using extracts derived from Xenopus oocytes elegantly recreated the cytoplasmic steps of the current Wnt/β-catenin pathway derived from genetic studies in model organisms, and also facilitated the development of kinetic modeling of the critical regulation of β-catenin stability by AXIN1 and APC.[43,44] These studies pinpointed cellular abundance of AXIN1 as the limiting step in the regulation of cellular β-catenin abundance by the so-called destruction complex comprised of AXIN1, APC and GSK3β, so it may not be all that surprising that AXIN1 abundance is a potential regulatory nexus of the crosstalk between Wnt/β-catenin and MEK/ERK signaling, as observed in *NRAS*- and *BRAF*-mutant melanomas (see chapter 2). It will be critical to identify the mechanism by which MEK/ERK pathway inhibitors significantly decrease AXIN1 abundance in some cell lines, while having little measurable effect in other cell lines. Our previous studies found that the proteasome inhibitor MG132 inhibits loss of AXIN1 protein due to WNT3A and MEK/ERK pathway inhibitor treatment in A375 melanoma cells,[14] suggesting that the loss of AXIN1 following WNT3A and MEK/ERK pathway inhibitor treatment in selected cell lines is mediated by proteasomal degradation. Consistent with a post-translational mechanism, changes in AXIN1 abundance were not correlated with changes at the transcriptional level.[14] A number of post-translational modifications of AXIN1 have previously been shown to regulate poly-ubiquitination and proteasomal degradation of AXIN1 (Figure 18). Finding the mechanism by

which MEK/ERK regulates AXIN1 proteasomal degradation may be a matter of identifying the changes in AXIN1 post-translational modification following MEK/ERK pathway inhibition.

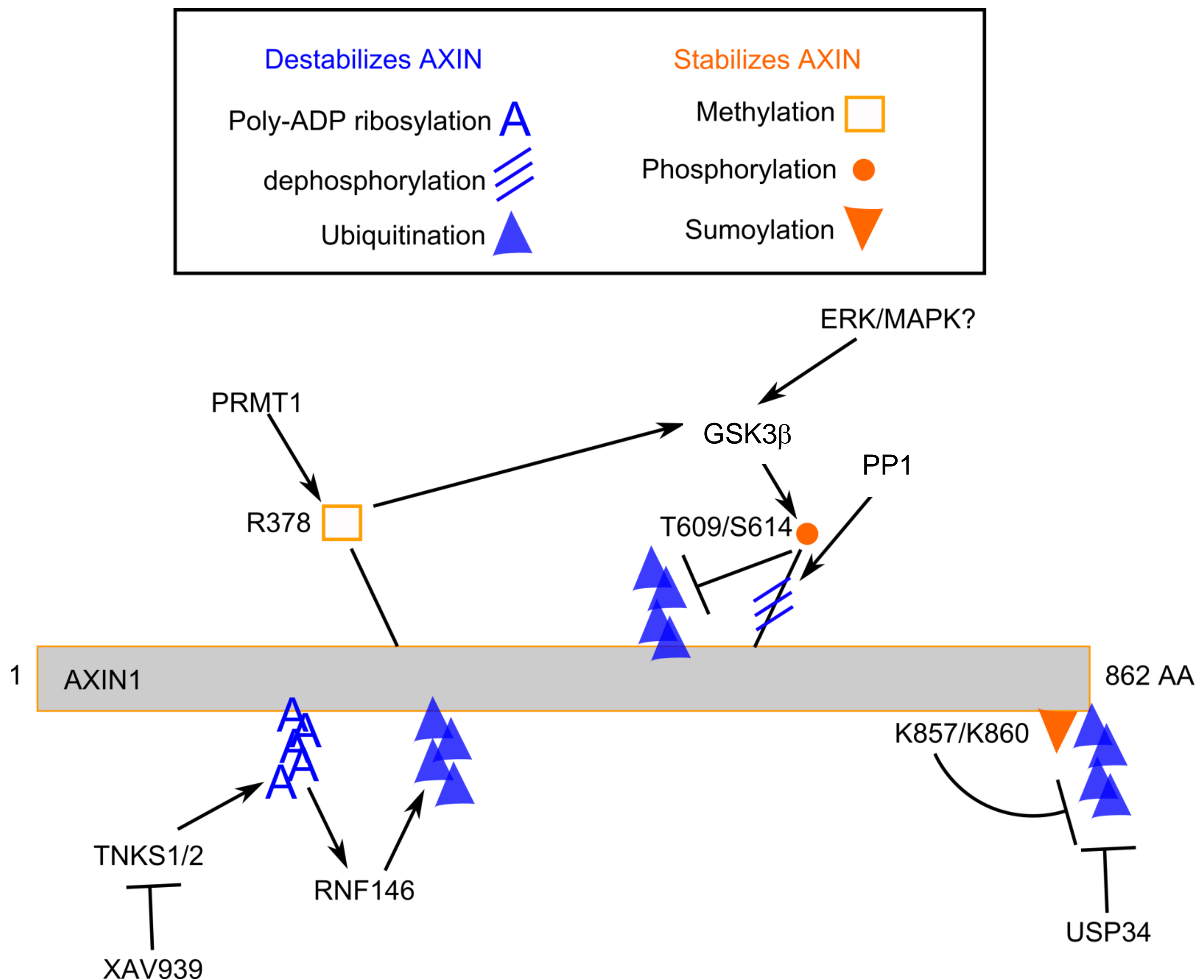



Figure 18 - Known mechanisms of AXIN1 degradation. PRMT1 is known to methylate AXIN1 at arginine 378. AXIN1 methylation increases GSK3β phosphorylation of AXIN1 at T609/S614, increasing AXIN1 stability. WNT3A treatment results in AXIN1 dephosphorylation, ubiquitination, and degradation. PP1 is known to dephosphorylate AXIN1 at the GSK3β phosphorylation sites. MEK is known to phosphorylate and activate GSK3β, potentially stabilizing AXIN. The enzymes TNKS1/2 PARsylate AXIN1 amino terminal region, promoting ubiquitination by the E3 ubiquitin ligase RNF146. The small molecule XAV939 inhibits TNKS1/2, increasing AXIN1 stability by decreasing its PARsylation. The C-terminal tail of AXIN1 is sumoylated. These SUMOylation events inhibit AXIN1 ubiquitination and degradation. Likewise, USP34 deubiquitinates AXIN1, promoting its stability.

In the absence of a Wnt ligand, glycogen synthase kinase-3β (encoded by GSK3B) phosphorylates AXIN1, promoting its stability.[41] We have observed this change in stability in apoptosis-sensitive melanoma lines (Figure 6C). In the presence of WNT3A, AXIN1 becomes dephosphorylated[41] and degraded.[42] Pulse-chase analysis of cells following treatment with the GSK3β inhibitor lithium chloride (LiCl) suggested that GSK3β inhibition decreased AXIN1 phosphorylation and decreased AXIN1 half-life.[42] Conversely, treating cells with a broad-spectrum phosphatase inhibitor increased AXIN1 phosphorylation and AXIN1 half-life.[42] Furthermore, mutation of AXIN1 at putative GSK3β phosphorylation sites to phospho-dead alanine decreased the half-life of AXIN1 relative to wild-type.[41] The F-box class of ubiquitin ligases that regulate the proteasomal degradation of other components of the Wnt/β-catenin signaling pathway identify their substrates by phosphorylation status, and these reported observations suggest that AXIN1 may be similarly targeted for degradation by changes in phosphorylation.

One possibility is that MEK/ERK signaling regulates AXIN1 phosphorylation via GSK3β. MEK has been shown to phosphorylate and activate GSK3β.[230] As mentioned above, active GSK3β increases AXIN1 stability.[41,42] Therefore, inhibiting MEK may inhibit GSK3β and decrease AXIN stability. Nonetheless, blocking MEK/ERK signaling using a MEK inhibitor measurably changes the phosphorylation state of many proteins both directly and indirectly in melanoma and it will be important to determine whether the differential regulation of AXIN1 by some of these downstream MEK/ERK-regulated effectors participates in determining the sensitivity of melanoma cell lines to apoptosis with inhibitors like vemurafenib.[213]

Phosphorylation of AXIN1 by GSK3β is also regulated upstream by methylation. Protein arginine methyltransferase 1 (encoded by PRMT1) promotes the methylation of AXIN1 in the GSK3β-binding domain at arginine-378. This modification increases GSK3β association with AXIN1, increasing phosphorylation of AXIN1, decreasing ubiquitination and promoting stability. It is possible that MEK/ERK signaling regulates methylation in some yet-undiscovered way.[231]

In addition to phosphorylation and methylation, there is strong evidence that C terminal SUMOylation promotes the stability of AXIN1. Deleting the SUMOylation site on the six C terminal amino acids of AXIN1 decreases the half-life of AXIN1 relative to wild-type. Furthermore, restoring SUMOylation to the C terminus of AXIN1 by addition of a heterologous SUMOylation site decreases AXIN1 ubiquitination and restores stability of AXIN1.[232] Monitoring SUMOylation of AXIN1 following MEK/ERK pathway inhibition may offer insight into the mechanism by which MEK/ERK signaling regulates AXIN1 stability.

AXIN1 stability is not only regulated by the cell prior to poly-ubiquitination, but is also regulated post-ubiquitination. RNF146 has been previously identified as an E3 ubiquitin ligase involved in PARsylation-dependent AXIN1 degradation, although its role downstream of MEK/ERK PATHWAY inhibition remains as yet uncharacterized.[233,234] As an endogenous counterbalance to AXIN1 degradation by PARsylation, de-phosphorylation, de-methylation and subsequent ubiquitination, the ubiquitin-specific protease USP34 has been shown to reverse ubiquitination of AXIN1, inhibiting its degradation. The transfection of cells with siRNA targeting USP34 increased AXIN1 ubiquitination, and decreased AXIN1 steady-state abundance.[235] It remains to be seen whether USP34 knockdown decreases AXIN1 in apoptosis-sensitive or apoptosis-resistant melanoma. However, a difference in USP expression or activity

could conceivably regulate differences in response to BRAF inhibition between apoptosis-sensitive and apoptosis-resistant melanoma cell lines.

## Identifying additional cancers where Wnt/β-catenin signaling promotes apoptosis

Is an apoptotic role for Wnt/β-catenin signaling exclusive to melanoma? Or, might other cancers undergo apoptosis in response to Wnt/β-catenin signaling? Our lab successfully identified a protective role for Wnt/β-catenin signaling in melanoma using the following foundational evidence. First, Wnt/β-catenin signal transduction components are rarely mutated in melanoma.[8,9] Second, Wnt/β-catenin signal transduction drives differentiation of melanocyte precursors into melanocytes. [63–69] Third, nuclear β-catenin is lost during melanoma progression. [72–74] Fourth, the negative regulator of Wnt/β-catenin signaling, WNT5A increases expression with disease progression.[72,236] Fifth, activation of Wnt/β-catenin signal transduction *in vitro* promotes differentiation or decreases proliferation. [13,15] Sixth, high levels of Wnt/β-catenin signal transduction promotes survival in melanoma. [13]

To identify potential cancers where Wnt/β-catenin signaling plays a protective role, I searched for cancers that met these six criteria. First, I utilized the COSMIC database (http://www.sanger.ac.uk/genetics/CGP/cosmic/) to determine the frequency by which WNT/β-catenin pathway components are mutated in a wide variety of tissues.[8,9] As expected, Wnt/β-catenin pathway components, including APC, are frequently found to be mutated in cancers of the large intestine within the COSMIC database (46%, Figure 19). In contrast, 16% of skin samples harbor mutations in Wnt/β-catenin signal transduction components.

The rate of Wnt/β-catenin pathway mutagenesis is not sufficient to predict a protective role for Wnt/β-catenin signaling in that tissue. Wnt/β-catenin signal transduction is known to play an oncogenic role in a number of tissues that show an even lower rate of Wnt/β-catenin pathway mutation than skin. For instance, breast cancers rarely harbor Wnt/β-catenin pathway mutations (4%, Figure 19). However, Wnt/β-catenin pathway is frequently activated in breast cancer by epigenetic means.[237] Furthermore, increased level of Wnt/β-catenin signal transduction predicts worse outcomes for breast cancer patients.[237]

In contrast to breast cancer, osteosarcoma meets many of the same criteria that originally identified a protective role for Wnt/β-catenin signaling in melanoma (Table 2). Osteosarcomas have a low rate of Wnt/β-catenin pathway mutation ( Figure 19 and Table 2). Wnt/β-catenin signal transduction drives differentiation of osteoblast precursors into osteoblasts.[238–241] Nuclear β-catenin is lost during osteosarcoma progression.[242] The negative regulator of Wnt/β-catenin signaling, WNT5A increases expression with osteosarcoma progression. [243,244] Activation of Wnt/β-catenin signal transduction *in vitro* promotes differentiation or decreases proliferation of osteosarcomas. [242] In patients, high levels of Wnt/β-catenin signal transduction promotes survival in osteosarcoma. [245]

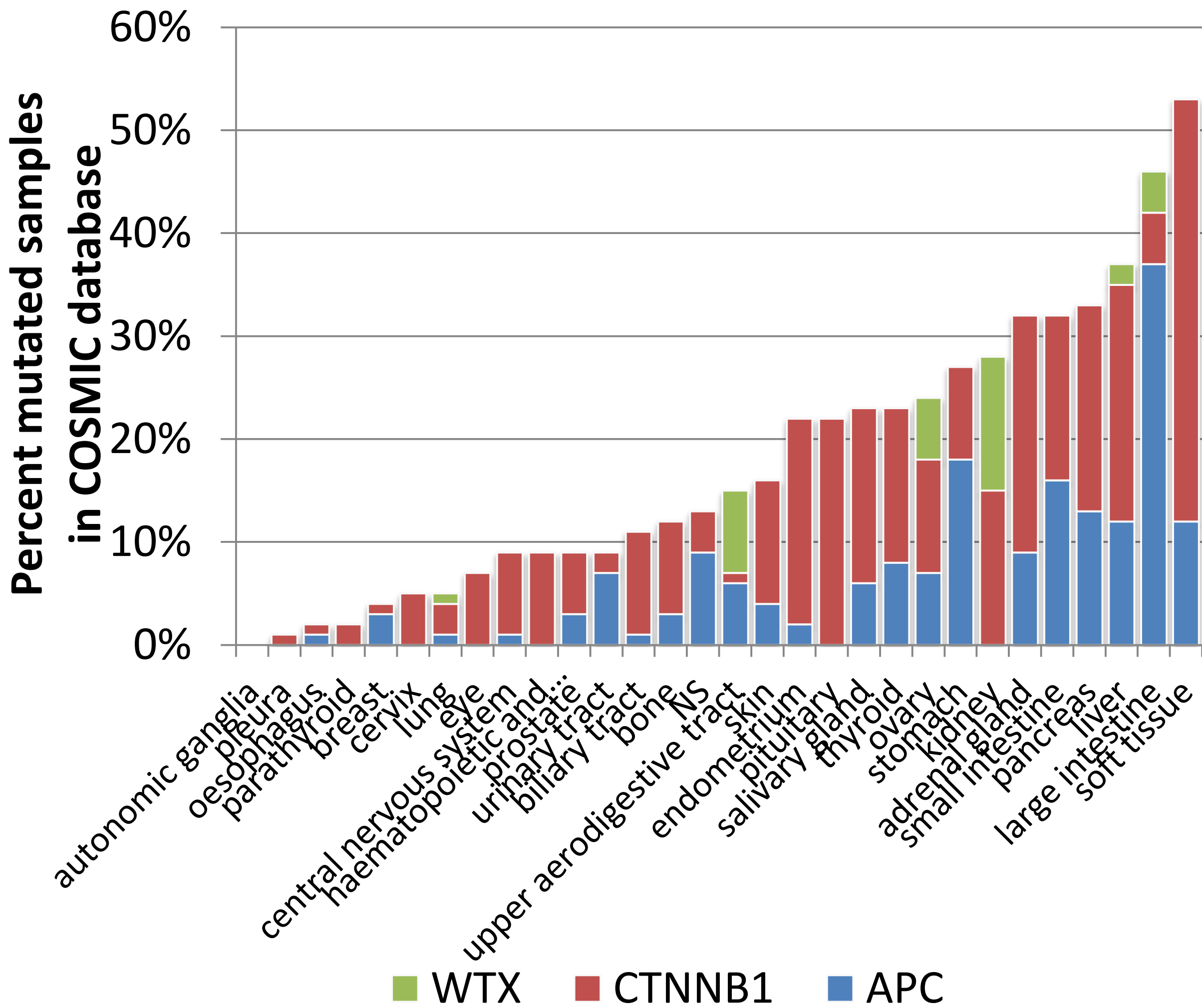



Figure 19 - Frequency of Wnt-activating mutations found in the COSMIC database, organized by tissue type. The frequency of mutations in WTX, β-catenin, and APC were cataloged as a percent of total samples tested for each tissue type. As expected, large intestine harbored frequent mutations in APC, β-catenin, and WTX (46%). Skin harbored fewer mutations Wnt-activating mutations(16%).

Table 2 – Multiple criteria support a protective role for Wnt/β-catenin signaling in Melanoma and osteosarcoma.

| Criterion | Melanoma | Osteosarcoma |
|---|---|---|
| Mutations in APC, β-catenin or WTX | 5%<br>(32 / 639) | 0%<br>(0 / 201) |
| Increasing Wnt5A with cancer progression | Yes | Yes |
| Wnt/β-catenin differentiates precursors | Yes | Yes |
| Loss of nuclear β-catenin during disease progression | Yes | Yes |
| Does Axin2 correlate with survival? | No | Yes |

Looking first at the rate of mutation in osteosarcoma, it is clear that bone cancers, in general, harbor a lower Wnt/β-catenin pathway mutation rate than skin cancers in the COSMIC database ( Figure 19). Even more surprisingly, 0% of osteosarcomas were found to have a Wnt/β-catenin pathway mutation (0 samples out of 201 sequenced), compared to melanoma, which still harbors about a 5% Wnt/β-catenin pathway mutation rate (32 samples out of 639 sequenced) ( Figure 19 and Table 2). Several independent analyses of osteosarcoma support the observation from the COSMIC database that osteosarcomas rarely harbor Wnt/β-catenin pathway mutations.[8,9] Interestingly, benign osteoblastomas often harbor Wnt/β-catenin pathway mutations, and show hyperactive Wnt/β-catenin pathway, indicating that Wnt/β-catenin pathway may actually prevent oncogenesis.[242] The evidence that Wnt/β-catenin pathway mutation rarely, if ever, occurs in osteosarcoma suggests that perhaps Wnt/β-catenin pathway activation selects against oncogenesis, or at least, does not play a role in promoting oncogenesis.

Similar to how Wnt/β-catenin signaling drives differentiation of neural crest cells to become melanocytes, [63–69] Wnt/β-catenin signaling is also required for differentiation of osteoblasts.[238–241] The differentiation role for Wnt/β-catenin signaling has been specifically demonstrated in genetic mouse models.[238] Conditional knockout of β-catenin in limb and head mesenchyme by Hill and colleagues demonstrated the requirement of Wnt/β-catenin signal transduction for osteoblast differentiation. [238] When β-catenin was knocked out in the limb and head mesenchyme, appendicular long bones were significantly shortened and lacked some distal structures. Furthermore, markers for Wnt/β-catenin transcription and mature osteoblast markers were absent in β-catenin mutant limbs. Subsequent *in vitro* experiments demonstrated that knockout of β-catenin from mesenchymal stem cells drove these cells down a chondrocyte lineage rather than an osteoblast lineage. [238]

The role for Wnt/β-catenin signaling in osteoblast differentiation has also been observed in human genetic disease. A patient with autosomal dominant osteopetrosis type II was found to have an in-frame deletion mutation of two amino acid residues in the Wnt-receptor LRP5.[246] *in vitro* analysis of this mutant LRP5 indicated a decreased inhibition of Wnt-LRP5 dependent signaling by the inhibitor proteins DKK1 and Sclerostin. This increase in bone density has been attributed to an increase in the number of osteoblasts relative to osteoclasts. [246]

As is the case in melanoma progression,[72–74] nuclear β-catenin is lost during osteosarcoma progression.[242] In a histochemical analysis of 52 osteosarcoma samples and 15 benign osteoblastoma samples it was observed that nuclear β-catenin is rare in osteosarcoma and common in benign osteoblastoma. 100% (15/15) of osteoblastoma samples were scored as positive for nuclear β-catenin. Only 10% (5/52) of osteosarcoma showed any nuclear β-catenin staining, and these were scored as weak nuclear staining compared to osteoblastoma.[242] This evidence indicates a potentially protective role for nuclear β-catenin signaling in osteosarcoma akin to melanoma, where nuclear β-catenin is observed in benign nevi, but is lost in malignant melanoma.

Multiple array datasets have observed that the inhibitor of Wnt/β-catenin signaling, *WNT5A*, increases during melanoma progression.[72,247] Likewise, WNT5A increases expression with osteosarcoma progression.[243,244] A study of 42 ostosarcoma patient biopsies revealed that WNT5A protein expression correlates with Enneking surgical stage (p=0.001), but not with age, gender or pathological type.[244] Highly invasive osteosarcoma sublines derived from the osteosarcoma cell line Hu09 express high levels of WNT5A relative to parental cells or cells with decreased invasion.[243] These highly invasive, WNT5A-expressing osteosarcomas metastasize significantly more frequently to the lung than parental or low WNT5A

osteosarcomas (200 metastases versus 50 metastases $p<0.01$). [243] Multiple negative regulators of Wnt/β-catenin pathway exist, including DKK1 and the soluble Frizzled related proteins (SFRPs).[248] It is intriguing that both melanoma and osteosarcoma upregulate WNT5A, as opposed to these other negative regulators. This observation could potentially indicate a common transcriptional program both cancers undergo during disease progression, and/or a specific role for WNT5A in disease progression in addition to Wnt/β-catenin pathway inhibition.

In mouse B16 melanoma, Wnt/β-catenin pathway activation drives the expression of multiple genetic markers of differentiation, as well as phenotypic markers including pigmentation.[13] Likewise, activation of Wnt/β-catenin signal transduction *in vitro* promotes differentiation or decreases proliferation of osteosarcomas. Treatment of a panel of four human osteosarcoma lines with a GSK3β inhibitor increased Wnt-dependent reporter activity in all cell lines, resulted in the decreased proliferation of two cell lines (MG-63 and U2OS) and the differentiation of two cell lines (HOS and SJSA-1). Both HOS and SJSA-1 cells exhibited increase alkaline phosphatase activity following 3 days of treatment with GSK3β inhibitors.[242]

My analysis of osteosarcoma patient data suggests high levels of Wnt/β-catenin pathway activation promote survival in osteosarcoma. In melanoma, it was observed that high nuclear β-catenin localization correlates to increased survival and decreased time to metastasis. [13] A similar relationship between nuclear β-catenin and survival or disease phenotype has not been observed with osteosarcoma. However, large panels of osteosarcoma linked to survival data have undergone microarray expression analysis.[245] One of those microarray datasets contained probes for the direct Wnt/β-catenin target gene *AXIN2*.[245] I segregated osteosarcomas in this dataset by tertile into *AXIN2* high, *AXIN2* medium, and *AXIN2* low, and performed Kaplan-Meier survival analysis on the upper and lower tertiles. Strikingly, patients expressing low levels of *AXIN2*

experience dramatically reduced survival (Figure 20). One limitation of these microarray analyses in comparison to nuclear β-catenin histology is the possibility that *AXIN2* expression is high in the cancer stroma, but not in the cancer tissue itself. Nonetheless, these data would point to a high level of Wnt/β-catenin signal transduction in the cancer tissue, and suggests that activating Wnt/β-catenin signaling has protective effects.

Surprisingly, *AXIN2* expression has not been shown to correlate with patient survival in melanoma (Table 2). Other correlates for Wnt/β-catenin signal transduction have been shown to correlate with patient survival,[13] but *AXIN2* gene expression by microarray has not. One technical possibility is that different microarray probes were used to for melanoma patients and osteosarcoma patients and that those probes resulted in different sensitivity to *AXIN2* expression. One biological possibility is that osteosarcomas express *AXIN2* with greater dynamic range than melanomas and therefore provide a more sensitive readout for level of Wnt/β-catenin signaling. Whatever the difference, it will be important to determine if *AXIN2* expression and Wnt/β-catenin pathway activation promotes survival in both melanoma and osteosarcoma.

The evidence above suggests that, as in melanoma, Wnt/β-catenin signaling has a protective effect in osteosarcoma. Wnt/β-catenin pathway members are rarely mutated in osteosarcoma ( Figure 19 and Table 2). Nuclear Wnt/β-catenin is rarely found in osteosarcoma. [242] GSK3β inhibitors differentiate osteosarcoma cells *in vitro* and suppress proliferation. [242] Patients expressing high levels of Wnt/β-catenin target genes show increased survival relative to those expressing low Wnt target genes (Figure 20). The characterization of Wnt/β-catenin signaling's protective role has laid the groundwork for identifying additional cancers wherein Wnt/β-catenin signaling acts in a protective manner. The parallels beween Wnt/β-catenin signaling in osteosarcoma and melanoma provide an enticing new target. The roadmap exists for

identifying additional cancers that are susceptible to WNT3A, it is now a matter of following up on that roadmap in a manner similar to the first part of my thesis.

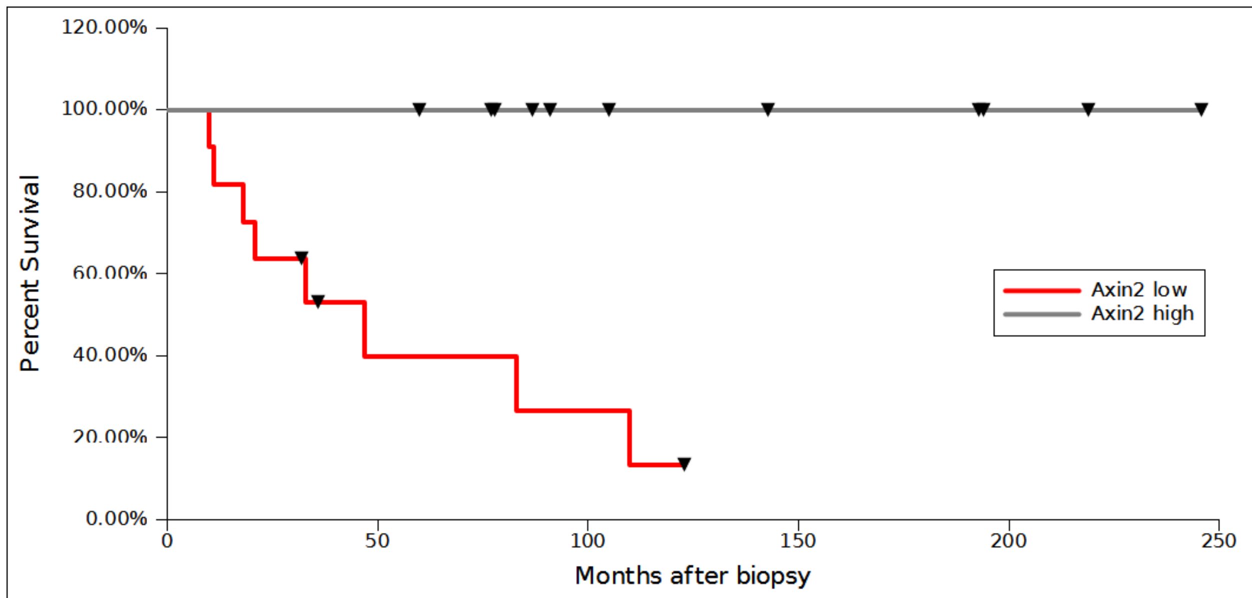



Figure 20 - High AXIN2 predicts survival in osteosarcoma. 34 osteosarcoma patients were biopsied and their osteosarcoma were analyzed for gene expression by microarray.[249] Patients were separated by expression of the Wnt/β-catenin target gene *AXIN2* into an upper tertile and a lower tertile (*AXIN2* low and *AXIN2* high). Osteosarcoma patients expressing low *AXIN2* have significantly decreased survival (P<0.001) with a median survival of 47 months. Median survival of *AXIN2* high expressing patients is >250 months.

## Identifying how FAM129B regulates Wnt/β-catenin signal transduction

As mentioned in Chapter 3, the mechanism by which FAM129B exerts its effect on Wnt/β-catenin signal transduction in melanoma and other cell types, its effect on TNFα-mediated apoptosis in HeLa cells, and its effects on invasion in melanoma remains largely unknown. Gaining a more direct understanding of the molecular mechanisms by which FAM129B exerts these effects will be important for explaining these cell-type and biological-process dependent differences in function.

FAM129B may regulate Wnt/β-catenin signaling as an intermediary between MEK/ERK signaling and Wnt/β-catenin signaling. MEK/ERK signaling negatively regulates Wnt/β-catenin signaling in A375 melanoma cells.[14,16] Furthermore, FAM129B is phosphorylated downstream of hyperactive MEK/ERK signaling in melanoma.[213] If MEK/ERK signaling regulates Wnt/β-catenin signaling via FAM129B, silencing FAM129B should inhibit synergy induced by MEK/ERK pathway inhibition. However, I observe that *FAM129B* siRNA regulates Wnt/β-catenin dependent reporter activity to the same degree in the presence or absence of targeted BRAF inhibitor PLX4720 (Figure 21).

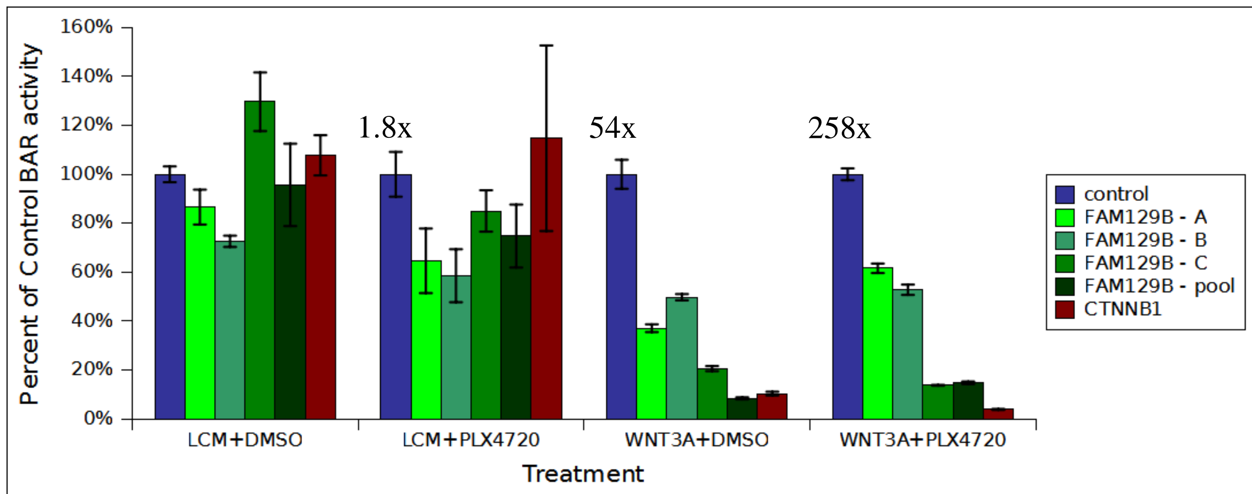


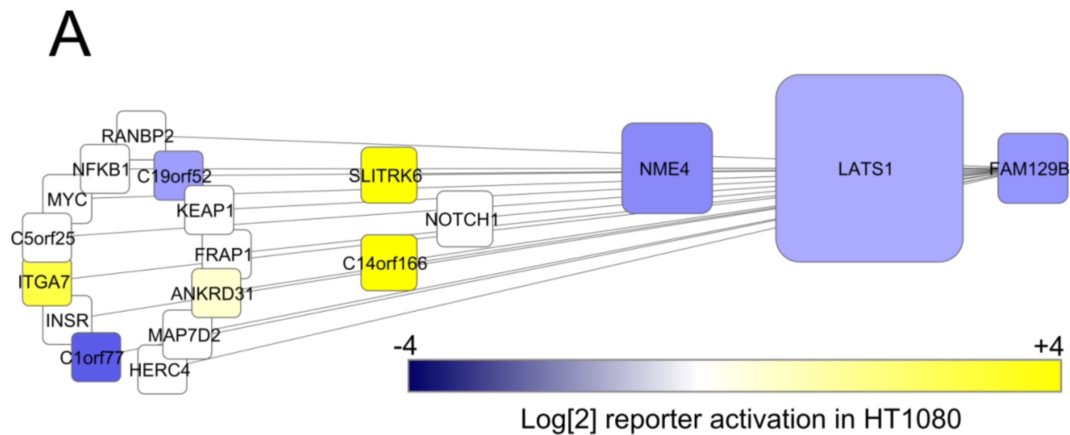

Figure 21 – MEK/ERK signaling does not negatively regulate Wnt/β-catenin signaling through FAM129B. As indicated above the control siRNA condition, PLX4720 treatment alone activates the BAR reporter 1.8 fold, WNT3A treatment activates the BAR reporter 54-fold, and WNT3A+PLX4720 activates the BAR reporter 258 fold. However, when each control condition is normalized to 100%, it is clear that *FAM129B* siRNA inhibit Wnt-dependent BAR reporter activity to the same extent in WNT3A treated cells as in WNT3A+PLX4720 treated cells.

While the above finding excludes the possibility that MEK/ERK signaling regulates Wnt/β-catenin signaling through FAM129B, it remains a possible that the effect of FAM129B on Wnt/β-catenin signaling requires ERK/MAPK phosphorylation. That possibility requires testing through rescue of *FAM129B* siRNA with phosphomutant forms of FAM129B. It might also be useful to test if *FAM129B* siRNA inhibit Wnt/β-catenin signaling in normal human melanocytes, which lack hyperactive MEK/ERK signaling. This type of experiment could provide corroborative evidence for a role of FAM129B phosphorylation in its regulation of Wnt/β-catenin signaling. This is a promising future direction for exploring the role of MEK/ERK signaling on FAM129B-dependent regulation of Wnt/β-catenin signaling.

Another approach for identifying the mechanism of FAM129B action is through protein-protein interaction. FAM129B contains no known catalytic domains,[213] so it likely exerts its effects as a scaffolding protein or adaptor protein, recruiting regulatory enzymes to specific complexes or locations within the cell. Indeed, FAM129B harbors a pleckstrin homology domain, which is known to bind phospholipids and anchor proteins to lipid bilayers.[250,251] Given that FAM129B localizes to the adherens junction,[213,252] this functional domain offers a biochemical mechanism for this localization. Furthermore, this localization may play a role in its function by interacting with proteins at the cell membrane. To identify FAM129B interactors, I performed multiple affinity purification: mass spectrometry experiments. These experiments yielded a number of putative FAM129B interactors including the large antigen tumor suppressor (LATS1) and the Kelch-like ECH-associated protein 1 (KEAP1, Figure 22A).

Overexpression of proteins can force interactions that do not occur under endogenous conditions. So, I used endogenous immunoprecipitation to test for the above predicted FAM129B interactions. To test if FAM129B interacts with the proteins identified by mass

spectrometry, I immunoprecipitated FAM129B and the predicted FAM129B interactors LATS1 and KEAP1 in A375 melanoma cells (Figure 22B). To test if FAM129B coimmunoprecipitates with these predicted interactors, I immunoblotted for FAM129B, LATS1, and KEAP. I also immunoblotted for β-catenin as it has previously been shown to colocalize with FAM129B at the adherens junction.[213,214] Immunoblot analysis indicates that FAM129B, LATS1, and KEAP1 antibodies immunoprecipitate their target proteins. However, immunoblotting could not detect FAM129B coimmunoprecipitation with LATS1 or KEAP1 via FAM129B purification, LATS1 purification, or KEAP1 purification. To test for any coimmunoprecipitation, I immunoblotted for the known LATS1 interactor MST1/2 (hippo). Surpisingly, FAM129B coimmunoprecipitates MST1/2 (Figure 22B). The discrepancy between FAM129B interaction with MST1/2 endogenously and LATS1 interaction when overexpressed could be explained a number of ways. First, it is possible that FAM129B interacts with LATS1 and MST1/2 under slightly different conditions not yet controlled for. One example is cell confluency, which regulates MST1/2 and LATS signaling.[253–255] Another possibility is that FAM129B overexpression forces a LATS1 interaction, when it endogenously interacts with MST1/2. A third possibility is that mass spectrometry is more sensitive to detecting LATS1 than it is MST1/2 and that both interactions were present in the mass spectrometry experiments. From the endogenous coimmunoprecipitation data and the FLAG-GFP-FAM129B immunoprecipitation data, I conclude that FAM129B coimmunoprecipitates with MST1/2 and possibly in a larger complex including LATS1.



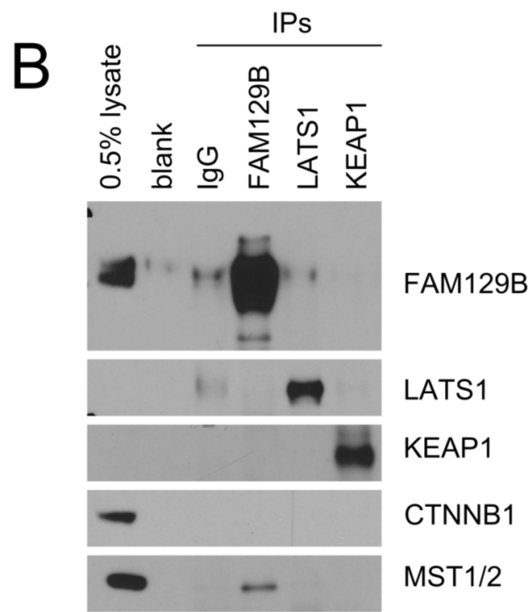




Figure 22 - Affinity purification / MS identifies MST1/2 (Hippo) as a FAM129B interacting protein. (A) FLAG-purification of FLAG-GFP-FAM129B unveils multiple potential FAM129B interactors. Nodes depict FAM129B interacting proteins identified by affinity purification: mass spectrometry. Node size indicates number of peptide identifications (LATS1=21 peptides, NME4 = 7 peptides, KEAP1 = 1 peptide). FAM129B is not drawn to scale (485 peptides). Node color indicates Log[2] reporter activation in HT1080 screen described in Figure 12. (B) FAM129B endogenously coimmunoprecipitates with MST1/2 (Hippo) in A375 melanoma cells. Endogenous FAM129B and predicted FAM129B interactors LATS1 and KEAP1 were coimmunnoprecipitated from A375 cells. Immunoblot for FAM129B, LATS1, and KEAP1 indicate that each antibody successfully immunoprecipitated their target. Furthermore, FAM129B was not found to coimmunoprecipitate with the predicted interactors LATS1, KEAP1, or the known Wnt/β-catenin pathway member, β-catenin. However, FAM129B was shown to coimmunoprecipitate MST1/2 (Hippo), the known LATS1 interactor.

The finding that FAM129B coimmunoprecipitates MST1/2 provides a potential mechanism whereby FAM129B regulates Wnt/β-catenin signal transduction (Figure 23). The kinases MST1/2 are known to positively regulate Wnt/β-catenin signal transduction. [254,256] Following cell-cell contact, MST1/2 phosphorylates LATS1, which in turn phosphorylates TAZ. TAZ phosphorylation prevents its localization to the nucleus and promotes its interaction with the Wnt regulatory component, disheveled.[254] This inhibits disheveled phosphorylation, promoting β-catenin degradation and preventing Wnt/β-catenin signal transduction. [254] Downstream of TAZ phosphorylation, YAP also regulates Wnt/β-catenin signal transduction. TAZ phosphorylates YAP, promoting its cytoplasmic localization. Nuclear YAP interacts with β-catenin, promoting transcription of target genes.[256] Through these two mechanisms, cell-cell contact inhibits YAP signaling as well as β-catenin signaling. One parsimonious mechanism by which FAM129B regulates Wnt/β-catenin signaling could be by inhibiting MST1/2 phosphorylation of LATS1. If *FAM129B* siRNA regulates Wnt/β-catenin signaling via MST1/2 / LATS/ TAZ / YAP signaling, then FAM129B siRNA treated cells should exhibit increased LATS1/2, TAZ, and YAP phosphorylation, increased TAZ-DVL coimmunoprecipitation, and decreased YAP- β-catenin coimmunoprecipitation. Furthermore, if FAM129B regulates Wnt/β-catenin signlaing by increasing MST1/2 activity, than suppression of MST1/2 or LATS1/2 activity by siRNA mediated knockdown should rescue the effect of *FAM129B* siRNA. Each of these predictions are testable, and should be tested to determine if FAM129B regulates Wnt/β-catenin signal transduction via the MST1/2 / YAP signaling pathway.

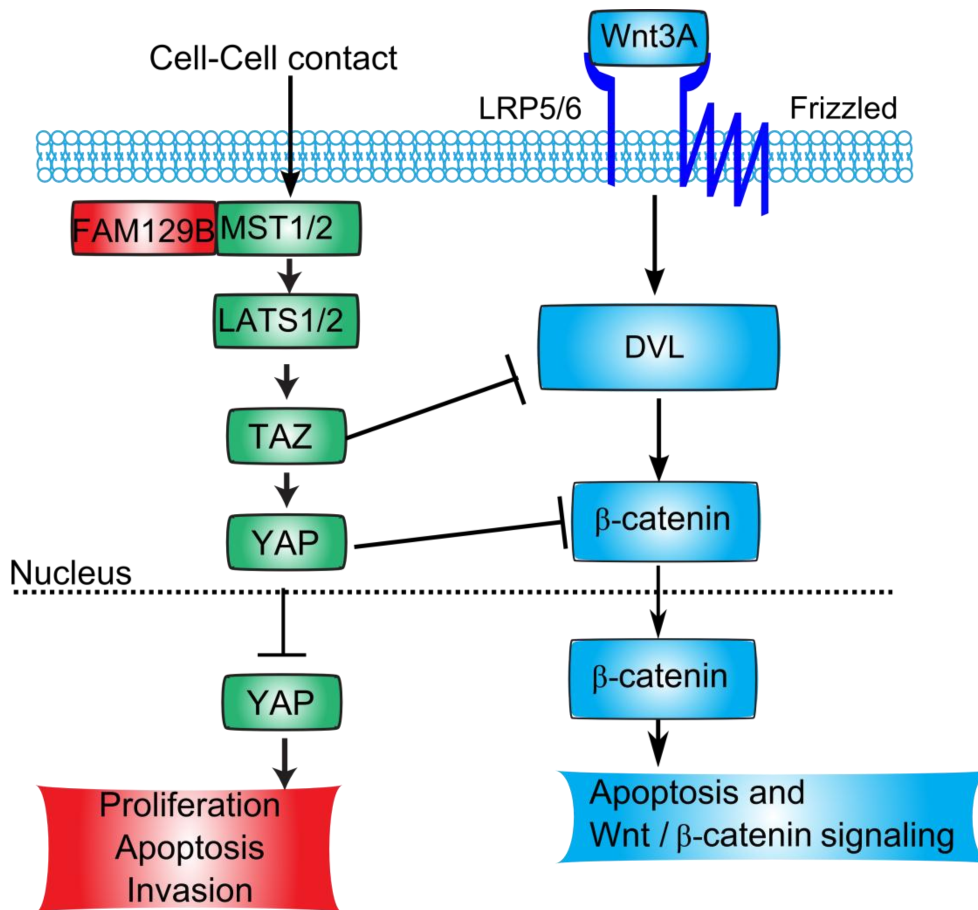



Figure 23 - Schematic depicting known regulation of Wnt/β-catenin signaling by MST1/2 signaling. FAM129B interacts with MST1/2. MST1/2 phosphorylates LATS1/2, which phosphorylates TAZ. TAZ interacts with disheveled (DVL), preventing DVL phosphorylation. DVL phosphorylation promotes Wnt/β-catenin signaling, and reduced DVL phosphorylation by phospho-TAZ inhibits Wnt/β-catenin signal transduction. Downstream of TAZ, the transcription factor YAP interacts with β-catenin to promote transcription. TAZ phosphorylates YAP, inhibiting its nuclear localization, and inhibiting its interaction with β-catenin.

As mentioned above, FAM129B regulates multiple phenotypes in addition to Wnt/β-catenin signal transduction. FAM129B may also regulate TNFα-mediated apoptosis and invasion by modulation of MST1/2 / YAP signaling. YAP is known to regulate apoptosis by interaction with p73 and increasing expression of the proapoptotic protein Bax.[257,258,255] If FAM129B silencing promotes TNFα-mediated apoptosis in HeLa cells via MST1/2 / YAP signaling, reduced nuclear YAP, and increased Bax should occur following FAM129B knockdown. FAM129B phosphorylation by MEK/ERK signaling is required for migration in melanomas.[213] YAP has previously been identified as a regulator of migration. siRNAs targeting *YAP* inhibit migration and invasion of gastric cancer cells.[253] If FAM129B promotes invasion through MST1/2 interaction, it must promote nuclear YAP. It is possible to test this hypothesis by monitoring the level of nuclear YAP in *FAM129B* siRNA-treated melanomas, or to measure the ratio of p-YAP to total YAP in *FAM129B* siRNA treated melanomas.

Of course, until further testing occurs it remains unknown whether FAM129B regulates Wnt/β-catenin signaling, invasion, and apoptosis solely through regulation of MST1/2 or whether it regulates these processes via alternative mechanisms. An important future direction for characterizing FAM129B will be to determine if it regulates these various processes via a unified mechanism or via multiple separate mechanisms.

Finally, the role FAM129B phosphorylation plays in regulating Wnt/β-catenin signaling remains unknown. While I used changes in phosphorylation as a screening approach to identify putative regulators of Wnt/β-catenin signaling, it would add a lot to know if FAM129B phosphorylation downstream of WNT3A treatment occurs, and if that change in phosphorylation affects its ability to regulate Wnt/β-catenin signal transduction.

## FAM129B does not regulate Wnt/β-catenin signal transduction in colorectal cancer

Considering the fact that hyperactive Wnt/β-catenin signaling drives the majority of colorectal cancers,[259] and given the observation that FAM129B positively regulates Wnt/β-catenin signal transduction in melanoma (see Chapter 3), it makes sense to determine if *FAM129B* siRNA inhibit Wnt/β-catenin signal transduction in colorectal carcinoma.

I first tested if *FAM129B* siRNA positively regulate Wnt/β-catenin signaling in DLD1 colorectal cancer cells. DLD1 cells harbor two mutations in *APC*, which renders Wnt/β-catenin signaling constitutively active.[260] To perform this test, I generated DLD1 colorectal cancer cells stably expressing the β-catenin activated reporter (BAR reporter) to detect the level of Wnt/β-catenin signaling by luciferase assay. Following hygromycin selection, I transduced these cells with either FLAG-GFP or FLAG-GFP-FAM129B and selected for GFP or FAM129B expression using puromycin. Finally, I treated these FLAG-GFP and FLAG-GFP-FAM129B expressing cells with a panel of siRNAs targeting *FAM129B* coding sequence (FAM129B A and B) or *FAM129B* 3' UTR (*FAM129B* UTR1 and UTR2), targeting β-catenin (*CTNNB1*), or targeting control. I observe that *FAM129B* siRNA inhibit Wnt-dependent BAR activity, but that FLAG-GFP-FAM129B overexpression cannot rescue this effect (Figure 24). Furthermore, I observe that coding sequence-directed *FAM129B* siRNA inhibit FLAG-GFP-FAM129B expression, but UTR-directed *FAM129B* siRNA do not (Figure 25). From these data, I conclude that FLAG-GFP-FAM129B does not rescue the effect of *FAM129B* siRNAs on Wnt-dependent transcription.

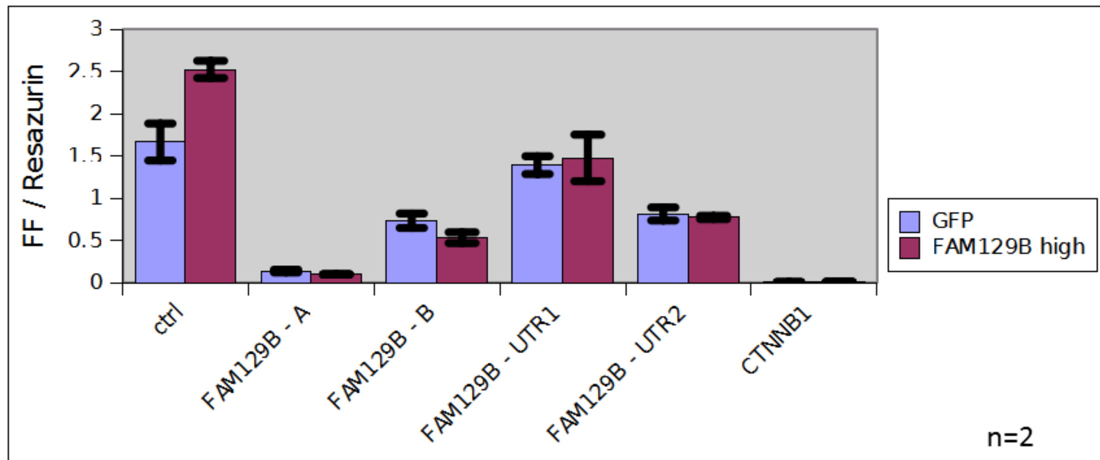



Figure 24 - FLAG-GFP-FAM129B does not rescue effect of *FAM129B* siRNA on WNT3A-dependent transcription in DLD1 cells. DLD1 colorectal BAR/Renilla cells were stably transduced with virus expressing FLAG-GFP (blue bars) or FLAG-GFP-FAM129B (maroon bars) and a separately expressed hygromycin resistance gene. Following hygromycin selection, cells were treated with siRNA for 72 hours (siRNA indicated on the X axis). Wnt-dependent reporter activity (FF) normalized to viability marker resazurin and is measured by the y-axis. Columns and bars represent mean and standard error of the mean for technical triplicates. Duplicate biological replicates were performed.

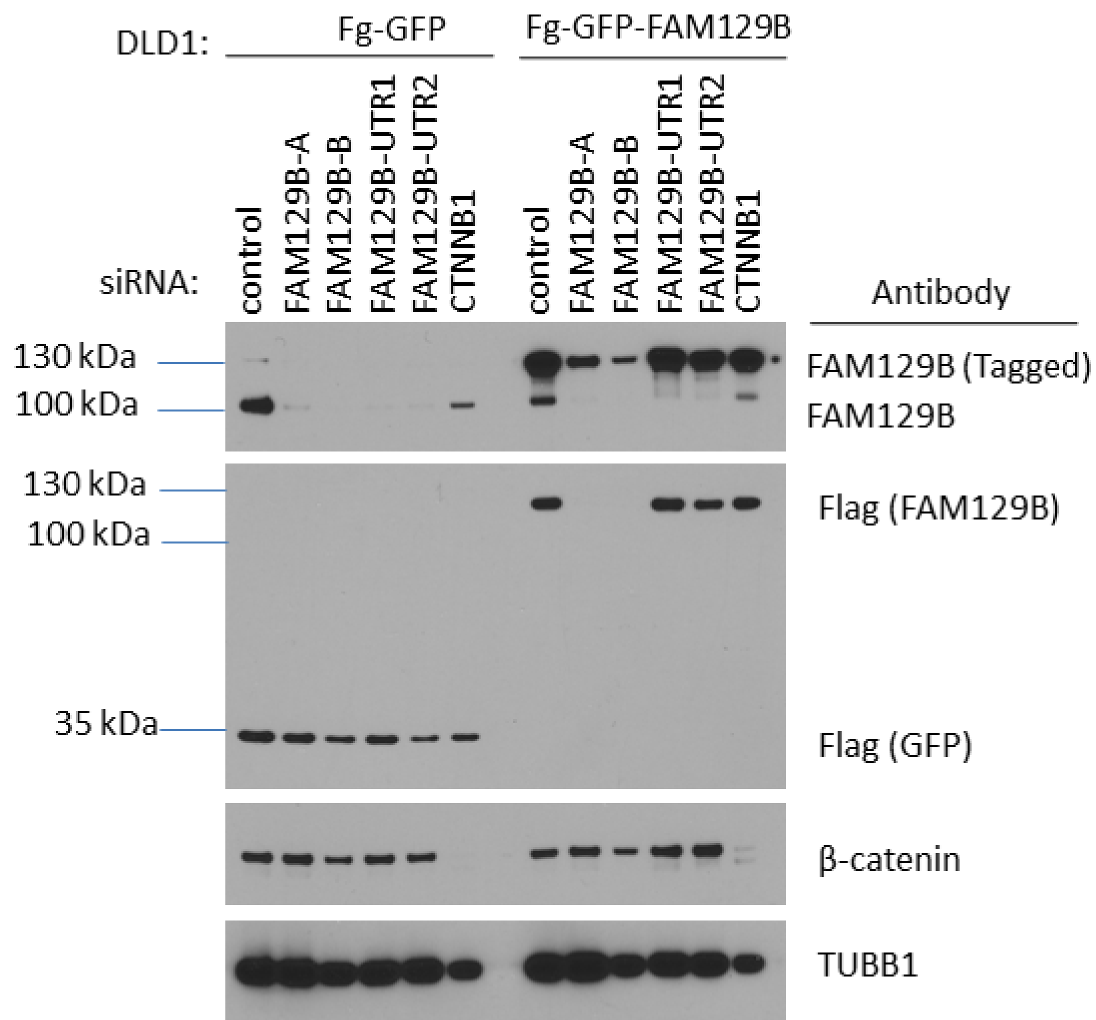



Figure 25 - Tagged FAM129B is knocked down by coding region siRNAS FAM129B-A and B, but not UTR-directed siRNAs Immunoblot analysis of GFP or FLAG-GFP expressing cells. Cells were treated with the indicated siRNA for 72 hr and lysed. Protein lysates from Figure 24 were separated by SDS PAGE and immunoblotted using FAM129B antibody, FLAG antibody, β-catenin antibody, and tubulin antibody.(Lanes 1-6) FAM129B A,B, and UTR siRNA inhibit endogenous FAM129B protein expression by FAM129B immunoblot. They do not impact FLAG-GFP expression, Tubulin, or β-catenin expression. *CTNNB1* siRNA does inhibit β-catenin expression. (lanes 8-13) All FAM129B siRNA inhibit expression a 100 kDa band representing endogenous FAM129B. A second band at 130 kDa is detected by FAM129B antibody and FLAG antibody and represents FLAG-GFP FAM129B. FAM129B targeting coding sequence (FAM129B A and B) knock down FLAG-FAM129B expression, but UTR siRNA1 and 2 do not. Once again *CTNNB1* siRNA knock down β-catenin and Tubulin serves as a loading control.

As an alternative approach, I tested if the mouse ortholog of FAM129B could rescue the effect of *FAM129B* siRNA on Wnt-dependent transcription. To test this hypothesis, I overexpressed the untagged mouse ortholog of FAM129B (msFAM129B) or GFP in DLD1:BAR reporter cells. To achieve varying degrees of FAM129B overexpression, I transduced the reporter cells with 500 ul of GFP or two doses of FAM129B (150 ul or 500 ul) and selected for expression using puromycin. I next treated reporter cells GFP cells FAM129B 150 ul cells and FAM129B 500 ul cells with three independent siRNA targeting human *FAM129B* (FAM129B A,B, or C), with a pool of these three siRNA (*FAM129B* pool), with a *CTNNB1* siRNA, or with control. I observed that each *FAM129B* siRNA inhibited Wnt-dependent transcription to varying degrees in the reporter alone cells and the GFP cells, but that the expression of GFP had no effect on Wnt-dependent transcription relative to control. Likewise, overexpression of msFAM129B does not rescue the effect of Wnt-dependent transcription by *FAM129B* siRNA (Figure 26). To determine if msFAM129B rescues the expression FAM129B, I performed immunoblot analysis. I observe that cells overexpressing msFAM129B show increased FAM129B immunoreactivity in the presence of *FAM129B* siRNAs (Figure 27). Because FAM129B antibody is raised against human FAM129B, the immunoreactivity is likely different between human and mouse, so it is impossible to directly compare the level of expression of human and mouse FAM129B protein expressed. Nonehteleess, these data show increased FAM129B immunoreactivity following msFAM129B overexpression. Therefore,these data suggest that msFAM129B expression does not rescue the effect of *FAM129B* siRNA. Taken together, the observations that FLAG-GFP-FAM129B or msFAM129B do not rescue the effect of *FAM129B* siRNA on Wnt-dependent transcription suggest that *FAM129B* siRNA regulate Wnt-dependent signal transduction via off-target effects.

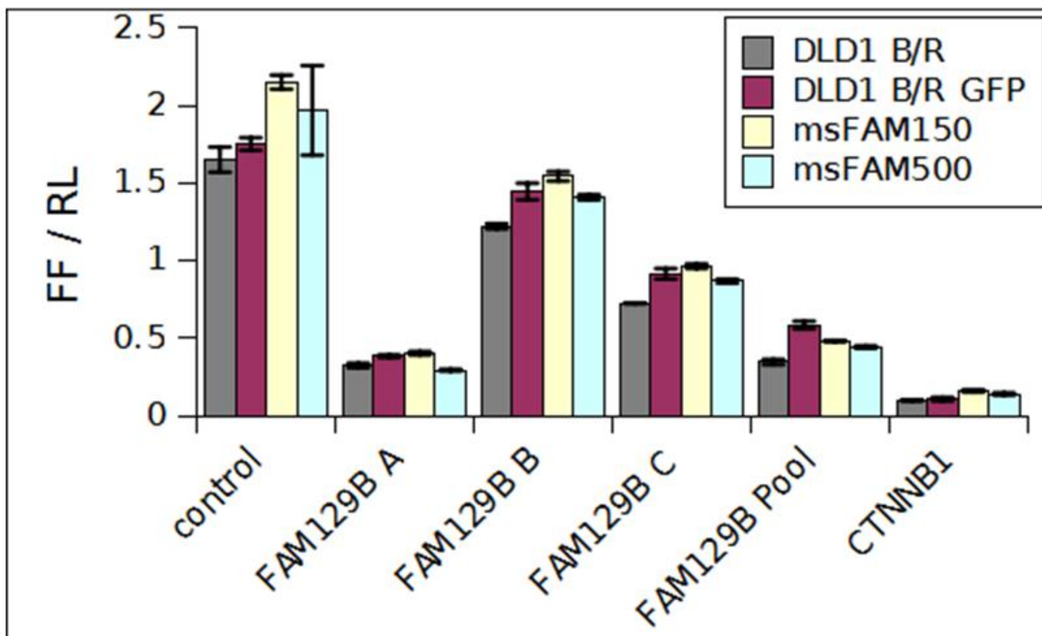



Figure 26 - stably expressed mouse FAM129B does not rescue the effect of FAM129B siRNA on Wnt-dependent transcription in DLD1 colorectal cancer cells. DLD1 colorectal BAR/Renilla cells (grey bars) were stably transduced with virus expressing FLAG-GFP (maroon bars) or low titer mouse-FAM129B (yellow bars), and high titer mouse-FAM129B (teal bars) and a separately expressed hygromycin resistance gene. Following hygromycin selection, cells were treated with siRNA for 72 hours (siRNA indicated on the X axis). Wnt-dependent reporter activity (FF) normalized to constitutively expressed renilla luciferase (RL) and is measured by the y-axis. Columns and bars represent mean and standard error of the mean for technical triplicates. Duplicate biological replicates were performed.

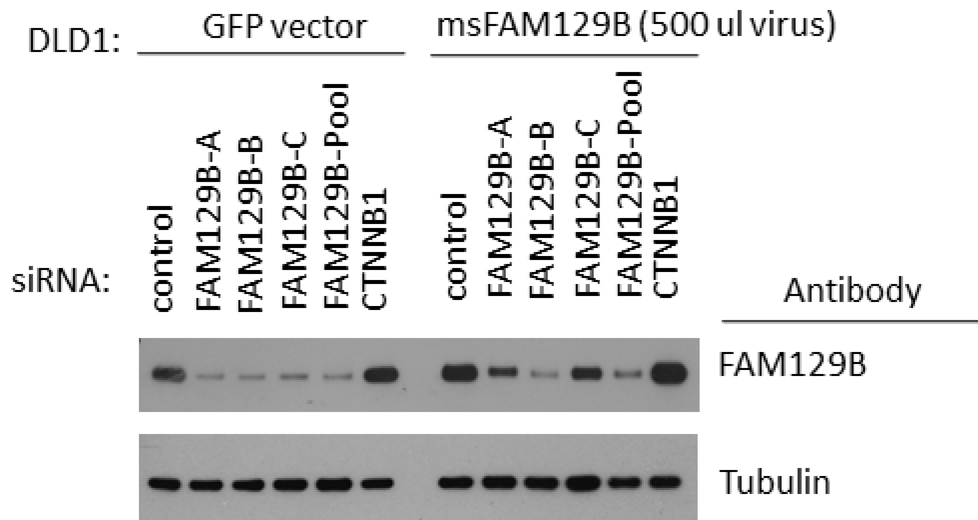



Figure 27 - mouse FAM129B rescues expression of FAM129B inhibited by FAM129B siRNA to varying degrees. Immunoblot analysis of GFP or msFAM129B expressing cells. Cells were treated with the indicated siRNA for 72 hr and lysed. Protein lysates from DLD1 cells in Figure 26 were separated by SDS PAGE and immunoblotted using FAM129B antibody and HSP90 antibody. (lanes 1-6) Cells overexpressing GFP. FAM129B A,B, C and pooled siRNAs inhibit endogenous FAM129B protein expression by FAM129B immunoblot. HSP90 serves as a loading control. (lanes 8-13). Cells overexpressing mouse FAM129B (msFAM129B). A band at the length of FAM129B is expressed to varying extents in FAM129B siRNA treated cells.

These data called into question the observation that *FAM129B* siRNA regulate Wnt-dependent transcription and cellular phenotypes in melanoma. To test if *FAM129B* siRNA regulate Wnt-dependent transcription in melanomas via on-target effects, I attempted to rescue the effect of *FAM129B* siRNA on Wnt-dependent transcription by transiently over expressing the Wnt-dependent transcriptional reporter (BAR reporter) as well as untagged *homo sapiens* FAM129B (hsFAM129B). Through these rescue experiments, I observed that FAM129B over-expression rescues the effect of *FAM129B* siRNA on the BAR reporter in A375 melanoma cells (Figure 28). Unfortunately, A375 melanoma cells have low transfection efficiency (<5%, data not shown) and rescue of FAM129B could not be determined by immunoblot. Nonetheless, from these data, I conclude that FAM129B regulates Wnt-dependent transcription in melanoma cells, but not in colorectal cancer cells.

FAM129B has cell-type specific effects on Wnt/β-catenin signal transduction. In chapter 3, I determined that *FAM129B* siRNA have a cell-type specific effect on Wnt/β-catenin signaling. In that chapter, I observed that while *FAM129B* siRNA inhibit WNT3A-dependent reporter activity in melanoma and fibrosarcoma cells, but not HEK293T cells. The observations in this section, that FAM129B likely does not regulate Wnt/β-catenin signaling in colorectal cancer, further demonstrates the cell-type specific effects of FAM129B on Wnt/β-catenin signaling. As a future direction, it will be important to determine if *FAM129B* siRNA regulates Wnt/β-catenin signaling in other cancers where hyperactive Wnt/β-catenin signaling promotes pathogenesis.

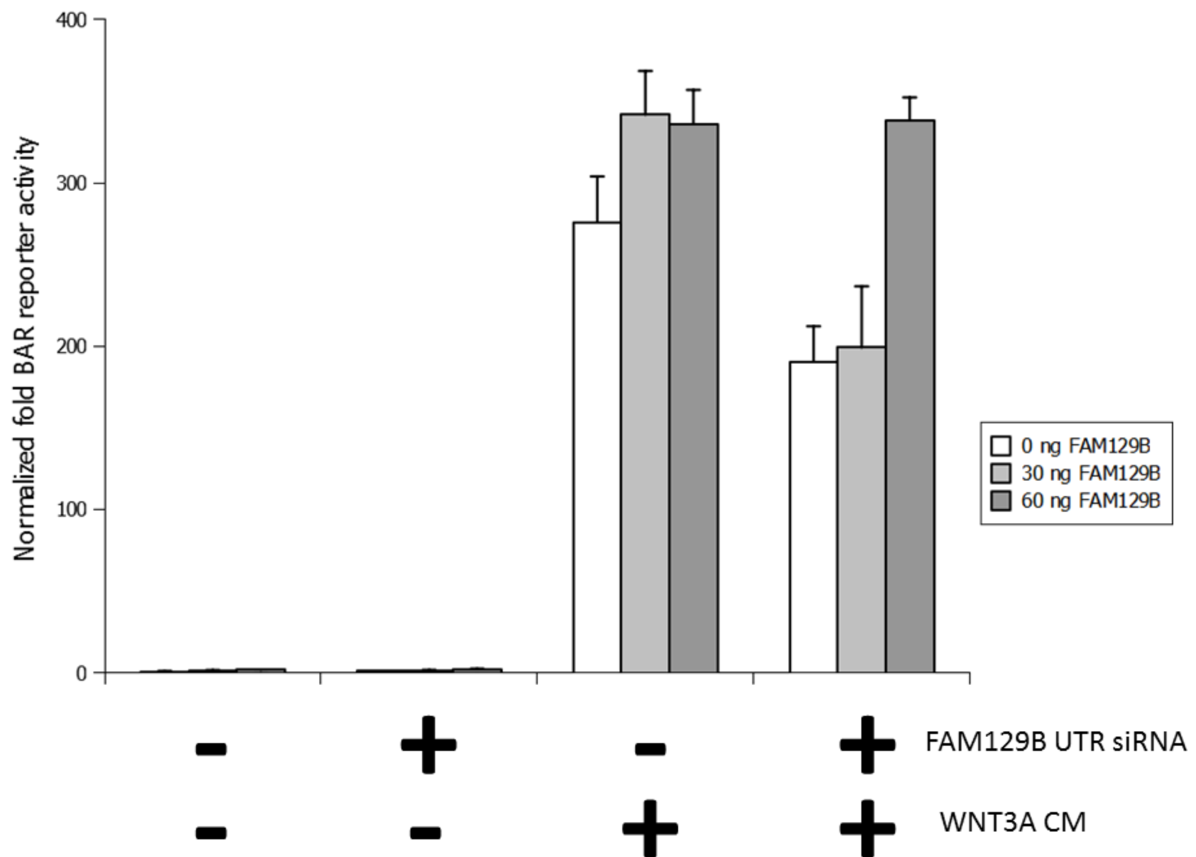



Figure 28 - Transient transfection of FAM129B rescues effect of FAM129B knockdown on Wnt-dependent transcription. A375 cells were reverse transfected with control or FAM129B UTR-directed siRNAs. After 48 hr, cells were transfected with beta-catenin activated reporter, renilla reporter and a dose of FLAG-FAM129B DNA (0 ng = white bars, 30 ng = grey bars, 60 ng = dark grey bars). After 12 hr, cells were treated with L-cell conditioned media or WNT3A cell conditioned media as indicated. After 15 hours Wnt-dependent transcription was measured and normalized for transfection efficiency by constitutive renilla luciferase expression.

## Identifying additional regulators of Wnt/β-catenin signal transduction in melanoma.

While it is important to characterize how FAM129B regulates Wnt/β-catenin signaling, especially if it offers insight into how MST1/2 /YAP signaling acts to regulate Wnt/β-catenin signaling, it is also important to identify negative regulators of Wnt/β-catenin signaling. After all, *FAM129B* knockdown inhibits the effect of WNT3A on apoptosis. Therapeutically, it will likely be more relevant to identify proteins, which enhance Wnt/β-catenin signaling and apoptosis when silenced or inhibited. A very narrow set of parameters identified FAM129B as a potential regulator of Wnt/β-catenin signaling and regulator of apoptotic response to WNT3A. FAM129B was identified as a potential regulator for a specific set of reasons. (1) Its phosphorylation state changed following 30 minutes of WNT3A treatment. (2) siRNA targeting FAM129B regulate Wnt/β-catenin signaling in fibrosarcomas. And, (3) my bioinformatic analysis identifying genes previously associated with melanoma identified FAM129B as one of 745 genes previously associated with melanoma. Furthermore, FAM129B was an interesting hit to follow up because it had been previously identified as a phosphoprotein downstream of MEK/ERK signaling.[213] While this approach identified a novel regulator of Wnt/β-catenin signaling that also regulates WNT3A's effect on apoptosis in melanoma, loosening original parameters may yield additional positive regulators and more negative regulators of Wnt/β-catenin signaling in melanoma that promote, rather than inibit, the apoptotic response to WNT3A when silenced or inhibited.

Several putative negative regulators of Wnt/β-catenin signaling arise as viable candidates from the combined functional screen and phosphoproteomic screen. Proteins identified as differentially phosphorylated in melanoma are, by definition, present in melanoma, so it is possible that they regulate Wnt/β-catenin signaling in melanoma in the same way as they do in

HT080 fibrosarcoma. RPRM, MTDH, CD3EAP, FUSIP1, BCAR3, and SNTB1 were all found to be change phosphorylation status immediately downstream of Wnt/β-catenin signaling, and are predicted to negatively regulate Wnt/β-catenin signal transduction >twofold.

Reprimo (RPRM) is a particularly interesting novel regulator of Wnt/β-catenin signal transduction. Little is known about the structure or function of this protein except that it harbors a putative transmembrane domain (hprd.org). siRNA targeting *RPRM* increased Wnt-dependent transcription 478% in HT1080s, and WNT3A treatment decreased RPRM phosphorylation greater than 4-fold at Serine 50 and Tyrosine 52 (Table 1). RPRM is a tumor suppressor gene directly targeted by p53, and when expressed promotes G2 cell-cycle arrest by inhibition cdc2 and CyclinB.[261,262] RPRM is frequently hypermethylated in a diverse array of cancers.[262262–276] It is unclear if *RPRM* methylation also has an effect on Wnt/β-catenin signal transduction. Considering the frequently oncogenic role of Wnt/β-catenin signaling in cancer, the further characterization of RPRM as a phospho-target and regulator of Wnt/β-catenin signaling might result in novel insight into how this protein suppresses growth. It would also be interesting to test RPRM against growth in melanoma, as well. If silencing RPRM promotes Wnt/β-catenin signaling in melanoma, then silencing it may also exhibit an antiproliferative effect. If RPRM negatively regulates Wnt/β-catenin signaling HT1080 cells, the original line screened, then it may have a pro-proliferative effect in that context. A comparison of the effect of RPRM in Wnt-driven and Wnt-suppressive cancers may lend insight in to the role this protein plays in regulating Wnt/β-catenin signaling.

While speculating about the possibility of other hits regulating Wnt/β-catenin signaling and its effect on apoptosis may prove successful, the utility of this approach remains somewhat unclear. While I originally combined phosphoproteomic data, with genome-wide siRNA screen

data, with a list of proteins previously connected to melanoma in an attempt to identify Wnt-pathway regulators in melanoma, it is possible that strategies other than those described in chapter 3 might identify novel Wnt-pathway regulators that strongly regulate apoptotic response.

There exist a number of negative pathway regulators, which were identified in HT1080 cells, that were not identified as Wnt-dependent phosphopeptides, but which were previously associated with melanoma. These include RAC1, CYR61, TBX2, PEPD, and CTAG1B. Each of these siRNAs increased Wnt/β-catenin reporter activity greater than *AXIN2* siRNA, and were previously implicated in melanoma according to our bioinformatic analysis. Furthermore, it remains possible that following up the HT1080 screen hits will identify more regulators of Wnt/β-catenin signaling relevant to the WNT3A response to apoptosis. After all, some of the siRNAs in this screen regulated Wnt/β-catenin signaling many-fold more than *AXIN2* but were not identified by any other combinatorial screening method. Nonetheless, these extremely potent siRNAs might target key regulators of Wnt/β-catenin signaling In the genome wide siRNA screen, siRNA targeting 473 different gene products regulate Wnt/β-catenin signal transduction greater than siRNA targeting *AXIN2* (greater than 5.1 fold). Any of these proteins could potentially regulate Wnt signaling potently enough to potentiate apoptosis following WNT3A treatment when silenced.

My experience working with siRNA screening data and my experience following up an siRNA screen hit has taught me (at least) two important lessons. First, siRNA screening has many false positives and can be overly sensitive, and second, to overcome false positives it is important to incorporate rescue experiments and tests for physiological relevance early in the process. I think early in my thesis research, I mistook the β-catenin activated reporter to be a highly specific reporter, when it is in actuality a highly sensitive reporter. This observation is

evident in the genome-wide siRNA screen results presented in Figure 13. siRNAs targeting just over 5100 gene products were identified to regulate this reporter greater than twofold. That's almost a quarter of all gene products known to be present in the human genome. A twofold effect was chosen because AXIN2 siRNA regulate the BAR reporter just over twofold, and is a known Wnt-regulatory protein. The observation that FAM129B siRNA has a less-than-twofold effect on other Wnt-dependent phenotypes calls into question whether or not a twofold effect on BAR reporter activity is physiologially relevant to a Wnt-dependent developmental process or disease. To better identify regulators of Wnt-dependent phenotypes from screening data, in the future, I would test for rescue of siRNA against Wnt-dependent transcription and also test for the effect of knockdown on a more physiologically relevant phenotype than wnt-dependent transcription (such as Wnt-dependent apoptosis or zebrafish embryonic development). Rescue experiments serve to rapidly confirm the effect of siRNA is likely not due to off-target effects. Gain-of-function experiments also offer the possibility of manipulating a given gene product (by point mutation or truncation) to gain a more refined understanding of function. More relevant experiments are initially more costly and time-consuming than siRNA mediated knockdown of cells in 2D culture, especially if one chooses to follow up a panel of potential regulators, but performing these experiments early saves time and money in the long run by focusing research energy on the most phenotypically relevant regulators of Wnt-signaling.

It's also important to note that a given screen might not answer the right question. My thesis research focused on identifying new regulators of Wnt/β-catenin signaling with the goal of better understanding and regulating Wnt-dependent apoptosis in melanoma. It was assumed that some regulators identified in HT1080 fibrosarcoma cells also regulate Wnt/β-catenin signaling in melanoma, and that these regulators affect wnt-dependent apoptosis. Screening melanoma cells

directly for Wnt-dependent apoptosis (or even Wnt-dependent gene expression) might increase the quality of data from a given screen.

While there is no shortage of potential regulators of Wnt/β-catenin signaling that have been identified by numerous screens and have been prioritized by various combinatorial approaches, the rate-limiting step continues to be the validation of these myriad exciting hits. What's needed is a strong set of secondary screens to confirm the phenotypic response of the siRNAs coupled to a strong and specific validation method to confirm the effect of siRNA is due to knockdown of the target. Our lab has successfully performed small scale screens to identify regulators of apoptosis using the casp-glo luminescence based caspase reporter. Identifying a novel negative regulator of Wnt/β-catenin signaling that also regulates apoptotic response to WNT3A. A secondary assay could transform the extant data predicting regulators of Wnt/β-catenin signaling to physiological regulators of other Wnt-dependent phenotypes.

## Chapter 5: Conclusion

The central goal of my thesis research was to investigate mechanisms of melanoma cell survival. Previously, our lab identified that melanoma survival depends on both MEK/ERK and Wnt/β-catenin signaling. I determined that the activation of Wnt/β-catenin signaling promotes apoptosis in *NRAS*- and *BRAF*-mutant melanomas. I went on to show that Wnt/β-catenin signaling likely mediates effects by decreasing AXIN1 stability in both *BRAF*- and *NRAS*-mutant melanomas. In a second line of experiments, I used a combinatorial screening approach to identify novel regulators of Wnt/β-catenin signaling in melanoma. I found that FAM129B regulates the ability of Wnt3A to activate a β-catenin responsive luciferase-based reporter and the transcription of Wnt/β-catenin target genes. In addition, FAM129B positively regulates the effect of WNT3A on apoptosis in cultured melanoma cells. Preliminary data indicate that FAM129B is phosphorylated following stimulation with Wnt3A and that FAM129B interacts with members of the oncogenic Hippo/WARTS signaling cascade.

My thesis research determined that the activation of Wnt/β-catenin signaling promotes apoptosis in both *NRAS*- and *BRAF*-mutant melanomas. I observed that *NRAS*-mutant SK-MEL-2 cells undergo apoptosis following co-treatment with WNT3A and the MEK inhibitor AZD6244 to a significantly greater extent than via either treatment alone. This observation is important because fewer treatments are currently available for patients with *NRAS*-mutant melanoma. Furthermore, patients with *NRAS*-mutant melanoma have a worse prognosis than those with a *BRAF*-mutant melanomas. *NRAS* mutation is an adverse prognostic indicator compared to wild-type melanoma with a hazard ratio >2.05. [99] The hazard ratio for *BRAF* is only 0.45.[99] 20% of patient samples harbor *NRAS*-mutant melanomas.[95,96] Adding those 20% to the 50% of

melanomas harboring *BRAF* mutations,[8,9] my data suggest a protective role for Wnt/β-catenin signaling in over 70% of all melanomas.

Wnt/β-catenin signaling likely mediates effects by decreasing AXIN1 stability in both *BRAF*- and *NRAS*-mutant melanomas. AXIN1 protein is lost in melanomas that undergo apoptosis in response to WNT3A treatment and MEK/ERK pathway inhibition (Figure 6). One consequence of AXIN1 loss is synergistic activation of Wnt/β-catenin gene expression.[200] In both *NRAS*- and *BRAF*-mutant melanoma, negative regulation of Wnt/β-catenin by MEK/ERK signaling predicts sensitivity to apoptosis (Figure 8). Loss of AXIN1 has previously been shown to increase transcription of Wnt/β-catenin target genes.[200] My data also demonstrate that loss of AXIN1 is sufficient to promote apoptosis in response to MEK/ERK pathway inhibition by AZD6244. Combination AZD6244 treatment and *AXIN1* siRNA treatment increases apoptosis in both *BRAF*- and *NRAS*-mutant melanomas that do not respond to combination WNT3A and AZD6244 treatment (Figure 7).

Because decreased *AXIN1* expression increases apoptosis by MEK/ERK pathway inhibitors, the mechanisms of AXIN1 stabilization and destabilization should be further resolved in melanoma. It remains to be determined if AXIN1 stability is regulated by known mechanisms. PARsylation, SUMOylation, ubiquitination, and phosphorylation all regulate AXIN1 stability.[41,42,231–235] Indeed, GSK3β phosphorylation stabilizes AXIN,[41,42] and MEK phosphorylates and activates GSK3β.[230] It is possible that MEK inhibitors decrease AXIN stability simply by inhibiting GSK3β phosphorylation of AXIN. This possibility and others need to be tested to determine why some melanomas undergo apoptosis in response to WNT3A while others do not.

Because my data demonstrate that Wnt/β-catenin pathway activation promotes apoptosis in multiple melanoma subtypes, the question of whether Wnt/β-catenin signaling promotes apoptosis in other cancers should be resolved. I identified osteosarcoma as a cancer with low Wnt/β-catenin pathway mutation frequency, [8,9] with decreased nuclear β-catenin during disease progression, [242] that differentiates following WNT3A treatment, [238–241] and where increased expression of Wnt/β-catenin target genes predicts favorable survival in patients (Figure 20). Further experimentation should be carried out to determine if Wnt/β-catenin pathway activation promotes apoptosis and suppresses tumor growth in osteosarcoma and other cancers.

Because Wnt/β-catenin signaling promotes apoptosis in a subset of *NRAS*- and *BRAF*-mutant melanomas, I dedicated the second part of my thesis research to identifying regulators of Wnt/β-catenin signaling in melanoma. Using combinatorial screening approaches, I identified FAM129B as a positive regulator of Wnt/β-catenin signal transduction (Figure 13). Subsequent loss of function experiments demonstrated that FAM129B regulates WNT3A-mediated target gene expression in a positive manner (Figure 14). Furthermore, loss of FAM129B decreases apoptosis in response to co-treatment with WNT3A and the targeted BRAF inhibitor PLX4720 (Figure 16). These findings demonstrated FAM129B to be a novel regulator of Wnt/β-catenin signal transduction as well as the apoptotic response to WNT3A in melanoma.

Preliminary data indicate that FAM129B is phosphorylated following stimulation with Wnt3A and that FAM129B interacts with members of the oncogenic Hippo/yorkie signaling cascade. Global phosphoproteomic screening determined that FAM129B is phosphorylated at serine 652 and at serine 668 following 30 minutes of WNT3A treatment. The role for this phosphorylation event remains unknown, but may explain a feedback mechanism for regulation of Wnt/β-catenin signaling via FAM129B. After all, many components of the Wnt/β-catenin

signal transduction cascade are regulated by phosphorylation following WNT3A treatment, including LRP5/6, disheveled, AXIN, APC, and β-catenin itself (see chapter 1). As another potentially related observation, endogenous FAM129B interacts with the kinases MST1/2 (Figure 22). This kinase regulates Hippo/yorkie signaling, which is known to regulate WNT/ β -catenin signaling via disheveled as well as interaction with β-catenin in the nucleus.[254,256] These preliminary observations may yield not only an explanation of how FAM129B regulates Wnt/β-catenin target gene expression, but also druggable targets for regulating Wnt/β-catenin signaling.

My thesis research expands our understanding of the mutational backgrounds behind which Wnt/β-catenin signaling regulates melanoma survival and cell death. Of the nearly 70,000 Americans diagnosed with melanoma in a given year, approximately 14,000 will harbor *NRAS* mutations.[2] The observation that Wnt/β-catenin signaling promotes apoptosis in *NRAS*-mutant melanoma offers potentially new strategies for these individuals. Furthermore, the observation that AXIN1 stability predicts apoptosis in response to Wnt/β-catenin signaling further reinforces the need to identify how AXIN1 stability is regulated in melanoma. I also further resolved the Wnt/β-catenin signaling pathway in melanoma by identifying FAM129B a positive regulator of cellular response to Wnt/β-catenin signaling, including apoptosis in response to WNT3A. Further resolution of how FAM129B regulates Wnt/β-catenin signaling and how the WNT/β-catenin pathway is regulated will hopefully allow therapeutic activation of this pathway in melanoma.

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## Supplementary Document 1

```
#Supplementary text 1 – Python script for listing disease-associated
                                                   genes
#Requires installation of Python v2.6 or later, and Biopython v1.55 or
                                                   later
#Also requires an internet connection, or local version of the NCBI
                                                   gene database.

import sys                                         # import system functions
                                                   from python
from Bio import Entrez                             # import Entrez search
                                                   functions from biopython
Entrez.email = "wconrad@u.washington.edu"
                                                   # Always tell NCBI who you
                                                   are when using esearch
disease = sys.argv[1]                              # The user enters their
                                                   disease term of interest

term1=disease+'[All Fields] AND (alive[PROP] AND "Homo
sapiens"[Organism])'
                                                   # This is the term that
                                                   entrez gene will search
handle = Entrez.esearch(db="gene", term=term1, retmax=800)
                                                   # the biopython esearch for
                                                   the above term is stored as
                                                   the variable "handle"
record = Entrez.read(handle)                       #the record created by the
                                                   term search is stored as
                                                   "record"
hitlist = record["IdList"]                         #the hitlist variable
                                                   collects all of the geneIDs
                                                   from the original search
newfile = open(disease+"_genes.txt", "w")          # a new tab-delimited text
                                                   file is created that uses
                                                   the search query as the
                                                   title and also the word
                                                   genes.
```

```
for id in hitlist:
 newfile.write(disease+"\t"+"lit"+"\t"+str(id)+"\n")
                                            # this loop enters the
                                            query, the designation lit
                                            for 'literature
                                            interaction' and the geneID
                                            as as string.
newfile.close()
print len(hitlist)                          #python displays the number
                                            of hits
```