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NF2-deficient cells depend on the Rac1-canonical Wnt signaling pathway to promote the loss of contact inhibition of proliferation

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Abstract

The neurofibromatosis type 2 (*NF2*) tumor suppressor gene encodes merlin, a membrane/cytoskeleton protein necessary for maintenance of contact inhibition of growth in cells. Biallelic inactivation of *NF2* is known to cause multiple cancers in both humans and mice. However, the mechanism through which merlin exerts its tumor suppressive function remains obscure. In this report we reveal that *NF2* knockout mouse embryonic fibroblasts (MEFs) lost contact inhibition of cell proliferation and contained significantly increased canonical Wnt signaling. Inhibition of Rac1, whose activity is inversely regulated by *NF2*, through the use of a dominant negative mutant, small hairpin RNA, or a small molecule inhibitor in the *NF2*-deficient cells, was able to suppress the elevated Wnt signals as shown by reduced activity of the T-cell factor 4 (TCF4) transcription factor. Dominant negative TCF4 or Rac1 mutant, as well as a small molecule inhibition of Wnt, were able to curb the *NF2* deficiency-elicited cell proliferation at the confluent state. Thus, Rac1-mediated canonical Wnt signaling is essential for the loss of contact inhibition in *NF2*-deficient cells.

Keywords

Rac1; NF2/merlin; neurofibromatosis type 2; wnt signaling; contact inhibition; proliferation

Introduction

The ezrin-radixin-moesin (ERM) family molecule, merlin, is the protein product of the *NF2* tumor suppressor gene which is inactivated in familial neurofibromatosis type 2 (NF2) as well as other types of sporadic tumors including schwannomas, mesotheliomas, and meningiomas. The *NF2* tumor suppressor gene is unique in that it exerts its tumor

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suppressive functions from the cell-environment interface rather than from within the cell to impact cell proliferation (McClatchey and Giovannini, 2005). A hallmark of *NF2*- deficient cells is the loss of contact inhibition of proliferation (Johnson *et al.*, 2002; Lallemand *et al.*, 2003; Morrison *et al.*, 2001). Several studies have proposed that the lack of association of merlin with Rac1 GTPase, CD44, or paxillin might be responsible for the destabilization of cell-cell contacts that allow for the loss of contact inhibition in *NF2*-deficient cells (Fernandez-Valle *et al.*, 2002; Morrison *et al.*, 2001; Okada *et al.*, 2005). A recent study has provided the first mechanistic insight by attributing this characteristic of *NF2*-deficient cells to their inability to silence EGFR signaling at confluency (Curto *et al.*, 2007). Despite these observations, our understanding of the mechanisms through which the loss of the cell membrane associated protein, merlin, transduces the growth signal to the nucleus that is necessary for cell proliferation at confluency remains rudimentary.

Rac1, a member of the Rho GTPase family of signal transducers, is a pleiotropic regulator of multiple signaling pathways including MAPK, JNK/SAPK, NF-kB, and PI-3K, allowing it to regulate a diverse set of cellular processes. Rac1 is a modifier of tumorigenesis and its overexpression or overactivation occurs in many tumor types (Fritz *et al.*, 1999; Jordan *et al.*, 1999). Studies have shown that Rac1 signaling is critical in both tumor formation and progression due to its involvement in cell migration, cell cycle progression, and Ras-induced foci formation (Sahai and Marshall, 2002). Because the Wnt signal transduction pathway has been established as a critical modifier of tumorigenesis, several studies have focused on linking Rac1 activity with Wnt signaling. Until recently it was thought that Rac1 may affect noncanonical, or β-catenin independent, Wnt signaling (Malliri and Collard, 2003) and is involved in canonical Wnt signaling only in cells containing stabilized pools of β-catenin (Esufali and Bapat, 2004). However, a recent work in osteoblast development indicates that Rac1 is critical for the nuclear translocation of β-catenin and subsequent activation of T-cell factor (TCF4) (Upadhyay *et al.*, 2008; Wu *et al.*, 2008). Yet, whether the physiologically relevant association of Rac1 with canonical Wnt signaling in osteoblasts is applicable to potential pathological context has not been investigated.

Although the functional relationship between merlin, Rac1, and its downstream effector PAK has been extensively studied, the contribution of Rac1 signaling to the loss of contact inhibition in *NF2*-deficient cells has not been demonstrated. It has been shown that levels of active Rac1 are increased in *NF2*-deficient cells (Ammoun *et al.*, 2008; Kaempchen *et al.*, 2003; Nakai *et al.*, 2006). Further, merlin knockdown promotes the recruitment of Rac1 to matrix adhesions which correlates with release of cells from contact inhibition (Okada *et al.*, 2005). Together, these studies suggest a mechanism by which Rac1 could modulate signaling to the nucleus in the context of *NF2*-deficiency.

In the present work, we provide the first evidence that Rac1-mediated canonical Wnt signaling contributes to the loss of contact inhibition in *NF2*-deficient cells. Specifically, we show that Rac1 is required for canonical Wnt signaling in the pathological context of *NF2* tumor suppressor loss. In addition, we found that loss of *NF2* increases the transcriptional activity of the nuclear pool of β-catenin in a Rac-dependent manner. One downstream consequence of this is increased amount and transcriptional activation of cell cycle promoting factors, *c-myc* and *cyclin D1*, which may be the mechanism by which *NF2*-

deficient cells are able to transduce signals from the cell membrane to the nucleus to achieve loss of contact inhibition. Our studies show that Rac1-mediated canonical Wnt signaling is critical for the loss of contact inhibition phenotype of *NF2*-deficient cells and the Rac1-Wnt signaling axis could serve as a new node of therapy for *NF2*-associated disease.

Materials and Methods

Cell culture, viral infection, and growth assays

NF2$^{flox2/flox2}$ MEFs were a kind gift from Dr. Marco Giovannini (House Ear Institute, Los Angeles, CA). Clones of spontaneously immortalized MEFs were selected and used throughout the study. All cells were maintained in DMEM supplied with 10% FBS, 2mM L-glutamine, and 100 U/mL penicillin/streptomycin at 37°C in air containing 5% CO_2. In this study, all primary cells were between passages 3 and 5. *NF2* deletion was achieved by infection with replication defective recombinant adenovirus expressing green fluorescent protein (Ad-GFP) or GFP in addition to Cre recombinase (Ad-GFP Cre). Adenovirus was used at approximately 2×10^7 virus particles per dish to achieve an infection efficiency of 90–95% as determined by GFP immunofluorescence. Cells were cultured for at least 4 days post-adenoviral infection prior to use, while the passage number and length of time post-infection remained consistent throughout all experiments unless otherwise stated. Infections of cells with retrovirus encoding MIEG3 or MIEG3-Rac1 N17 or lentivirus encoding shRNA to Rac1 or scrambled control (Sigma PLKO shGly plasmid) were performed according to standard protocol and cells were sorted based on GFP (MIEG3) or YFP (shRNA) expression 48 hours post infection. Cell growth assays were performed by trypsinization and counting by trypan blue exclusion every three days. All assays were performed at sub-confluency unless otherwise stated. For studies in the confluent condition, 4×10^6 cells were seeded in a 10cm tissue culture dish, whereas sub-confluent studies were performed with approximately 5×10^5 cells in the same size dish.

Genomic PCR analysis of recombination and quantitative PCR

Genomic PCR analysis was performed to verify adenoviral-Cre-mediated recombination in primary and immortalized MEFs. Total genomic DNA was extracted using the Blood and Tissue DNA kit (Quiagen, Valencia, CA) according to the manufacturers protocol and amplified using the PCR conditions and the following primers as previously described (Giovannini *et al.*, 2000): P4) 5’ CTT CCC AGA CAA GCA GGG TTC, P5) 5’ GAA GGC AGC TTC CTT AAG TC, P6) 5’ CTC TAT TTG AGT GCG TGC CAT G. RNA was extracted using the RNeasy mini kit (Quiagen, Valencia, CA) and cDNA was synthesized from 1μg of RNA with the SuperScript RT-PCR system (Invitrogen, Carlsbad, CA) according to the manufacturers protocol. cDNAs were amplified by quantitative PCR using TaqMan gene expression assays for *cyclin D1*, *c-myc*, and *gapdh* with TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) according to the manufacturers protocol. Assays were run on an ABI Prism 7900 HT sequence detection system (Applied Biosystems, Foster City, CA).

Transfection, Reporter assay, and BrdU incorporation

Cells were cotransfected with either TCF4-luciferase (TOPflash), TCF4-mutant-luciferase (FOPflash) (Upstate biotechnology, Lake Placid, NY), or cyclin D1-luciferase (generously provided by Dr. Karen Knudsen, Thomas Jefferson University, Philadelphia, PA.) reporter plasmids and a plasmid encoding beta-galactosidase for normalization using FuGENE 6 Transfection Reagent (Roche, Nutley, NJ). 48 hours post-transfection, reporter activity was measured by Luciferase assay kit (Promega, Madison, WI) following manufacturer's protocol. Wnt3A (R&D Systems, Minneapolis, MN) stimulation for reporter assay was performed for 8 hours prior to cell harvest and NSC23766 treatment lasted 24 hours prior to harvest. All reporter assays were performed on nonconfluent cells unless otherwise stated and at least three times in triplicate. Untreated control cells were normalized to 1. Cells were transfected with pSFG or pSFG-dnTCF4 plasmids (a kind gift of Dr. Fanxin Long, Washington University, St. Louis, MO) and subjected to TCF4 -reporter assay as described above. For S phase analysis, cells were transfected with MIEG3, MIEG3-Rac1 N17, pSFG or pSFG-dnTCF4 and H2B-GFP and sorted by flow cytometry based upon GFP expression and were then cultured for 24 hours. Next, cells were labeled with BrdU (GE Healthcare, Little Chalfont Buckinghamshire, UK) for 1 hour prior to fixation and staining for BrdU/ PI FACS analysis as previously described (Bosco *et al.*, 2004) using a FACSCanto flow cytometer and FACSDiva software (BD Biosciences, San Diego, CA). PKF115-584 was kindly provided by Novartis (Basel, Switzerland) and used at 10μM in cell cuclture for 18 hours.

Immunoblotting and immunofluorescence

Whole-cell lysates were prepared by cell extraction using lysis buffer containing 20 mM Tris-HCl (pH 7.6), 100 mM NaCl, 10 mM $MgCl_2$, 1% Triton X-100, 0.2% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL of leupeptin, 1 μg/mL of aprotinin, and 1 mM dithiothreitol for 30 min. Equal amounts of protein, as determined by Bradford assay, were resolved by SDS-PAGE. Specific proteins were detected by standard immunoblotting procedures using the following primary antibodies: (Thermo Fisher Scientific, Fremont, CA,1:500 dilution) Cyclin D1 Ab-3 (Cell Signaling, Danvers, MA, 1:500 dilution) phospho-PAK1 (Thr 423)/PAK2 (Thr 402), phospho-Akt (S473), merlin (9168), (BD Biosciences, San Diego, CA, 1:500 dilution) Rac1, β-catenin (610153), (Millipore, Billerica, MA, 1:500) active β-catenin (05-665), (Abcam Inc., Cambridge, MA, 1:500) phospho-β-catenin Y142 (27798-50), (Sigma-Aldrich, St. Louis, MO, 1:500) Actin. Wnt3A (R&D Systems, Minneapolis, MN) stimulation for immunoblot was performed for 1 hour prior to cell harvest. For immunofluorescence, cells were cultured on coverslips prior to fixation with 3.7% formaldehyde (Sigma-Aldrich). Cells were permeabilized using .5% triton X-100 (Sigma-Aldrich, St. Louis, MO) and stained for immunofluorescence using the previously mentioned antibody for β-catenin and 4',6-diamidino-2-phenylindole (Invitrogen, Carlsbad, CA).

Results

The phenotypes of NF2 loss in primary cells are maintained in immortalized NF2-deficient MEFs

In the literature many of the seminal studies that define the role of merlin in maintaining cellular contacts have been performed in primary mouse embryonic fibroblasts (MEFs). These works have been the cornerstone of our understanding of the tumor suppressor merlin. In this study, primary *NF2*$^{flox2/flox2}$ MEFs were infected with an adenovirus encoding Cre recombinase (Ad-GFP Cre) or a control adenovirus (Ad-GFP), and the cells were harvested for genomic PCR of the *NF2* gene to validate functional deletion 96 hours post infection. Concurrently, spontaneously immortalized clones were derived from these two cell types. The genomic deletion of *NF2* in the immortalized cells was confirmed by the smaller size of the PCR product upon infection with Cre recombinase (442 bp as compared to 338 bp) and was comparable to the deletion evident in the primary lines (Figure 1A, left panel). By immunoblotting for merlin, we show an efficient loss of the merlin protein following infection in the immortalized cells (Figure 1A, right panel). To ensure that our immortalized cell model has maintained the published phentoypes of *NF2*-deficiency in primary MEFs, contact inhibiton and adherens junction stability were examined. *NF2*-deficiency indeed conferred loss of contact inhibition of proliferation in the immortalized cells as shown by growth assay (Figure 1B). Similarly, at confluency destabilization of adherens junctions in immortalized *NF2*-/- cells was reflected by a reduction in the level and diffused distribution of β-catenin at the cell junction as well as the cytoplasm (Figure 1C). These phenotypes are similar to what was reported in the primary cell cultures in Lallemand et al. (Lallemand *et al.*, 2003). Although the localization of β-catenin to adherens junctions is disrupted in the *NF2*-deficient cells upon confluency, the total amount of cellular β-catenin remains similar in both cell types as assessed by immunoblot (Figure 1D). These data are also in agreement with previous findings by Curto et al (Curto *et al.*, 2007) that *NF2*-deficiency results in a loss of contact inhibition and reduced adherens junction, and thus validate our immortalized cell model.

NF2-deficiency increases canonical Wnt signaling

Next we sought to understand whether *NF2*-deficient cells could have an increase in nuclear β-catenin activity since the junctional levels are reduced, yet the total levels remain similar to that of wild type cells. Nuclear β-catenin has a relatively fast turnover that is difficult to detect and we, similar to Curto et al (Curto *et al.*, 2007), did not identify significant differences in the protein levels of the nuclear fraction of β-catenin by Western blotting (data not shown). Therefore, we instead measured transactivation of the TCF4 transcription factor by reporter assay since it is directly responsive to nuclear β-catenin. In both primary (Figure 2 left panel) and immortalized cells (Figure 2 right panel), we observed a four- to six- fold increase in TCF4 transcription factor activity (TOPflash) when *NF2* was excised, whereas the TCF-mut showed minimal activation in all samples (FOPflash). Because the hyperactive Wnt signaling and the loss of contact inhibition phenomena are shared between the primary and immortalized cells, all subsequent experiments are carried out with the immortalized cell line.

Rac1 is necessary for the elevated Wnt activity in NF2-/- cells

In order to probe the signaling events that contribute to the Wnt pathway hyperactivation in the absence of merlin, *NF2*-proficient and -deficient cells were harvested for immunoblot. Similar to what has been shown in Schwann cells (Ammoun *et al.*, 2008; Kaempchen *et al.*, 2003; Nakai *et al.*, 2006), active Rac1 signaling was increased in the *NF2*-deficient cells as two downstream effectors of Rac1, PAK and Akt, became hyperactivated (Figure 3A). Previous studies by the Clevers lab revealed that active β-catenin, or the fraction that is dephosphorylated on ser37 and Thr 41, is indicative of the transcriptionally active portion of β-catenin in the cell (van Noort *et al.*, 2002). In addition, recent reports have shown that phospho-β-catenin Y142 is a phosphorylation event which marks dissociation of β-catenin from adherens junctions leading to a switch toward its nuclear accumulation (Aberle *et al.*, 1996; Brembeck *et al.*, 2004; Sampietro *et al.*, 2006). In the absence of merlin, we found that the levels of nuclear β-catenin, as determined by both "active" or NH_2-terminally dephosphorylated β-catenin and phospho-β-catenin Y142, were elevated as compared to total β-catenin levels. Concomitantly, increased levels of one of the TCF4 transcription factor targets, cyclin D1, was evident in the *NF2*-deficient cells, further confirming the elevated canonical Wnt signaling in these cells (Figure 3A). To verify that the heightened level of cyclin D1 protein in the absence of merlin was transcriptionally active, we performed a reporter assay with *NF2*-proficient and -deficient cells transfected with the cyclin D1-luc reporter. *NF2*-/- cells displayed a 3.5 fold increase in cyclin D1 transcriptional activity as compared to control cells (Figure 3B). Together, these results indicate that Rac1 and canonical Wnt signaling are significantly increased in the absence of merlin.

To dissect the functional relationship between Rac1 and the canonical Wnt pathway, Rac1 activity was inhibited by either using the dominant negative Rac1 mutant, (Rac1 N17), the Rac1 specific small molecule inhibitor (NSC23766), or lentiviral shRNA to Rac1, matched with a scrambled control (shSCR). A TCF4-luciferase reporter assay was performed in wild type cells that had been transduced with a retrovirus encoding either the MIEG3-Rac1 N17 mutant or control MIEG3 alone. This was also executed in cells transduced with lentivirus expressing YFP- tagged shRac1 or shSCR. Cells were sorted by flow cytometry for GFP expression (encoded by MIEG3 virus) or YFP expression (encoded by sh lentivirus) prior to transfection with the reporter constructs and a subset were treated with 100 μM NSC23766 24 hours prior to harvest and/or 100ng/ml Wnt3a 8 hours prior to harvest. As expected, treatment of control MIEG3 infected cells with Wnt3a significantly induced TCF4 reporter activity more than 8 fold over untreated cells (results were nearly identical with shSCR controls and are not shown). This induction of TCF4 transactivation was abrogated by pharmacological, shRNA, and dominant negative Rac1 inhibition (Figure 4A, left panel). Wild type cells transduced with lentivirus encoding YFP-tagged shSCR or shRac1 were sorted based upon YFP expression and immunoblotted to validate the Rac1 gene knockdown prior to use in the aforementioned luciferase reporters (Figure 4A, right panel). The results of these reporter assays are in agreement with a recent study in the osteoblast model (Wu *et al.*, 2008) indicating that Rac1 activity is a critical component of the canonical Wnt signaling pathway.

To understand the contribution of Rac1 to the regulation of the canonical Wnt pathway in a pathological context, similar experiments were performed in Ad-GFP or Ad-GFP Cre infected *NF2*$^{flox2/flox2}$ immortalized MEFs. Wnt3a stimulation resulted in heightened TCF4 transactivation, and this effect was increased in the absence of *NF2*. However, TCF4 transactivation was completely blocked in *NF2*-/- cells by Rac1 targeting either through the use of the dominant negative Rac1 mutant, MIEG3-Rac1 N17, or shRac1. This inhibitory effect of Rac1 targeting on TCF4 transctivation was also evident on the Wnt3a stimulated wild type cells (Figure 4B). This result indicates that Rac1 is necessary for the elevation of canonical Wnt pathway signaling in the absence of merlin.

The *c-myc* and *cyclin D1* genes are well characterized downstream targets of the Wnt pathway. To verify the enhancing effect of Rac1 on Wnt signaling in the *NF2*-deficient cells, we performed quantitative PCR to determine the relative abundance of both genes with respect to a *GAPDH* control. Ad-GFP or Ad-GFP Cre infected cells were transduced with MIEG3-Rac1 N17 or MIEG3 alone and sorted based upon GFP expression. Cells were then plated at confluency to determine the impact of Rac1 blockade on the induction of *c-myc* and *cyclin D1*, which are important for cell proliferation and therefore could exhibit inappropriately high levels upon loss of contact inhibition. As shown in Figure 4C, the levels of both genes are elevated in the control infected *NF2*-/- cells, and dominant negative inhibition of Rac1 was able to reduce the expression of both genes to levels comparable to those in *NF2*-proficient cells. This data indicates that Rac1 contributes to the increase in *c-myc* and *cyclin D1* in the *NF2*-/- cells under confluent conditions.

Wnt signaling contributes to the loss of contact inhibition in NF2-deficient MEFs

We next sought to determine the effect of the increased β-catenin activity on the loss of contact inhibition of proliferation of the *NF2*-deficient cells by using a dominant negative mutant of TCF4 (dnTCF4). DnTCF4 is an N-terminal deletion of TCF4 that is unable to bind β-catenin yet retains the ability to bind to DNA, thus is effective in blocking transcriptionally active β-catenin from binding DNA (Tetsu and McCormick, 1999). First, we either transfected Ad-GFP or Ad-GFP Cre infected cells with pSFG-control DNA or pSFG-dnTCF4 and sorted the cells by flow cytometry, prior to performing a TCF4 reporter assay. dnTCF4 was found to efficiently block TCF4 transactivation in response to Wnt3a stimulation and restored the level of TCF4 hyperactivation in *NF2*-/- cells to levels comparable to the wild-type cells (Figure 5A), indicating that Wnt pathway inhibition was functional. Second, we tested whether dnTCF4 was able to inhibit the elevated TCF4 activity in the *NF2*-/- cells at confluency. As shown in Figure 5B, upon dnTCF4 expression, the TCF4 transcriptional activity in the confluent *NF2*-deficient cells was attenuated approximately 10-fold. These results suggest that the loss of contact inhibition in *NF2*-deficient cells at confluency is associated with Wnt pathway hyperactivation. Third, we have examined the cell proliferative activity in the confluent state using a BrdU incorporation assay. Not surprisingly, the number of Ad-GFP infected cells that were able to enter S phase was greatly reduced as compared to the Ad-GFP Cre infected controls at confluency, and dnTCF4 did not significantly effect the ability of the Ad-GFP infected cells to enter S-phase. In contrast, the Ad-GFP Cre infected cells exhibited ~60% decrease in proliferative capacity following dnTCF4 transfection (Figure 5C). The results of these Wnt signaling inhibition

experiments indicate that merlin-deficient cells depend on Wnt/TCF4 signaling to achieve the loss of contact inhibition. To directly test the requirement of active Rac1 for loss of contact inhibition in the merlin-deficient cells, Ad-GFP or Ad-GFP Cre infected cells were transduced with a virus encoding either MIEG3 alone or MIEG3-Rac1 N17. Similar to Figure 5C, BrdU/ PI analysis revealed that Ad-GFP infected cells are contact inhibited and their cell cycle progression was thus unaffected by dominant negative Rac1 (Figure 5D). However, Ad-GFP Cre infected cells exhibited a two fold increase in BrdU incorporation at confluency which was abrogated by the addition of Rac1 N17, indicating that Rac1 activity significantly contributes to loss of contact inhibition in *NF2*-/- cells. When dnTCF4 and Rac1 N17 were cointroduced into the *NF2*-/- cells, a similar degree of inhibition of BrdU incorporation at confluency was evident as compared to dnTCF4 or Rac1 N17 expression alone (data not shown). These data further suggest that Rac1 and β-catenin signal in the same pathway to impact the loss of contact inhibition in *NF2*-/- cells.

Small molecule inhibition of wnt-signaling suppresses the loss of contact inhibition of *NF2* cells

To demonstrate the therapeutic relevance of the proposed dependence of *NF2*-deficient cells on the Rac mediated Wnt pathway to achieve loss of contact inhibition, a natural small molecule inhibitor of the Wnt pathway, PKF115-584 (Lepourcelet *et al.*, 2004) was utilized. Similar to the dnTCF4 mutant, the PKF115-584 small molecule prevents the formation of the β-catenin-TCF4 complex, thereby inhibiting its ability to bind DNA (Lepourcelet *et al.*, 2004). Treatment of the MEF cells in our study with 10μM PKF115-584 for 18 hours was able to abrogate expression of the β-catenin downstream target, cyclin D1, with minimal toxicity and thus is the dose we proceeded with in further experiments. Consistent with what has been observed in acute myeloid leukemia cell lines (Minke *et al.*, 2009), inhibition of β-catenin expression itself was also evident following treatment with the Wnt inhibitor (Figure 6A, right panel). To determine the effectiveness of PKF115-584 on TCF4 transactivation, Ad-GFP and Ad-GFP Cre infected *NF2* floxed MEFs were treated with DMSO control or 10μM PKF115-584 for 18 hr in combination with Wnt3a stimulation in the final 8 hours prior to harvesting for TCF4 reporter assay. PKF115-584 was able to significantly inhibit the activation of TCF4 in response to Wnt3a, independent of the activity of *NF2* (Figure 6A, left panel). To specifically examine the ability of PKF115-584 to block Wnt signaling in *NF2*-deficient cells at confluency, the Ad-GFP and Ad-GFP Cre infected cells were seeded into confluent conditions and treated with PKF115-584 or vehicle control prior to the TCF4 reporter assay. The small molecule inhibitor was effective in blocking the hyperactivation of TCF4 in the *NF2*-deficient cells at confluency, and showed minimal effect on the already reduced level of TCF4 transactivation in *NF2*-proficient cells (Figure 6B). Lastly, to verify that such Wnt pathway inhibition can affect cell cycle progression at confluency, *NF2*-proficient and –deficient cells cultured in confluent conditions were treated with vehicle control or PKF115-584 for 18 hr, pulsed with BrdU for the final 1 hr, and harvested for BrdU FACS analysis. As shown in Figure 6C, PKF115-584 completely blocked the ability of the Ad-GFP Cre infected cells to proliferate at confluency. These results suggest that the dependence of *NF2*-deficient cells on Wnt signaling for the loss of contact inhibition could be useful in reversing this phenotype.

Discussion

Loss of contact inhibition of cell proliferation is the signature phenotype of cells lacking the tumor suppressor merlin. Associated with loss of contact inhibition, merlin-deficient cells are known to contain defective adherens junctions at confluency with unstablized cadherin-catenin complexes (McClatchey and Giovannini, 2005). The issue of how proliferative signals arise from the sites of cell-cell junctions and are transduced to the nucleus to promote inappropriate cell cycle progression remains unresolved. Recent evidence in primary MEFs has implicated persistent EGFR signaling in the inappropriate proliferation of *NF2*-/- cells, suggesting that merlin restrains EGFR activity at the cell membrane (Curto *et al.*, 2007). Our current work provides new evidence that the relationship of merlin with EGFR is not the only mechanism by which *NF2*-/- cells are able to escape contact inhibition.

Similar to the observations in Curto et al. (Curto *et al.*, 2007), we show that the total level of β-catenin is unaltered by the loss of merlin. But in contrast to that study, we found a significant increase in transcriptionally active β-catenin upon merlin deletion. This is established by several means. First, we found that increased TCF/LEF transcription factor activity (Figure 2A), which is a direct downstream target of activated β-catenin in the nucleus, exists in the absence of merlin. Second, *NF2*-/- cells contained an increase in both active β-catenin, and phospho-β-catenin Y142 levels (Figure 3A), which have been shown to be markers for β-catenin that is being translocated from the cell membrane to the nucleus. Third, elevations in the mRNA (Figure 4C) and transcriptional activity (Figure 3B) of downstream targets of activated β-catenin were evident in the absence of merlin. Thus, we believe that deregulated canonical Wnt signaling is associated with *NF2* loss of function. To examine whether the increased nuclear β-catenin activity could be a causal event in the ability of *NF2*-/- cells to inappropriately proliferate at confluency, we were able to restore basal levels of TCF/LEF transcriptional activity to the *NF2*-/- cells either through the use of a dominant negative TCF4 construct or a small molecule Wnt pathway inhibitor, PFK115-584 (Figure 5B, Figure 6B). Suppressing either the elevated TCF or Rac1 activities in the merlin-deficient cells significantly reduced the capacity of *NF2*-deficient cells to enter S phase at confluency (Figure 5C–5D, Figure 6C). Collectively, our results show that the elevated nuclear β-catenin activity in *NF2* deficient cells contributes to the growth phenotype of the cells at confluent state.

Like many classical tumor suppressors, merlin's activity is governed by its phosphorylation state. Several seminal papers in the study of neurofibromatosis type 2 have linked merlin activity to its phosphorylation induced by Rac1-GTP and its downstream effector PAK (Kissil *et al.*, 2002; McClatchey and Giovannini, 2005; Shaw *et al.*, 2001). The ser518 phosphorylation of merlin weakens its self- and cytoskeletal- associations, inactivates its growth suppressive properties, and potentiates Rac-dependent signaling (Gutmann *et al.*, 1999; Kissil *et al.*, 2002; Xiao *et al.*, 2002). This opposing relationship between Rac1 and merlin is strengthened by observations that overexpression of merlin inhibits the Rac1 signaling axis, specifically PAK1 and JNK, and downregulates cyclin D1 (Kaempchen *et al.*, 2003; Kissil *et al.*, 2002; Xiao *et al.*, 2002). Moreover, it is also established that *NF2*-/- cells contain very high levels of active Rac1 (Ammoun *et al.*, 2008; Kaempchen *et al.*, 2003; Nakai *et al.*, 2006). Thus, in light of the recent evidence that Rac1 is critical for canonical

Wnt pathway signaling (Wu *et al.*, 2008), we have examined the relationship between active Rac1 and the elevated Wnt signaling in the context of *NF2*-deficiency induced inappropriate proliferation. We first confirmed that Rac1 is necessary for canonical Wnt signaling in the *NF2* loss of function context, a pathologically relevant model system (Figure 4A), as previous experiments in osteoblasts and NIH 3T3 cells do not address such a relevance (Wu *et al.*, 2008). Next, we found that Rac1 activity was indeed responsible for the elevation in canonical Wnt signaling in *NF2*-deficient cells, as Rac1 inhibition with a dominant negative mutant, small molecule inhibitor, or shRNA eliminated TCF4 transcriptional activation (Figure 4B), and transcription of downstream targets *c-myc* and *cyclin D1* (Figure 4C). Although our data do not define a mechanism through which Rac1-GTP can impact Wnt signaling in the context of merlin loss, it could be envisioned to occur through various Rac effectors, such as the MAPK, Akt, or JNK (Chadee *et al.*, 2006; Tang *et al.*, 2007; Wu *et al.*, 2008). Because the inhibitory effect of Rac1 N17 together with dnTCF4 on the confluent *NF2*-/- cell proliferation was comparable to that by Rac1 N17 or dnTCF4 alone (Figure 5; data not shown), it is likely that Rac1 and TCF4 activities do not synergize and may act as a part of the same pathway containing *NF2*-Rac1-β-catenin-contact inhibition.

Recent advances in the study of *NF2* have indicated that merlin is a tumor suppressor that is capable of modulating a wide range of signaling pathways that influence cell growth, motility and apoptosis. Similar to our results in MEFs, a recent study in high grade human gliomas has also concluded that *NF2* loss results in an increase in Wnt signaling, specifically TCF transcription factor activity (Lau *et al.*, 2008). This work found that merlin acts to increase expression of molecules that inhibit canonical Wnt signaling (*Dkk-1* and *Dkk-3*) and decrease expression of molecules that activate Rac1/ RhoA signaling (*Vav3*) (Colomba *et al.*, 2008; Lyons and Burnstein, 2006). With the glioma model in mind, it is possible that elevated levels of active Rac1 in the absence of merlin are due to unrestrained Vav3 activity in *NF2*-associated tumors as well and could serve as another potential point of therapeutic intervention in the disease.

Because schwannomas are benign and relatively slow-growing they do not respond well to traditional chemotherapy and often result in morbidity. Treatment strategies are centered on local tumor control by repeated surgeries and radiation which can have detrimental effects on the nerves and are made more challenging by tumor location and multiplicity. Thus, new pharmacological treatment strategies are urgently needed. Because NF2 patients typically suffer from schwannomas and meningiomas, further study is necessary in these disease models in order to apply our observations. In this context, our work provides new insight into the tumor suppressive activity of merlin and suggests that targeting the Rac-Wnt pathway could serve as a novel therapeutic strategy for neurofibromatosis type 2 associated disease.

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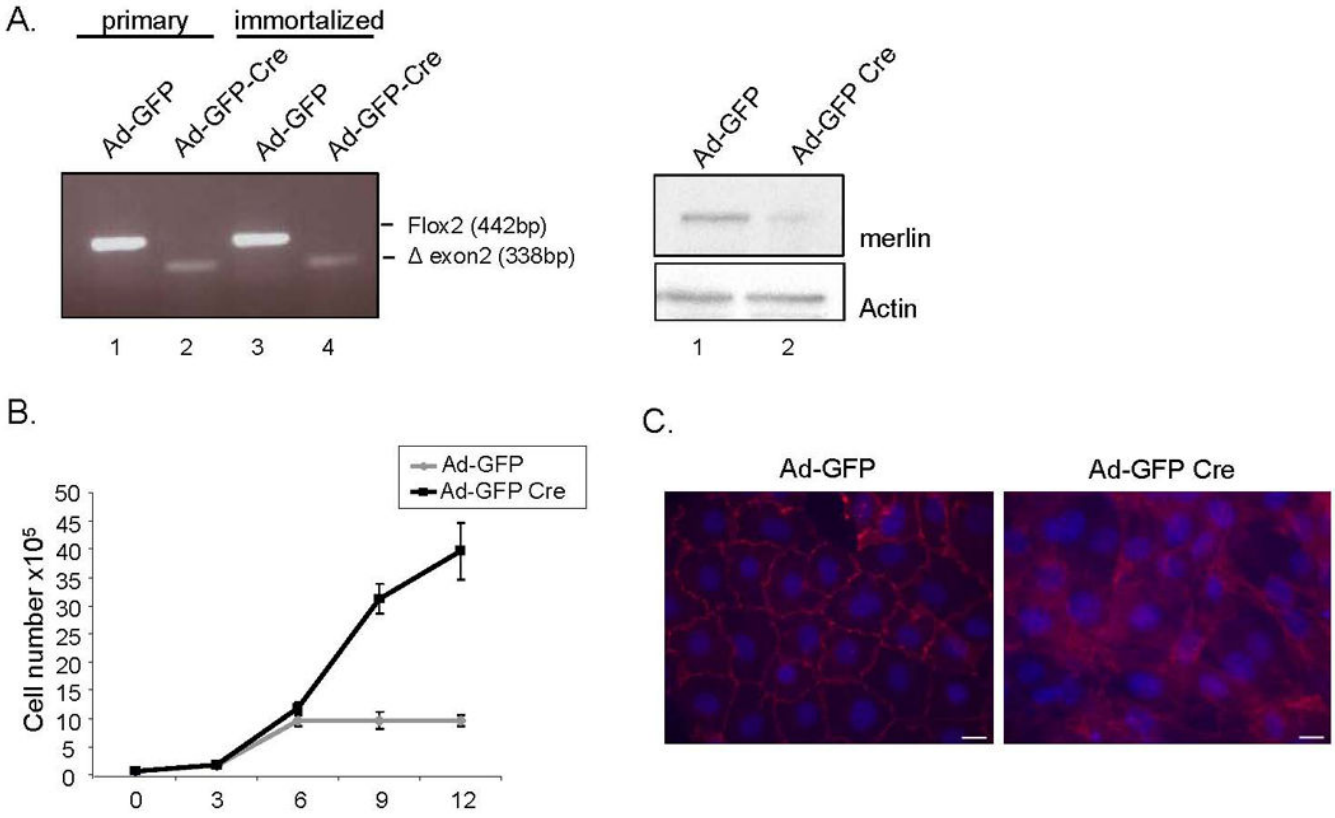


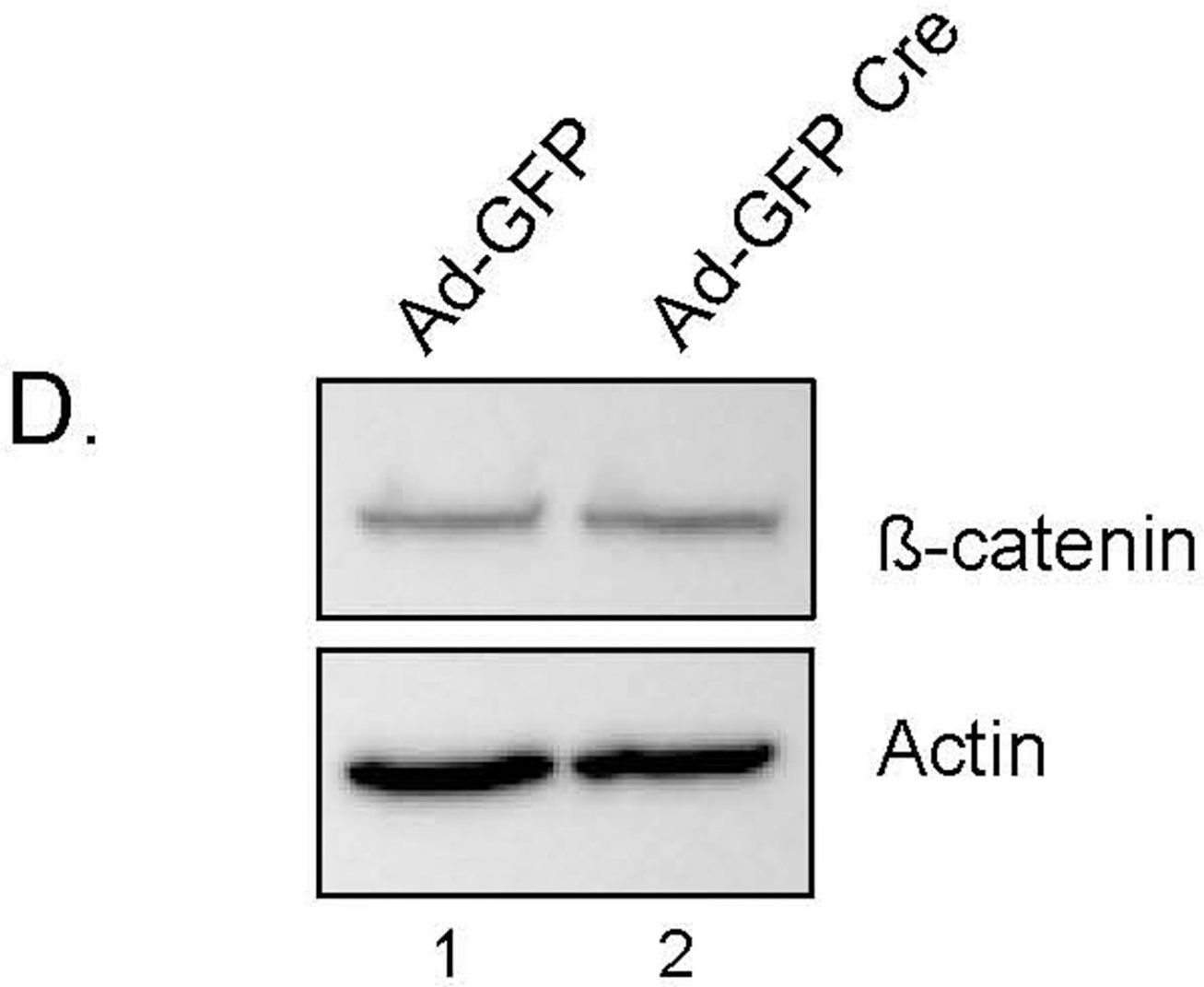


Figure 1. *NF2*-deficiency confers a loss of contact inhibition and adherens junction instability in immortalized MEFs

(A) Primary and immortalized MEFs were infected with adenovirus encoding GFP or GFP-Cre. Cells were harvested 4 days post infection and genomic DNA was purified for PCR of the *NF2* gene (left panel) or cells were processed for immunoblot with an antibody to merlin (right panel). Actin served as a loading control. (B) Immortalized cells from A were plated at a concentration of 1×10^5 and counted every 3 days. (C) Immortalized cells from A were plated on coverslips, grown to confluency, and starved of serum for 48 hours prior to immunofluorescent staining for β-catenin (red). DAPI is a nuclear stain (blue). Images were taken at equal exposures. (D) Cells from A were harvested for immunoblotting for the total levels of β-catenin. Actin served as a loading control.

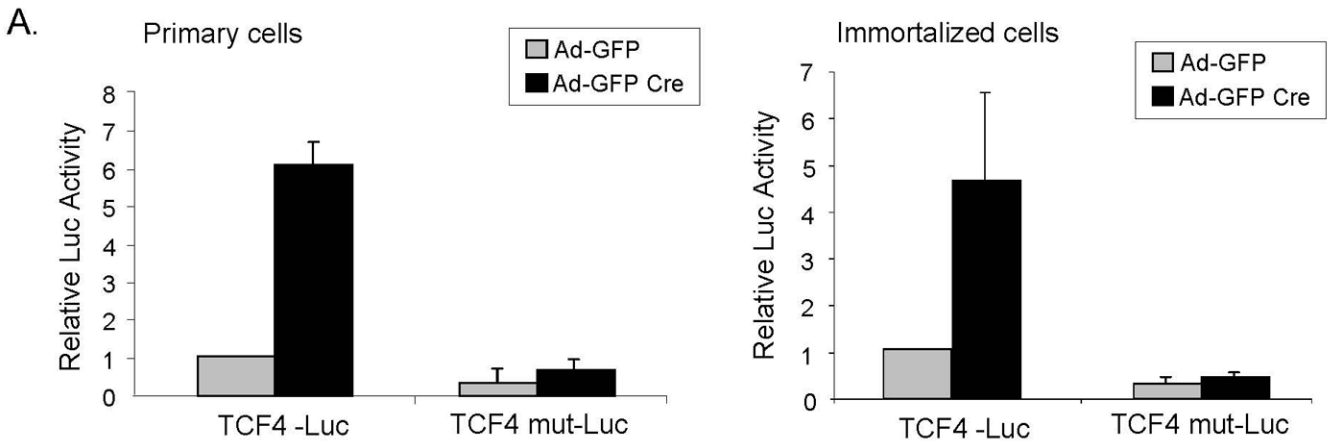


Figure 2. ***NF2*** **loss results in an increase in Wnt signaling**

Ad-GFP or Ad-GFP Cre infected primary (left panel) or immortalized (right panel) MEFs were transfected with TCF4-luc (TOPflash) or TCF4-mut-luc (FOPflash) and β-gal plasmids and luciferase reporter assay was performed.

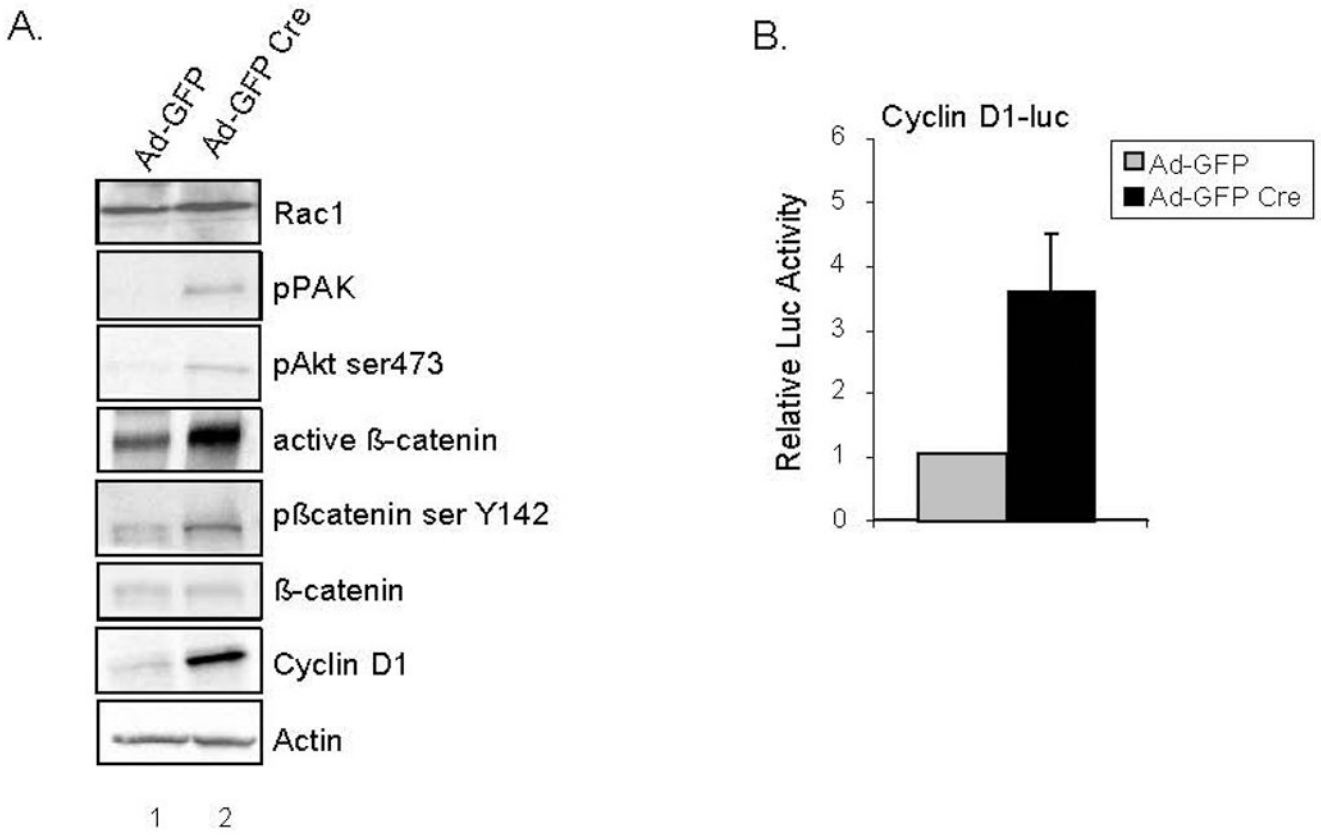


Figure 3. Wnt and Rac1 signaling are elevated in *NF2*-/- cells

(A) Immortalized cells infected with Ad-GFP and Ad-GFP Cre were harvested and a Western blot was performed to analyze the levels of total Rac1, pPAK, pAkt ser 473, active β-catenin, pβ-catenin Y142, total β-catenin, and cyclin D1. Actin served as a loading control. (B) Cells from A were transfected with cyclin D1-luc and β-gal plasmids prior to harvest for reporter assay.

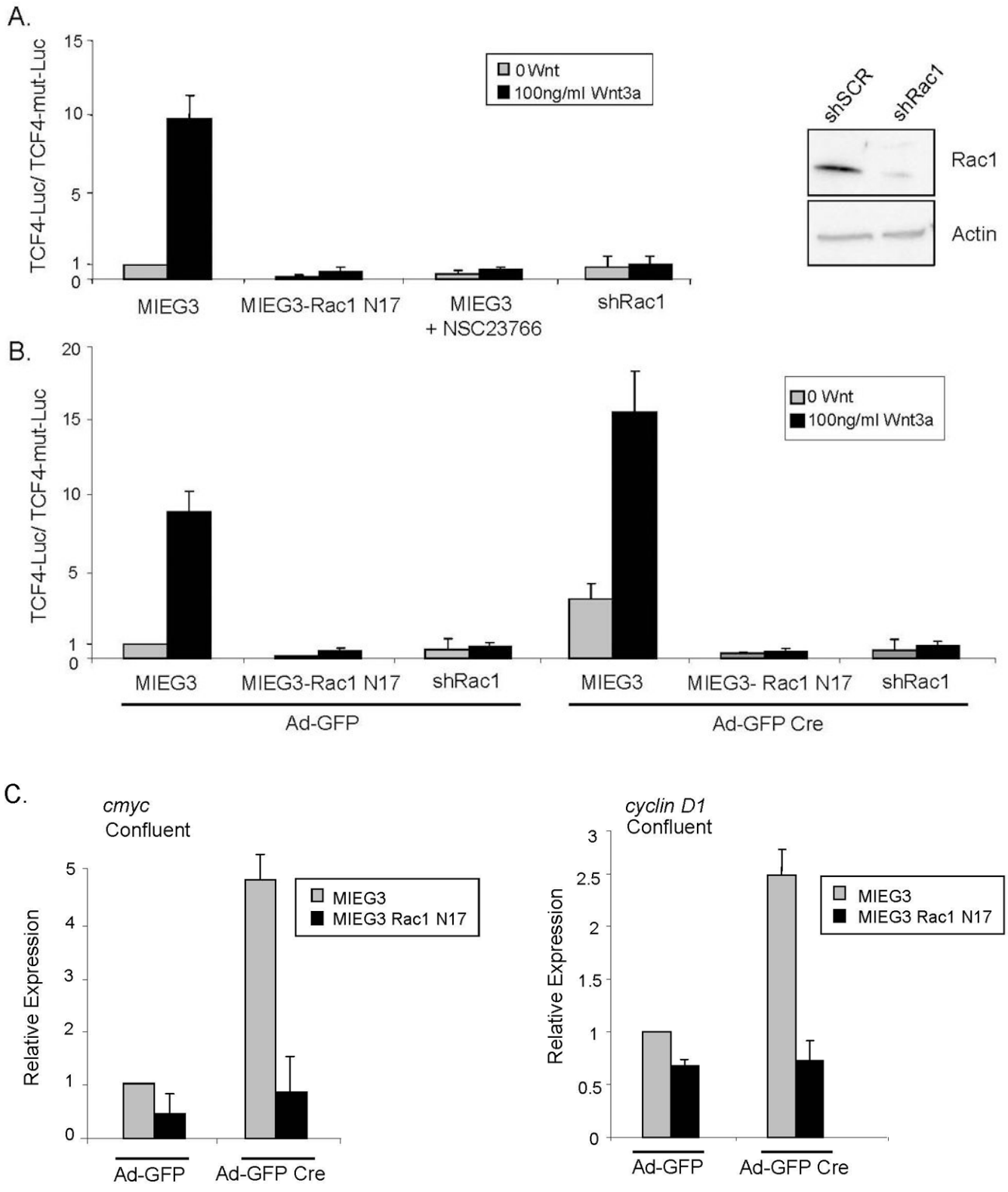
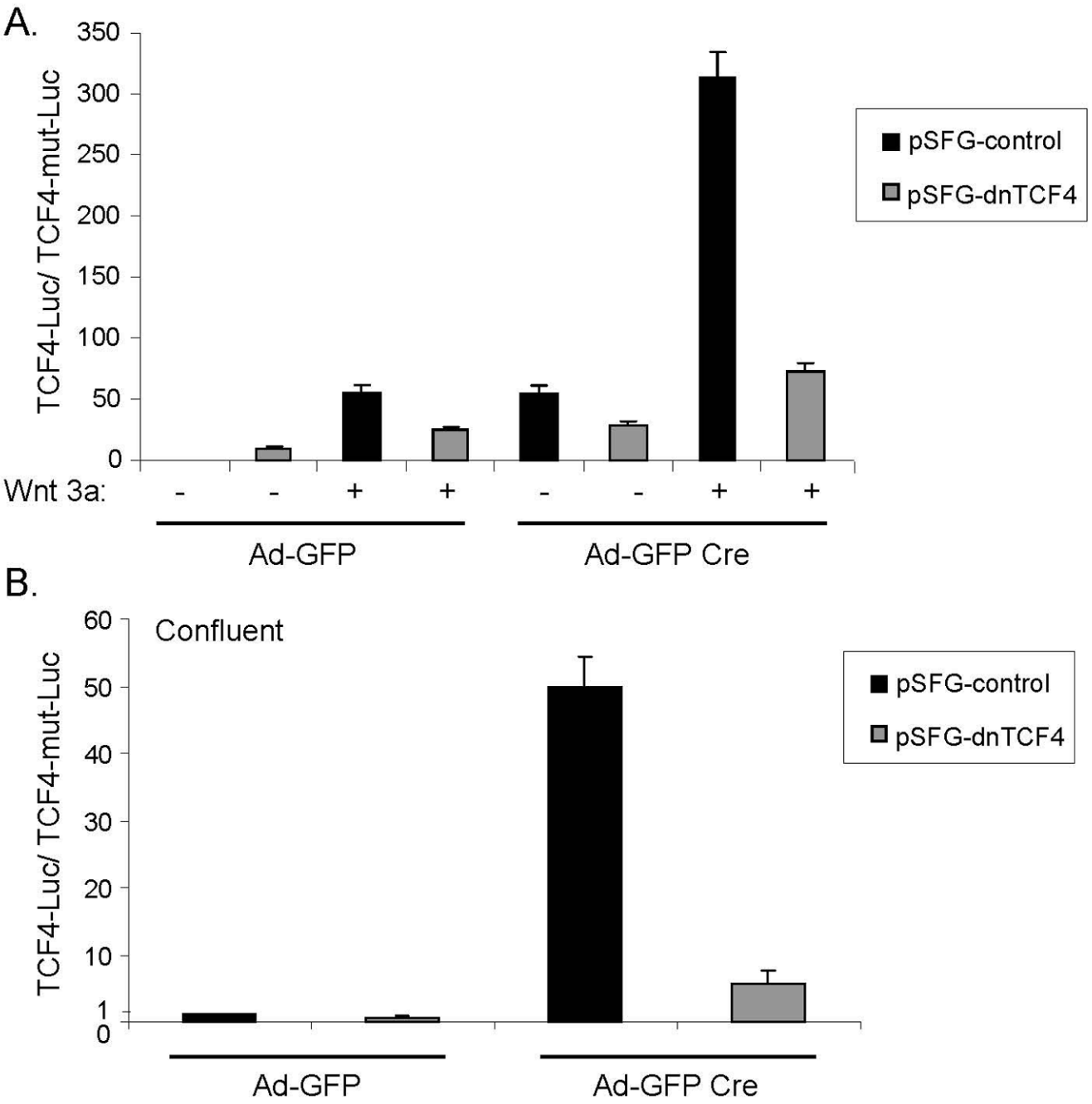


Figure 4. Rac1 activity is necessary for elevated Wnt signaling in *NF2*-deficient MEFs

(A) Wild type MEF cells were transduced with retrovirus expressing either MIEG3 control or a MIEG3-Rac1N17 mutant and sorted by flow cytometry based on GFP positivity. Cells were then transfected with TCF4-luc (TOPflash) or TCF4-mut-luc (FOPflash) and β-gal plasmids and were stimulated with Wnt3a for 1 hour prior to harvest for reporter assay. Cells were treated with NSC23766 for 24 hours prior to reporter assay harvest where indicated. Data is represented as TCF4-luc/TCF4-mut-luc and the activity of the untreated MIEG3 infected cells are set to 1. (B) Ad-GFP or Ad-GFP Cre infected immortalized MEFs were subsequently infected with retrovirus encoding MIEG3, or MIEG3-Rac1 N17 and sorted by GFP expression 48 hours later. This population of cells was transfected with TCF4-luc (TOPflash) or TCF4-mut-luc (FOPflash) and β-gal plasmids and stimulated with Wnt3a prior to harvesting for reporter assay. Data is represented as TCF4-luc/TCF4-mut-luc and normalized to the activity of the untreated MIEG3, Ad-GFP infected cells. (C) Retrovirally transduced cells from B were harvested at confluency for quantitative PCR analysis of *c-myc* (left panel) and *cyclin D1* (right panel) with respect to a *GAPDH* control.



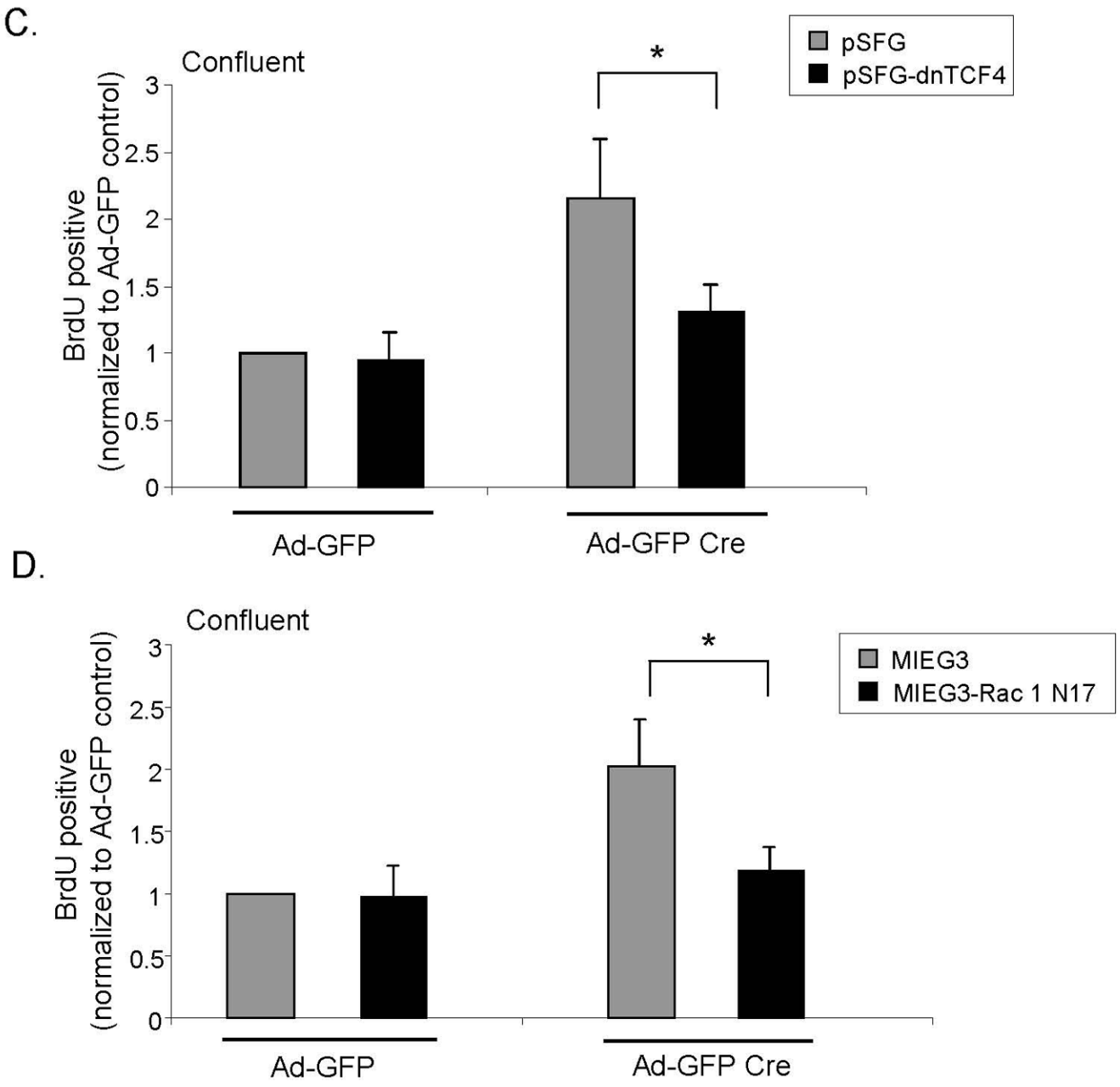
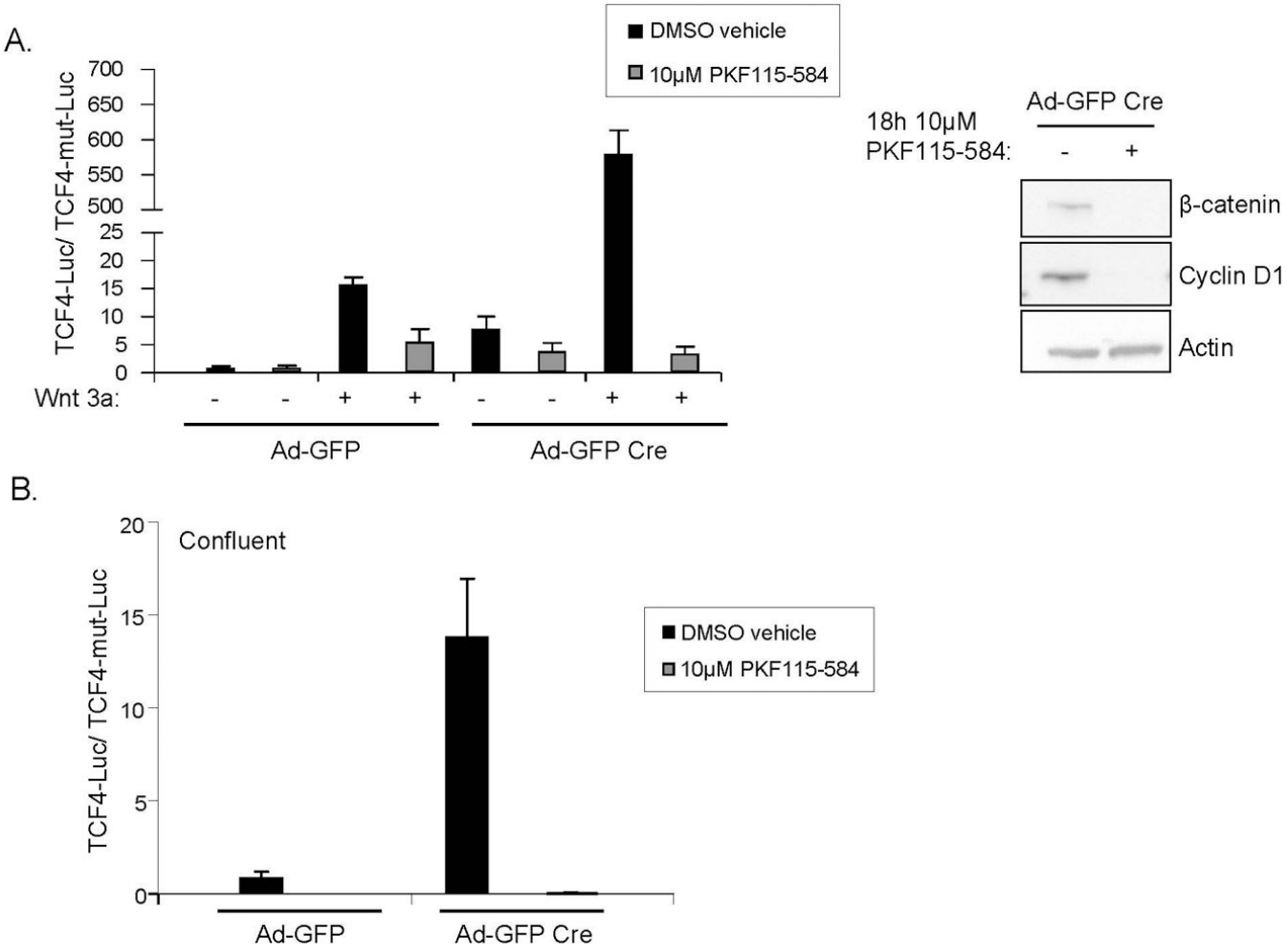


Figure 5. Wnt signaling contributes to the loss of contact inhibition in *NF2*-deficient MEFs

(A) Ad-GFP or Ad-GFP Cre infected MEFs were cotransfected with TCF4-luc (TOPflash) or TCF-mut-luc (FOPflash), β-gal, and pSFG-control or pSFG-dnTCF4 prior to Wnt stimulation and reporter assay. Data is represented as TCF4-luc/TCF4-mut-luc and normalized to the activity of the untreated pSFG-control, Ad-GFP infected cells. (B) Cells from A were grown to confluency prior to harvest for luciferase reporter assay and the activity of the pSFG-control, Ad-GFP infected cells was set to 1. (C) Cells from A were grown to confluency and pulsed with BrdU for 1 hour prior to fixation and staining for BrdU/ PI followed by FACS analysis. Data is normalized to the Ad-GFP pSFG-control

infected cells which is set to 1, and a student t-test was run to determine statistical significance $p<.05$. (D) Ad-GFP or Ad-GFP Cre infected cells were cotransfected with MIEG3 alone or MIEG3-Rac1 N17, and sorted based on GFP expression. Sorted cells were plated at confluency and pulsed with BrdU for 1 hour prior BrdU/ PI analysis as in C. Data is normalized to the Ad-GFP control MIEG3 infected cells which is set to 1, and significance was determined by student t-test ($p<.05$).



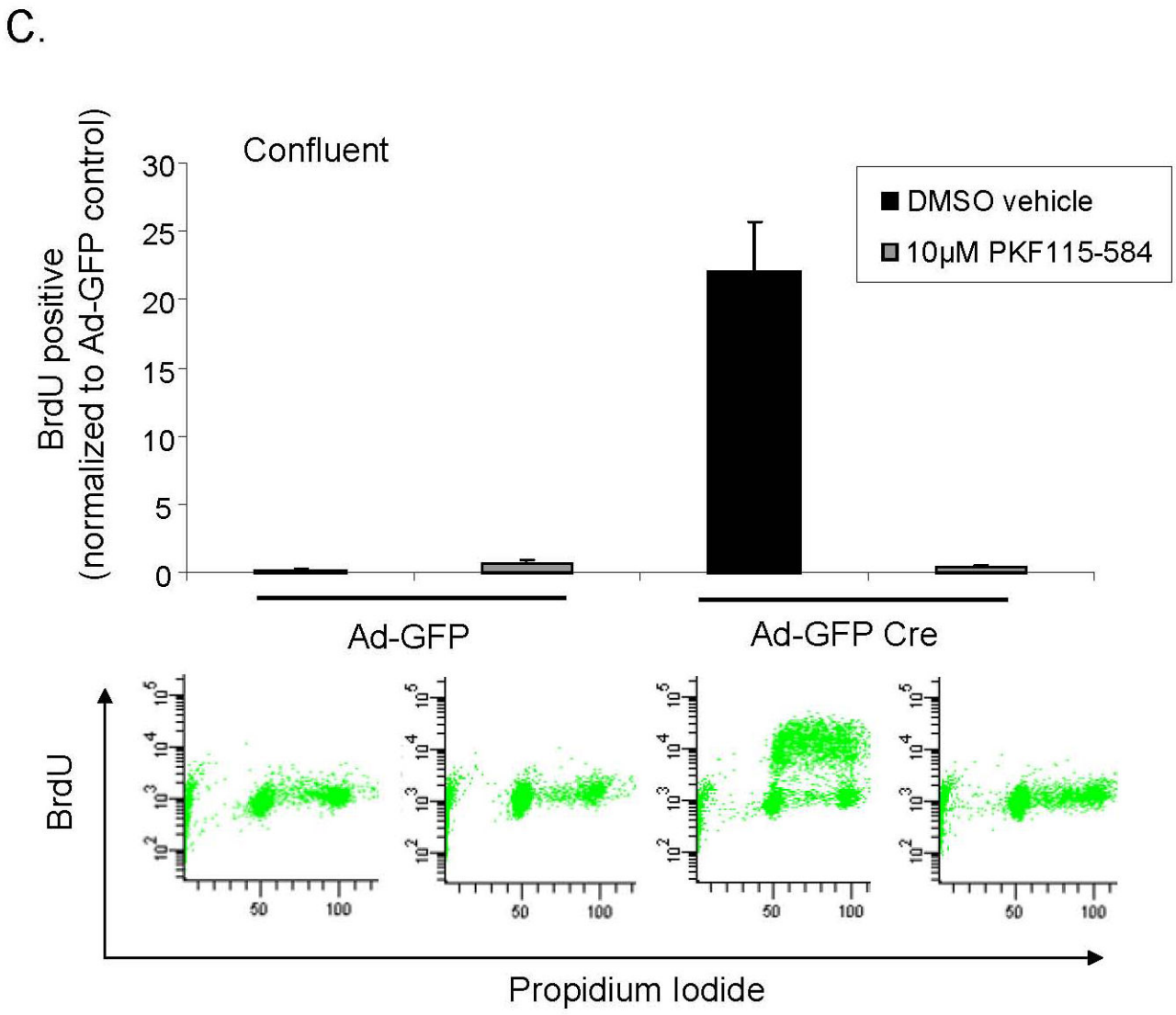


Figure 6. Small molecule inhibition of Wnt signaling restores *NF2*-deficient cells elevated TCF4 activity and inappropriate proliferation to levels comparable to wild type cells

(A) Ad-GFP or Ad-GFP Cre infected MEFs were cotransfected with TCF4-luc (TOPflash) or TCF-mut-luc (FOPflash), and β-gal, prior to 24 hour treatment with 10μM PKF115-584, Wnt stimulation and reporter assay. Data is represented as TCF4-luc/TCF4-mut-luc and normalized to the activity of the untreated DMSO vehicle control, Ad-GFP infected cells (left panel). Ad-GFP Cre infected MEFs were treated with 10μM PKF115-584 for 18 hours prior to harvest for immunoblot to verify inhibition of β-catenin and cyclin D1. Actin served as a loading control. (B) Cells from A were grown to confluency prior to harvest for luciferase reporter assay and normalized to the activity of the untreated DMSO vehicle control which was set to 1. (C) Upper panel: Ad-GFP or Ad-GFP Cre infected MEFs were grown to confluency, treated with either DMSO vehicle or 10μM PKF115-584, and pulsed with BrdU for 1 hour prior to harvest for BrdU/ PI analysis. Data is normalized to the Ad-

GFP infected, DMSO treated control cells which are set to 1. Lower panel: Representative histograms from FACS analysis are displayed.