RESEARCH ARTICLE

FAM129B is a novel regulator of Wnt/β-catenin signal transduction in melanoma cells [version 1; referees: 1 approved, 1 approved with reservations]

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Abstract

The inability of targeted BRAF inhibitors to produce long-lasting improvement in the clinical outcome of melanoma highlights a need to identify additional approaches to inhibit melanoma growth. Recent studies have shown that activation of the Wnt/β-catenin pathway decreases tumor growth and cooperates with ERK/MAPK pathway inhibitors to promote apoptosis in melanoma. Therefore, the identification of Wnt/β-catenin regulators may advance the development of new approaches to treat this disease. In order to move towards this goal we performed a large scale small-interfering RNA (siRNA) screen for regulators of β-catenin activated reporter activity in human HT1080 fibrosarcoma cells. Integrating large scale siRNA screen data with phosphoproteomic data and bioinformatics enrichment identified a protein, FAM129B, as a potential regulator of Wnt/β-catenin signaling. Functionally, we demonstrated that siRNA-mediated knockdown of FAM129B in A375 and A2058 melanoma cell lines inhibits WNT3A-mediated activation of a β-catenin-responsive luciferase reporter and inhibits expression of the endogenous Wnt/β-catenin target gene, AXIN2. We also demonstrate that FAM129B knockdown inhibits apoptosis in melanoma cells treated with WNT3A. These experiments support a role for FAM129B in linking Wnt/β-catenin signaling to apoptosis in melanoma.
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Introduction
The incidence of melanoma continues to rise across the U.S. at a rate faster than any other cancer. Malignant melanoma has a poor prognosis with a 5-year survival rate of only 15%. The recently approved therapeutic, vemurafenib, extends median patient survival by 7 months. This major advance raises expectations that even greater rates of survival might be attainable with combination therapies.

Activation of the Wnt/β-catenin pathway decreases tumor growth and cooperates with ERK/MAPK pathway inhibitors to promote apoptosis in melanoma. Analysis of melanoma tumor samples show a positive correlation between nuclear β-catenin staining and decreased tumor depth, increased patient survival and increased time to metastasis. Moreover, treatment with WNT3A-containing conditioned media or stable overexpression of WNT3A in mouse B16 or human A375 melanoma cells reduces cell number in vitro. All organs of mouse B16 or mouse xenografts of human A375 cells overexpressing WNT3A decrease tumor size compared to control. Recently, we found that activation of Wnt/β-catenin signaling concurrent with the inhibition of the ERK/MAPK pathway synergistically elevates apoptosis in a subset of BRAF- and NRAS-mutant cultured human melanoma cells. Given the interaction between Wnt/β-catenin signaling and pathways known to be critical for melanoma pathogenesis, the identification of Wnt/β-catenin regulators might prove to be informative in developing novel approaches to treat this disease.

In the present study, we identify novel regulators of Wnt/β-catenin signaling in melanoma by performing a large-scale small-interfering RNA (siRNA) screen of a Wnt/β-catenin responsive reporter in human HT1080 fibrosarcoma cells, and by identifying siRNA targets that are also regulated by ERK/MAPK signaling and that have been previously associated with melanoma. By integrating these three approaches, we identified FAM129B as a potential regulator of Wnt/β-catenin signaling. FAM129B is a 746 amino acid protein that contains an amino-terminal pleckstrin homology (PH) domain and a differentially phosphorylated carboxy-terminal region. FAM129B is known to inhibit TNFα-dependent apoptosis in HeLa cells. FAM129B is expressed in melanoma and promotes tumor cell invasion into collagen matrices in an ERK/MAPK phosphorylation-dependent manner. In the present study we demonstrate that FAM129B promotes Wnt/β-catenin signal transduction in melanoma and that reducing levels of FAM129B with siRNA reduces the ability of WNT3A to increase apoptosis in melanoma cells.

Results
Phosphoproteomic and siRNA screens identify FAM129B as a regulator of Wnt/β-catenin signaling
In order to identify novel regulators of Wnt/β-catenin signaling, we performed a siRNA screen. We used HT1080 cells stably transduced with a luciferase reporter of β-catenin-mediated transcription (BAR). We screened 28,044 pools of siRNAs. 19,490 gene products were targeted by one or more siRNA pool. Cells were transfected with siRNAs and treated with WNT3A-conditioned media to activate the reporter. BAR activity was normalized to the activity of Renilla luciferase driven by the constitutive TK promoter to control for total cell number. siRNAs targeting positive control proteins such as the known Wnt/β-catenin inhibitor, AXIN2, modulated BAR activity by at least 2.0 fold with a p-value less than 0.01 (Figure 1b). Using this as a criterion, we found that 10,215 siRNA pools regulated BAR activity. Of the 19,490 gene products targeted by one or more siRNA in our screen, we identified 5189 gene products for which every given siRNA significantly regulated BAR activity (Data File 1).

To refine the results of our large-scale siRNA screen, we performed an integrative analysis of our siRNA screen regulators by cross-referencing these regulators with a list of genes previously identified in melanoma, and a list of gene products phosphorylated downstream of MEK and ERK in melanoma. First, we identified 12 proteins in common between the siRNA and phosphoproteomic screens (Figure 1a and Data File 3a). Next, we generated a list of melanoma-associated genes using a custom biopython script (Data File 2 and Query Script). We identified 745 melanoma-associated genes by querying the NCBI gene database. Of these, one gene (FAM129B) encoded a protein that was differentially phosphorylated following MEK inhibition (Figure 1 and Data File 3a) and 123 were gene targets of siRNA pools that regulated Wnt/β-catenin signaling (Figure 1b, and Data File 3b). Finally, we discovered FAM129B as the only melanoma-associated gene that both modulated Wnt/β-catenin signaling and was phosphorylated following MEK activation, (Figure 1b and Data File 3a).

Validation of FAM129B as a regulator of Wnt/β-catenin signaling
The siRNA screen suggested that FAM129B is a regulator of Wnt/β-catenin signaling. In order to confirm this possibility, we designed three independent siRNAs targeting FAM129B. First, we confirmed that all three siRNAs inhibit expression of FAM129B protein in HT1080 fibrosarcoma, A2058 melanoma and A375 melanoma cells (Figure 2a). Next, we asked whether the siRNAs inhibited the ability of WNT3A to activate BAR. Indeed, we found that each FAM129B siRNA reduced the ability of WNT3A to activate BAR in all three cell lines (Figure 2b). We also tested whether FAM129B siRNAs reduce the ability of WNT3A to activate BAR. Indeed, we found that each FAM129B siRNA reduced the ability of WNT3A to activate BAR in all three cell lines (Figure 2b). We also tested whether FAM129B siRNAs reduce the ability of WNT3A to activate BAR. Indeed, we found that each FAM129B siRNA reduced the ability of WNT3A to activate BAR in all three cell lines (Figure 2b). We also tested whether FAM129B siRNAs reduce the ability of WNT3A to activate BAR. Indeed, we found that each FAM129B siRNA reduced the ability of WNT3A to activate BAR in all three cell lines (Figure 2b). We also tested whether FAM129B siRNAs reduce the ability of WNT3A to activate BAR. Indeed, we found that each FAM129B siRNA reduced the ability of WNT3A to activate BAR in all three cell lines (Figure 2b). We also tested whether FAM129B siRNAs reduce the ability of WNT3A to activate BAR. Indeed, we found that each FAM129B siRNA reduced the ability of WNT3A to activate BAR in all three cell lines (Figure 2b). We also tested whether FAM129B siRNAs reduce the ability of WNT3A to activate BAR. Indeed, we found that each FAM129B siRNA reduced the ability of WNT3A to activate BAR in all three cell lines (Figure 2b). We also tested whether FAM129B siRNAs reduce the ability of WNT3A to activate BAR. Indeed, we found that each FAM129B siRNA reduced the ability of WNT3A to activate BAR in all three cell lines (Figure 2b). We also tested whether FAM129B siRNAs reduce the ability of WNT3A to activate BAR. Indeed, we found that each FAM129B siRNA reduced the ability of WNT3A to activate BAR in all three cell lines (Figure 2b). We also tested whether FAM129B siRNAs reduce the ability of WNT3A to activate BAR. Indeed, we found that each FAM129B siRNA reduced the ability of WNT3A to activate BAR in all three cell lines (Figure 2b).
Figure 1. FAM129B is identified as a putative regulator of Wnt/β-catenin signaling using large-scale siRNA screen integrated with phosphoproteomic 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 siRNAs were plotted against the p-value of that treatment. If, for a given gene, all siRNAs targeting that gene showed a twofold change in normalized reporter activity and a p-value <0.01, that gene was classified as a hit. This screen identified 5,189 gene products as hits, which are depicted in dark grey. Overlapping phosphoproteomic data from Old WM *et al.* (2009) are depicted in light grey. The known regulator of Wnt/β-catenin signaling, AXIN2, is indicated, as is FAM129B (right panel). Data plot is the same as the left panel with melanoma-associated genes plotted in light grey instead. (b) Venn diagram depicting overlaps between phosphoproteomic dataset, siRNA screen and melanoma-associated genes. 12 protein targets overlap between the phosphoproteomic hits and the siRNA screen, 1 protein target overlaps 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.
Figure 2. FAM129B positively regulates Wnt/β-catenin signal transduction in a panel of three cell lines. (a) Immunoblots show three independent siRNAs reduce steady-state levels of FAM129B following 72 hr treatment with 20 nM siRNA. The beta-tubulin immunoblot serves as a control. Three independent siRNAs targeting FAM129B inhibit FAM129B expression in HT1080 (left), A2058 (middle), and A375 cells (right). (b) FAM129B siRNA inhibit WNT3A-dependent luciferase reporter activity (BAR reporter) normalized to constitutively expressed Renilla luciferase in HT1080 (left), A2058 (middle), and A375 cells (right). (c) FAM129B siRNA inhibit Wnt-dependent AXIN2 expression in HT1080 (left), A2058 (middle), and A375 cells (right) relative to beta-actin mRNA expression by qPCR. Columns and error bars represent mean and SEM, respectively. Data are representative of at least three separate biological replicates. *p<0.05 by unpaired, two-tailed T-test.
activation of the BAR reporter by WNT3A across a wide range of
doses (Figure 3a). FAM129B siRNA has only negligible effects on
TNFα-dependent NFκB reporter activity (Figure 3b). While this
result does not allow the conclusion that FAM129B functions solely
as a modulator of β-catenin signaling, this result does suggest that
FAM129B is not required for activation of all pathways.

**FAM129B regulates WNT3A-mediated apoptosis in A375
melanoma cells**

The combined treatment with WNT3A protein and compounds that
inhibit ERK/MAPK signaling synergizes to induce robust apopto-
sis in cultured melanoma cells. If FAM129B is required for
Wnt/β-catenin signaling, then FAM129B loss of function should
inhibit this synergy. We monitored apoptosis in A375 melanoma cells
by western blot for cleaved caspase-3 and immunofluorescence stain-
ing for TUNEL (terminal deoxynucleotidyl transferase-mediated de-
oxuryridine triphosphate nick end labeling). As previously reported,
A375 cells treated with control siRNA and the combination of WNT3A
and PLX4720 exhibit robust levels of cleaved caspase-3 (Figure 4a).
siRNA mediated knockdown of FAM129B decreases the levels of
cleaved caspase-3 in response to WNT3A siRNA (Figure 4a–4c).
Moreover, when measuring WNT and PLX4720-dependent apopto-
sis by TUNEL staining, we found that siRNA mediated FAM129B
knockdown reduced the number of TUNEL positive cells as compared
to control siRNAs. Collectively, these results show that FAM129B
is required for the synergy between Wnt3A and PLX4720 to induce
melanoma apoptosis.

**Materials and methods**

**Plasmids**

Detailed information on the β-catenin activated reporter plasmid
(pBARLS) has been previously described. Briefly, the reporters
are generated from lentiviral plasmids that contain 12 TCF/LEF
binding sites (5′-AGATCAAAGG-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. These reporters were generated by Travis L. Biechele in
the lab of Randall T Moon as previously published.

**Cell lines and cell culture**

Human A375 and A2058 cells were a generous gift from Cassian
Yee (Fred Hutchinson Cancer Research Institute, Seattle, WA).
HT1080 cell lines were purchased from the American Type Culture
Collection (ATCC, Manassas, VA). Stable reporter lines were gener-
ated as previously described. Cell lines were cultured in a Thermo
Forma steri-cult humidified incubator (#3310, Thermo Scientific,
Rockford, IL) at 37°C and 5% CO₂. All cell lines were cultured in
Dulbecco’s Modified Eagle’s Medium (DMEM, #11965–084 In-
vitrogen, St. Louis, MO) 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.

Control (LCM) and WNT3A-conditioned media (WNT3A CM)
used to activate the Wnt/β-catenin signaling pathway were prepared
as previously described. 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 St. Louis, MO, product 472301) or 2 μM PLX4720 (Syman-
ness, Timaru New Zealand SY-PLX4720).

**Discussion**

We combined phosphoprotoemics and siRNA screening to identify
novel regulators of Wnt/β-catenin signaling in human melanoma. We
focused on FAM129B, a previously identified protein that has not
previously been linked to Wnt/β-catenin signaling. Using independent
siRNAs, we confirmed that FAM129B is required for Wnt3A to acti-
vate a β-catenin dependent reporter and reduces the ability of Wnt3A
to enhance the expression of the β-catenin target gene Axin2. We
demonstrated that loss of function of FAM129B inhibits the apop-
tosis of melanoma cells induced by the combined treatment with
WNT3A and PLX4720.

FAM129B siRNAs suppress apoptosis in melanoma cells treated
with WNT3A and PLX4720. This result was surprising given that
the transfection of FAM129B siRNA in HeLa cells promotes in-
creased apoptosis in response to TNFα and cyclohexamide. The
discrepancy between the ability of FAM129B siRNAs to suppress
Wnt-dependent apoptosis in melanoma and the ability of these
siRNA to promote TNFα-mediated apoptosis in HeLa remains un-
resolved, although it does suggest that FAM129B may function in
a manner that is dependent on cellular context. Alternatively, the
differences in apoptotic response with FAM129B loss of function
may merely reflect the regulation of Wnt/β-catenin signaling in these
two cell types. Uncovering the underlying roles of FAM129B in
the cell may well illuminate how FAM129B exerts these opposing
effects on apoptosis in response to different stimuli. Future studies
should probe the role, if any, of TNFα/NFκB in melanoma apoptosis
and the cross-talk between Wnt/β-catenin and TNFα/NFκB signal-
ing in cell lines, such as HeLa, that respond to TNFα by apoptosis.

Data showing positive regulation of Wnt/β-catenin signal
transduction by FAM129B siRNA using transcriptional reporter
assay, target gene expression, and apoptosis assay

7 Data Files

http://dx.doi.org/10.6084/m9.figshare.705153
Figure 3. **FAM129B** siRNA regulate Wnt-dependent transcriptional reporter, but not TNFα/NFκB dependent reporter. (a, left panel) pooled FAM129B siRNAs inhibit Wnt-dependent BAR reporter activity over a wide range of doses. Increasing doses of WNT3A d increases activation of the BAR reporter (normalized to constitutive Renilla luciferase in control treated cells). WNT3A does not activate the reporter in the presence of FAM129B or CTNNB1 siRNAs. (Right panel) A375 cells were treated with siRNAs as indicated and treated with an EC50 dose of WNT3A (50 ng/ml). All FAM129B siRNA and positive control CTNNB1 siRNA inhibit Wnt-dependent BAR reporter activity. (b) The same experiment was carried out as in (a, left panel) in A375 lines TNFα/NFκB reporter. Data in the left panel indicate dose-dependent activation of the NFκB reporter by TNFα. However, FAM129B siRNAs do not inhibit the activation of the TNFα/NFκB reporter. (Right panel) FAM129B siRNA do not regulate activity of the NFκB reporter activated by 1.5 ng TNFα/ml in A375 cells. High dose TNFα (10 ng/ml) does differentially activate the reporter. Data represent 3 separate biological replicates. *p<0.05 by unpaired, two-tailed T-test.
Figure 4. FAM129B positively regulates Wnt/β-catenin-dependent apoptosis in A375 melanoma. (a) FAM129B siRNA inhibits Wnt-dependent apoptosis 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 µM PLX4720, and L-conditioned or WNT3A-conditioned media for 24 hr as indicated. 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. (b) FAM129B siRNA inhibits Wnt-dependent apoptosis as quantified by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) immunofluorescence (IF). A375 melanoma cells were treated as above, fixed and stained using TUNEL. Percent TUNEL positive cells calculated as a percent of DAPI positive cells. (c) 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.
siRNA transfection and low throughput reporter assays

Approximately 200,000 A375, A2058, or HT1080 cells (as estimated by hemocytometer counts) were reverse transfected at a final dose of 20 nM siRNA in 6-well format using 5 µl RNAi max/well (Invitrogen, Grand Island, NY). Medium GC universal stealth control siRNA was used as a negative control (Cat. No. 29395–112, Invitrogen, Grand Island, NY). Invitrogen’s stealth siRNA targeting FAM129B were designed using the BLOCK-it RNAi designer and are described below. The sequence for “FAM129B A” is UCACG-GACAUAGGCUACGUCAU. The sequence for “FAM129B B” is ACUGAGGGCGAGAUCUUCAU. The sequence for “FAM129B C” is CAGCAGCAGAUUGAUGUUGGCA. As a positive control for inhibition of Wnt/β-catenin signal transduction by siRNA, we used silencer select siRNA targeting CTNNB1 with the sequence GGUGGUGUUAAUAAGGCUATT (Invitrogen, Grand Island, NY).

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 indicated conditions, and luciferase activity was measured 15 hours later with a Dual-Luciferase Reporter Assay kit (Promega, Madison, WI) and an Envision multilabel plate reader (PerkinElmer, Waltham, MA) 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 µg of RNA was reverse transcribed using Fermentas’ RevertAid M-MuLV Reverse Transcriptase (Fermentas, Glen Burnie, MD). QPCR was performed on a Lightcycler 480 (Roche, Indianapolis, IN) using Lightcycler 480 SYBR Green 1 master mix (04707516001 Roche, Indianapolis, IN). The following primers were used for qPCR: “AXIN2 F” CTCCCCACCTT-GAATGAAGA and “AXIN2 R” TGGCTGGTGCAAAGACATAG; and, “ACTB F” AGAGCAAGAGAGGCATCCT 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 all purchased from Sigma, St. Louis, MO). For monitoring cleaved caspase-3, 90% confluent 12-well plates were treated for 24 hr with the indicated conditions described in the “cell lines and cell culture” section. Media were collected and cells were rinsed once with indicated conditions described in the “cell lines and cell culture” section. Cells were lysed on-plate in 100 µl 1× RIPA lysis buffer containing protease and phosphatase inhibitors (Complete EDTA-free and PhoStop by Roche, Indianapolis, IN). Cells were disrupted by scraping of a 1000 µl pipette tip against the plate. Apoptotic cells present in the media and PBS wash were centrifuged at 300 g, rinsed once with PBS, and lysed with the RIPA buffer collected from the plate lysis. Cell lysates were transferred to a nitrocellulose membrane (162–0115, Bio-Rad, Hercules, CA) using IDEA scientific GENIE transfer apparatuses (Idea Scientific, Minneapolis, MN). Blots were probed using polyclonal rabbit anti FAM129B (#HPA023261 Sigma, St. Louis, MO), monoclonal mouse anti Tubulin (#T7816 Sigma, St. Louis, MO), monoclonal mouse anti β-catenin (C2206 Sigma, St. Louis, MO), polyclonal Rabbit anti cleaved-caspase-3 (#9661 cell signaling). Rabbit anti ERK1/2 (#9102 cell signaling, Danvers, MA), Rabbit anti phospho ERK1/2 (#9211 cell signaling, Danvers, MA).

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 the “cell lines and cell culture” section. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was performed using an in situ cell death detection kit (Roche, Indianapolis, IN). 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 (Sigma, St. Louis, MO) in 0.1% sodium citrate (Sigma, St. Louis, MO) 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, Grand Island, NY). 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.

Author contributions

WC, RTM, AC and JB conceived the study. MF, SM, and NC automated the large-scale screen. MBM, MAC, BR, and WTA designed and carried out the large scale screen. WC carried out the research in melanoma cells and identified melanoma-associated genes. WC prepared the first draft of the manuscript. AC, JB, and RTM contributed to the experimental design and preparation of the manuscript. All authors were involved in the revision of the draft manuscript and have agreed to the final content.

Competing interests

No competing interests were disclosed.

Grant information

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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References

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1. The title is appropriate for the study.
2. The experimental design and methods are appropriate for the topic being studied.
3. The information and data provided is enough for replicating the experiment.
4. The conclusions are correct but need additional data to substantiate it. In this article, the authors have identified FAM129B as a positive regulator of Wnt/beta-catenin signalling in melanoma cells. They also demonstrate that FAM129B is required for Wnt3a-induced apoptosis in vitro.

However, there are three important pieces of information missing that the authors should address:
- Can this effect be replicated in vivo in animal models?
- Does FAM129B expression correlate with beta-catenin activation and disease progression in melanoma patients?
- What is the mechanism by which FAM129B regulates Wnt signalling?

I would like the authors to do a bit more work on answering at least one of the three questions raised. They should also discuss the other two questions in the discussion section. From my standpoint it seems the easiest thing to do would be to compare FAM129 expression in various tumor samples and correlate them to Beta-catenin activation (nuclear localization) and growth pattern (tumor depth).

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Competing Interests: No competing interests were disclosed.

Author Response 10 Oct 2013
William Conrad, University of Washington, Seattle, USA

Dear Dr. Apte,

We appreciate your thorough reading of our manuscript, as well as the thoughtful questions that you have raised in your review. We have outlined a response to specific questions below:

1. “Does FAM129B expression correlate with beta-catenin activation and disease progression in melanoma patients?”
This is a very interesting question, and one which we had sought to address using previously published data from defined cohorts of melanoma patients. We analyzed the abundance of FAM129B present in three different cohorts of melanoma (Figure 5a in version 2) and observed a significantly higher abundance of FAM129B mRNA in a melanoma cohort associated with higher invasiveness and relatively lower Wnt/β-catenin signaling. From this observation, we concluded that FAM129B abundance did not predict Wnt/β-catenin signaling in melanoma patients. This conclusion was supported by a lack of significant correlation between levels of FAM129B and expression of the established Wnt/β-catenin target gene, AXIN2, within this cohort (data not shown). We have included a brief discussion of these results in our revision in order to clarify the fact that we do not have data that supports a clear role of direct regulation of Wnt/β-catenin signaling by FAM129B in melanoma patients.

2. “Can this effect be replicated in vivo in animal models?”
We did attempt to address this question, but despite repeated attempts we were unable to stably knock down FAM129B using lentiviral shRNA in melanoma cells. As a result, we were not able to generate cell lines that could be used to address this question in murine xenografts.

3. “What is the mechanism by which FAM129B regulates Wnt/β-catenin signaling?”
Unfortunately, we have been unable to solve the specific mechanism by which FAM129B regulates Wnt/β-catenin signaling. We had hoped that a proteomics-based approach identifying proteins that interact with FAM129B might prove informative, but to date we have not solidified any mechanistic links based on the hits identified with this strategy. While our efforts to validate this network are currently too preliminary for publication, we have (in the spirit of open access) included our FAM129B protein interaction network in its entirety in order to allow other readers to potentially gain insight and generate hypotheses regarding the role of this protein in regulating cellular signaling (Figure 5b).

**Competing Interests:** No competing interests were disclosed.
caspase cleavage. I feel that the authors could provide a more in-depth discussion on their findings and place them in the context of the background information provided in the introduction part;

How do the authors consider this candidate relevant for melanoma treatment?

How do the authors integrate the beneficial effects of enhanced Wnt responsiveness (this report) and the previously described enhanced tumour cell invasion (ref 20) of FAM129B expressing melanoma cells?

It would be helpful for the reader to include a model which shows how Wnt and ERK pathways modulate apoptosis in melanoma cells and where FAM129B would be placed in these events. Also in the legend of Fig. 2a it remains unclear whether endogenous levels of FAM129B are depicted.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

**Competing Interests:** No competing interests were disclosed.

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**Author Response 10 Oct 2013**

William Conrad, University of Washington, Seattle, USA

Dear Dr. Maurice,

We appreciate your thorough reading of our manuscript, as well as the thoughtful questions that you have raised in your review. We have outlined a response to specific questions below:

1. **“How do the authors consider this candidate relevant for melanoma treatment?”**
   It is unclear how FAM129B is relevant for melanoma treatment at this point. Our data identifies FAM129B as a regulator of Wnt/β-catenin dependent phenotypes in melanoma cells, while previous studies had already identified FAM129B as a downstream protein regulated by ERK/MAPK signaling. Given the importance of both of these signaling pathways in melanoma biology, we hypothesize that FAM129B could be an important regulatory nexus between Wnt/β-catenin and ERK/MAPK signaling, although at this point we have been unable to draw a direct connection in patient tumors. Thus, at this point it is difficult to make any conclusions on the role of FAM129B’s role in melanoma treatment without being overly speculative.

2. **“I feel that the authors could provide a more in-depth discussion on their findings and place them in the context of the background information provided in the introduction part”**
   We have added some more content to the discussion, and we hope that these changes have improved the manuscript.

3. **“How do the authors integrate the beneficial effects of enhanced Wnt responsiveness (this report) and the previously described enhanced tumour cell invasion (ref 20) of FAM129B expressing melanoma cells?”**
   You have raised a very interesting and relevant point regarding the difficulties in broadly generalizing results obtained in cultured cell systems across a disease. Our evaluation of published data (presented in this revision) also shows increased expression of FAM129B in a cohort previously defined as more invasive, and with lower activation of Wnt/β-catenin
signaling (Figure 5a). These data would suggest that in patients, the effect of FAM129B on melanoma cell invasion may supersede any effects on regulating Wnt/β-catenin signaling.

4. In response to your helpful suggestion, we have added a model which shows how Wnt/β-catenin and ERK/MAPK pathways modulate apoptosis in melanoma cells (Figure 5c).

5. Thank you for your careful reading of the figure legends, we have edited the legend of Figure 2a to clarify that our western blot of FAM129B is for endogenous FAM129B protein levels.

**Competing Interests:** No competing interests were disclosed.