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NF-κB inducing kinase (NIK) modulates melanoma tumorigenesis by regulating expression of pro-survival factors through the β-catenin pathway

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Abstract

Nuclear factor-κB (NF-κB) inducing kinase (NIK) is a MAP3K that regulates the activation of NF-κB. NIK is often highly expressed in tumor cells, including melanoma, but the significance of this in melanoma progression has been unclear. Tissue microarray analysis of NIK expression reveals that dysplastic nevi (n=22), primary (n=15) and metastatic melanoma (n=13) lesions showed a statistically significant elevation in NIK expression when compared to benign nevi (n=30). Moreover, when shRNA techniques were used to knock-down NIK, the resultant NIK-depleted melanoma cell lines exhibited decreased proliferation, increased apoptosis, and reduced tumor growth in a mouse xenograft model. As expected, when NIK was depleted there was decreased activation of the non-canonical NF-κB pathway, while canonical NF-κB activation remained intact. NIK depletion also resulted in reduced expression of genes that contribute to tumor growth, including *CXCR4, c-MYC* and *c-MET*, and pro-survival factors such as BCL2 and survivin. These changes in gene expression are not fully explained by the attenuation of the non-canonical NF-κB pathway. Shown here for the first time is the demonstration that NIK modulates β-catenin mediated transcription to promote expression of survivin. NIK-depleted melanoma cells exhibited down-regulation of survivin as well as other β-catenin regulated genes including *c-MYC*,

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Conflict of Interest

The authors declare no conflict of interest.

Author Contribution

Designed experiments and wrote paper: YMT, AR. Performed experiments: YMT, YS, JY, RS. Analyzed data: YMT, JY, RS. shared reagents: SN. Contributed human tissue samples for TMA: AB, CS and CM.

c-MET and CCND2. These data indicate that NIK mediates both β-catenin and NF-κB regulated transcription to modulate melanoma survival and growth. Thus, NIK may be a promising therapeutic target for melanoma.

Keywords

NIK; β-catenin; melanoma; tumorigenesis; survival factors

Introduction

Melanoma is a deadly disease and number of efficacious therapies to treat malignant melanoma is limited. Thus, finding new targets and understanding molecular differences in melanoma is essential for the development of novel cancer therapeutics. Similar to other cancers, malignant melanoma results from genetic and molecular changes, such as gain-of-function mutation in BRAF ($BRAF^{V600E}$), N-RAS ($N\text{-}RAS^{Q61KorR}$), loss of $p16^{INK4a}$, or activation of c-MET (Chin et al., 1998; Davies et al., 2002). Likewise, many major signaling pathways such as MAPK, β-catenin, Akt/PTEN, and NF-κB pathways are mis-regulated in melanoma. However, until recently, use of this information for targeted therapy in melanoma has met with only limited success (Sullivan & Atkins, 2009). The remarkable 50% response rate currently observed in patients with metastatic melanoma with the $BRAF^{V600E}$ mutation (Chapman et al., 2011; Puzanov & Flaherty, 2010) was possible due to new advances in our understanding of molecular pathways crucial for melanoma tumorigenesis.

Ongoing research has shown that various combinations of gene mutations, deletions and amplifications result in the formation of several subtypes of melanoma lesions, with different responses to therapy. Identification of the molecular pathways associated with each individual's melanoma tumor will provide key insights for better classification of melanoma and more appropriate, personalized therapeutic intervention. It is increasingly apparent that NF-κB activation is one of the molecular changes important for melanoma growth (Devalaraja et al., 1999; Dhawan & Richmond, 2002; Lev et al., 2003; Yang & Richmond, 2001; Yang et al., 2010). Consequently, targeting NF-κB is an effective tool for inhibiting the growth of melanoma tumors with constitutive NF-κB activation (Yang et al., 2006; Yang et al., 2007; Yang et al., 2010).

The term nuclear factor-κB (NF-κB) is used to denote a family of transcription factors which play crucial roles in the development of cancer due to its regulatory function in inflammation, proliferation, apoptosis and survival. Mammalian NF-κB members include p65 (RelA), p105/p50, RelB, p100/p52 and c-Rel. The regulation of NF-κB activity is achieved by modulation of two distinct pathways: the canonical and the non-canonical pathways. The canonical pathway of NF-κB is regulated by the IκB kinase (IKK) complex, containing IKKα, IKKβ and IKKγ. The IKK complex phosphorylates inhibitor of κBs (IκBs), which usually sequester NF-κB members in the cytoplasm, thereby inhibiting the nuclear translocation of the transcription factors. Upon phosphorylation, IκBs are degraded by the proteasome, releasing NF-κB complexes to function as transcription factors.

The non-canonical pathway of NF-κB is regulated by IKKα and NF-κB inducing kinase (NIK). Though NIK activates both IKKα and IKKβ, it preferentially phosphorylates IKKα (Ling et al., 1998). NIK, also known as MAP3K14 located on the human chromosome 17q21, was first identified as a kinase which activates NF-κB upon TNF and IL-1 receptor ligation, leading to activation of the canonical pathway (Malinin et al., 1997). However, later studies suggest that NIK is dispensable for activation of the canonical NF-κB pathway (Smith et al., 2001; Yin et al., 2001). NIK and IKKα phosphorylate p100, an IκB for this pathway. Upon phosphorylation, p100 undergoes partial proteolysis to generate a 52kD protein (p52) which can dimerize with RelB, and translocate to the nucleus to activate transcription of RelB/p52 responsive genes.

Previous research has shown that alterations in the NIK gene, NIK activity or NIK protein expression occur in hematological cancers and that targeting NIK reduces tumor cell survival (Annunziata et al., 2007; Conze et al., 2010; Keats et al., 2007; Saitoh et al., 2008). While the importance of NIK in tumorigenesis of solid tumors has been reported (Neely et al., 2010; Saitoh et al., 2010), its role in solid tumors is less well-understood and detailed mechanisms of how NIK modulates tumor growth are not fully explored.

We have previously shown that a higher amount of NIK co-immunoprecipitates with the IKK complex in melanoma compared to melanocytes (Dhawan & Richmond, 2002). Constitutive activation of the IKK complex has been identified in melanoma (Yang & Richmond, 2001), explaining the enhanced expression of NF-κB-regulated chemokines linked to melanoma growth (Balentien et al., 1991; Devalaraja et al., 1999; Lev et al., 2003; Wang et al., 2000). Since NIK can activate the IKK complex, we asked whether NIK may also contribute to the tumorigenesis of melanoma. In the study described herein we establish the functional consequence of NIK up-regulation in melanoma by showing that NIK contributes to melanoma growth and modulates the expression of pro-survival factors, such as BCL2 and survivin. Interestingly, while deficiency of NIK decreases activation of the non-canonical NF-κB pathway, it does not attenuate canonical NF-κB activity. However, NIK depletion reduces occupancy of β-catenin on the survivin promoter, resulting in reduced survivin expression and enhanced tumor cell apoptosis. These data suggest NIK may be a promising therapeutic target for melanoma.

Materials and Methods

Cell culture

Melanoma cells were cultured in DMEM/F12 containing 10% FBS at 37°C with 95% air/5% CO_2 in a water jacketed incubator. Normal human epidermal melanocytes were obtained from Vanderbilt Skin Research Core and cultured in Media 254 containing supplements for melanocytes (Cascade Biologics S-002-5) under the same conditions. Melanoma cell lines were obtained from ATCC and were tested for mycoplasma monthly.

Generating NIK knock down cell lines

Melanoma cells (Hs294T or WM115) were infected with lentivirus carrying NIK shRNA, (Sigma Mission shRNA pLKO.1_733 or_731) or IKKα shRNA (Sigma Mission shRNA

pLKO.1_508) and each shRNA vector also encoded a puromycin resistance marker. Non-silencing shRNA (ns) in pLKO vector containing shRNA insert that does not target human genes was used as a mock shRNA control. Forty-eight hours after infection, cells were cultured with puromycin (Sigma P9620) at a concentration of 2ug/ml to select knock-down cells.

Cytokine array

Non-silencing or knock-down cells were cultured for 24 h in equal volumes of serum free DMEM/F12 to obtain conditioned media. Cytokine array (RayBioTech: AAH-CYT-G2000-8) was performed according to the user manual using 50μl of collected conditioned media. Serum- free media was used as a control to determine the background level of cytokines/chemokines.

BrdU incorporation

BrdU incorporation was performed using the BrdU staining kit (BD Biosciences 559619). Briefly, cells were cultured to 70-80% confluency, and incubated with BrdU for 4 h in serum containing media. Following BrdU incorporation, cells were fixed, permeabilized, stained with FITC-conjugated anti-BrdU antibody, according to the manufacturer's protocol and analyzed by flow cytometry (Beckman Coulter, CA).

Annexin V staining

Non-silencing and knock-down cells were plated at equal density and treated with TNFα (1000ng/ml) (PeproTech 300-01A) where indicated. After 8h of TNFα treatment, the Annexin V staining assay was performed as described in the user manual (TACS Annexin V 4830-01-K R&D Systems) then samples were subjected to flow cytometry analysis (Beckman Coulter, CA).

Tumorigenic study in mice

Non-silencing control or NIK knock down WM115 cells (3×10^6 per mouse) were subcutaneously injected into athymic-Foxn1^{nu} nude mice (Harlan Laboratories) and allowed to grow until palpable tumors developed (about two to three weeks). Tumors were measured every two days with a micro-caliper. Three weeks from the day of initial tumor detection (or when tumors reached 1.5 cm in one dimension), mice were sacrificed and final tumor volumes were measured by HBSS volume displacement. All experiments performed with mice were according to the guidelines approved by Vanderbilt University School of Medicine Animal Care and Use Committee.

Statistical analysis

Non-parametric statistical analyses (Mann-Whitney or ANOVA) were performed using the Graphpad Prism software (La Jolla, CA). Statistically significant differences are assumed if the p-value ≤ 0.05.

Supplemental Methods

Methods for Western blot, nuclear and cytoplasmic extraction, IKK kinase assay, qRT-PCR, chromatin immunoprecipitation (ChIP), cell cycle analysis, immunofluorescence staining of tissue microarray, immunohistochemistry and immunofluorescence staining are described in Supplementary Materials.

Results

To determine whether NIK levels are elevated in melanoma, we examined NIK expression in tissue microarray of nevi (n=30), dysplastic nevi (n=22), primary melanoma (n=15) and metastatic melanoma (n=13) lesions by immunofluorescence staining. Intriguingly, NIK expression is higher in dysplastic nevi and in primary and metastatic melanoma tissues compared to benign nevi ($p<0.05$) (Figures 1A and B). Likewise, Western blot analysis on lysates of 10 human melanoma patient tumors and 4 melanoma cell lines (Hs294T, SKMel5, SKMel28, WM115) showed that NIK protein expression was elevated in some melanoma tissues (M2, M5, M6, M7) and all melanoma cell lines compared to normal human epidermal melanocytes (NHEM) (Figures 1C and D). Nevertheless, NIK mRNA expression in melanoma cells was not significantly up-regulated in melanoma (Figure S1A), suggesting that NIK over-expression may be due to protein stability.

To understand the importance of NIK over-expression in melanoma tumorigenesis, NIK was knocked down in two melanoma cell lines, Hs294T (derived from a metastatic melanoma) and WM115 (derived from a primary melanoma) with NIK targeting shRNA. Western blot and quantitative RT-PCR (qRT-PCR) analyses showed that NIK expression was efficiently reduced in Hs294T and WM115 (Figures 1E and F). However, NIK knock-down was gradually lost within the polyclonal population of Hs294T and WM115 cells selected to stably express the shRNA (Figure 1G).

Generation of clonal and polyclonal NIK knock-down cells

To prevent the growth selection against NIK depleted cells in polyclonal cultures, a clonal population of NIK deficient WM115 cells was selected by in-cell-Western assay. The efficiency of the NIK silencing in WM115 clones was confirmed by Western blot after treatment with the proteasome inhibitor (MG132) which inhibits the degradation of NIK (Figure S1B). This NIK depleted WM115 clone was used to examine the functional significance of NIK in melanoma cells. In some experiments, polyclonal NIK knock-down WM115 cells [shNIK1(p)] and NIK knock-down Hs294T cells were included to further confirm the effects of NIK depletion.

NIK deficiency in melanoma cells decreased proliferation, increased apoptosis and decreased cell cycle progression

Consistent with phenotype of compromised growth, NIK deficient Hs294T and WM115 cells exhibited a significant lag in growth compared to non-silencing control cells (Figure 2A). Both Ki67 and BrdU staining suggest that the percentage of cells undergoing proliferation was lower in knock-down populations than in the non-silencing controls (Figures 2B and C). This phenotype was confirmed in WM115 with another shRNA

targeting NIK (shNIK2) (Figure S1C) and in another melanoma cell line, Hs294T (Figure 2B).

To determine whether NIK deficiency affects apoptosis, Annexin V and propidium iodide (PI) staining were performed. For these experiments, only polyclonal NIK knock-down cells in early passages were used, as we reasoned that the clonal knock-down cell line had already developed a mechanism to maintain equilibrium between survival and death. At a basal level, the percentage of apoptotic cells was two-fold higher in the knock-down population than in the control (Figure 2D, white bars). Interestingly, knocking down IKKα in WM115 did not induce apoptosis (Figure S1D), suggesting that NIK-mediated apoptosis is not likely to result from decreased IKKα activity. It has been documented that NF-κB activation and NIK activity protect cells from TNFα-induced cell death (Malinin et al., 1997; Van Antwerp et al., 1998; Wang et al., 1996). Consistent with previous studies, we observed that TNFα treatment markedly increased the percentage of apoptotic cells in NIK deficient population (Figure 2D, dark bars). In addition, NIK knock down WM115 cells exhibited a delay in cell cycle progression compared to the control, most prominent at 12h after the release from thymidine block. At 12h after release, non-silencing cells had completed the cell cycle whereas, a significant percentage of NIK depleted cells were still S and G2/M phases (Figure 2E).

NIK depletion in WM115 cells reduced xenograft tumor growth in nude mice

In agreement with *in vitro* results, depletion of NIK significantly decreased melanoma tumor growth and burden in nude mice ($p<0.05$) (Figures 3A and B). NIK deficiency in tumor samples was confirmed by both Western blot and qRT-PCR analyses (Figures 3C and D). Immunostaining of Ki67 or cleaved-caspase 3 was lower in NIK knock-down tumor sections than non-silencing control tumor sections ($p<0.05$) (Figures 3E and F). These data suggest that tumors from NIK knock-down WM115 grew and turned over slower than control tumors. In summary, NIK deficiency substantially attenuated melanoma tumor growth of WM115 cells both *in vitro* and *in vivo*.

NIK depletion altered expression of genes important for melanoma tumor growth

To elucidate potential mechanisms by which depletion of NIK contributed to reduced tumorigenicity of melanoma cells, the global gene expression profile of polyclonal and clonal NIK knock-down WM115 cells as compared to the non-silencing control was examined by gene expression microarray analyses. While results of these experiments suggested clear differences in the expression of many genes, the differences were not statistically significant when stringent analyses ($p<0.05$) were applied, possibly due to the variations between polyclonal and clonal knock-down populations. However, using less stringent criteria for analysis ($p<0.15$), several genes that play a crucial role in tumor growth or progression (*CXCR4*, *c-MYC*, *c-MET*, *CCND2*, *LIN28B*) (Pastorino et al., 2003; Puri et al., 2007; Scala et al., 2006; Scala et al., 2005; Viswanathan et al., 2009) were markedly reduced in NIK knock-down cells (Table 1). NIK knock-down also up-regulated expression of genes such as XIAP-associated factor 1 (*XAF1*), which plays a negative role in cancer cell survival (Plenchette et al., 2007). Similar trends of gene expression were observed with control and NIK depleted melanoma tumor samples (Table 1). Follow up analysis of

selected genes by qRT-PCR revealed statistically significant major changes in expression for several genes identified by microarray analyses (fold changes relative to the non-silencing control): *c-MYC*(-3.39), *c-MET*(-4.30), *CXCR4*(-620.35), *CCND2*(-58.08), *XAF1*(5.78) and *LIN28B*(-3.2×10^7) with $p<0.05$ for all genes) (Table 1). Taken together, our data demonstrate that NIK depletion altered the expression of several genes crucial for tumor growth.

NIK depletion in WM115 did not reduce the canonical NF-κB activation

Interestingly, both IKK kinase activity and nuclear phospho-p65 (Ser536) suggest that the canonical NF-κB activity was not diminished by NIK knock-down under basal or TNFα stimulated conditions (Figures 4A and B). Similarly, the basal level of some NF-κB-regulated chemokines and cytokines (IL-6, IL-8, CCL2 and CXCL1) in the conditioned media from either WM115 clonal or polyclonal knock-down cells were not consistently reduced between these two cell lines as compared to the control (Figures 4C and D). Above data suggest that NIK depletion did not significantly diminish the canonical NF-κB activation in WM115 melanoma cells.

NIK depletion decreased the non-canonical NF-κB activation

In contrast to the canonical NF-κB activation, depletion of NIK in WM115 cells reduced the basal processing of p100 to p52 and nuclear localization of p52 (Figures 4E and F). NIK deficient cells still responded to the ligation through LTβR although to the less extent (Figure 4F). Knock-down of NIK in another melanoma cell line, Hs294T, resulted in a similar phenotype (Figures 4E and F).

To determine whether NIK deficiency in melanoma cells exhibited expected changes in the gene expression profile associated with NIK activity, we compared the gene expression from NIK knock-down WM115 cells to the gene expression pattern from lymphoma, where cIAP2-MALT1 fusion protein induces NIK activation (Rosebeck et al., 2011). In agreement with their observations, genes (such as *TLR4*, *IL1β*, *PTX3*) up-regulated in cIAP2-MALT1 negative tumors (NIK low) were increased in NIK deficient melanoma, whereas genes (such as *HMOX1*, *PIM2*, *CXCR4*) up-regulated in cIAP2-MALT1 positive tumors (NIK high) were decreased in NIK deficient cells compared to the control ($p<0.05$ except *PTX3*) (Figure 4G). Based on above data, we conclude that knocking-down NIK reduced the non-canonical NF-κB activation in melanoma cells.

NIK knock-down attenuated the expression of pro-survival factors

One of the mechanisms by which tumor cells escape apoptosis is by up-regulating the expression of survival factors. To understand the apoptotic phenotype of NIK depleted cells, protein expression of a panel of pro-survival factors was examined. Some anti-apoptotic proteins such as BCL-XL and cFLIP expression remained unchanged (Figure 5A). However, pro-survival proteins such as cIAP1, survivin, and BCL2 were markedly decreased in NIK knock-down WM115 cells (Figure 5A). Depletion of NIK in Hs294T cells also diminished survivin protein levels (Figure 5A). qRT-PCR demonstrates that there is no difference in *cIAP1* mRNA in NIK-depleted melanoma cells, though both *BCL2* and *survivin* mRNA levels were significantly diminished (Figure 5B). While NIK depletion may affect cIAP1 at

a post-translational level, the above data suggest that NIK regulates pro-survival proteins BCL2 and survivin at the transcriptional level. A similar decrease in protein expression and mRNA levels of pro-survival factors was observed in *Nik*$^{-/-}$ MEFs, suggesting that NIK regulates the expression of these genes and the regulation is not melanoma specific (Figures 5C and D).

NIK modulated melanoma tumorigenesis by regulating β-catenin activity

Although NIK knock-down WM115 cells exhibited decreased non-canonical NF-κB activity, this pathway has not been directly linked to the expression of pro-survival proteins such as survivin. Therefore, we postulated that NIK modulates the expression of survivin through an alternative pathway. Firestein *et. al.* have identified NIK (MAP3K14) as one of the kinases that regulates β-catenin activation in colorectal cancer cells (Firestein et al., 2008). To explore the possibility that NIK may modulate β-catenin mediated transcription, we examined the mRNA expression of *AXIN2* and *TCF7*, two genes typically targeted by β-catenin, in both NIK knock-down melanoma cells and *Nik*$^{-/-}$ MEFs by qRT-PCR. Data show that deficiency of NIK reduced expression of these two β-catenin regulated genes (Figures 6A and B). In addition, microarray analysis confirmed by qRT-PCR of NIK knock-down melanoma cells shows significant reduction of β-catenin targets including c-*MYC*, *c-MET* and *CCND2*, further supporting the concept that NIK can modulate β-catenin activity (Table 1) (Boon et al., 2002; Cole et al., 2010; He et al., 1998).

In addition, the expression of pro-survival factors such as survivin can be transcriptionally regulated by β-catenin (Zhang et al., 2001). Thus, we determined whether NIK modulates survivin expression through affecting β-catenin transcriptional activity using ChIP analysis to monitor the presence of β-catenin at the survivin promoter. Both NIK knock-down melanoma cells and *Nik*$^{-/-}$ MEFs exhibited a decreased β-catenin occupancy at the promoter of the survivin gene (Figures 6C and D), supporting the notion that NIK regulates survivin expression, and possibly other β-catenin targets such as c-*MYC*, *c-MET* and *CCND2*, through β-catenin activity.

To understand how NIK may affect β-catenin activity, nuclear and cytoplasmic extraction and immunofluorescence staining of β-catenin was performed in NIK knock-down melanoma cells and *Nik*$^{-/-}$ MEFs. The ratio of nuclear to cytoplasmic pool of β-catenin is less in NIK deficient cells as shown by both analyses (Figures 6E and F), implying that NIK regulates sub-cellular localization of β-catenin.

Discussion

In this study, we demonstrate for the first time that NIK regulates melanoma tumor cell growth and survival. We also demonstrate that the mechanism for this effect on tumor growth involves NIK regulation of expression of pro-survival genes in part through modulation of β-catenin transcriptional activity. NIK depletion decreases proliferation and increases apoptosis in melanoma cells *in vitro* and sensitizes melanoma cells to TNFα-induced apoptosis. NIK knock-down cells also exhibit a defect in cell cycle progression. Although the canonical NF-κB pathway is not decreased in NIK knock-down cells, pro-survival proteins such as BCL2, cIAP1 and survivin are diminished, suggesting that the

canonical NF-κB pathway lacks the cyto-protective function in response to TNFα when pro-survival effectors are absent. Other studies have also reported that cell death can be induced by TNFα in the presence of NF-κB activation when other survival signals such as those of cIAPs are disrupted in cancer cells (Petersen et al., 2007; Varfolomeev et al., 2007; Vince et al., 2007).

Consistent with *in vitro* data, the melanoma tumor xenograft data show that NIK silencing markedly decreases tumor cell proliferation (based upon Ki67 staining) and melanoma tumor growth. Despite the significant decrease in tumor volume, NIK deficient tumors showed diminished cleaved caspase 3 staining, suggesting the following possibilities: 1) cancer cells undergoing apoptosis were already eliminated by the time tumors were harvested; or 2) the turn-over rate of melanoma cells in the rapidly growing control tumors was faster due to the limited nutrients in the microenvironment. The delay in cell cycle observed in NIK-depleted cells also likely contributes to the decreased tumor growth. Overall, NIK depletion markedly decreases the net melanoma tumor growth.

Similarly, studies of multiple myeloma, T-cell leukemia, and lymphoma suggest that NIK mediates proliferation and survival and as a result, depleting NIK in these cancers decreased tumor growth (Annunziata et al., 2007; Conze et al., 2010; Demchenko et al., 2010; Keats et al., 2007; Saitoh et al., 2008). Consistent with the previous literature, our data clearly demonstrate that NIK contributes to melanoma tumor growth. However, which downstream effectors of NIK regulate tumorigenesis has remained unclear. Here, we demonstrate that NIK regulates expression of genes that are pro-tumorigenic and pro-survival.

Gene expression data from NIK deficient melanoma cells suggest that NIK modulates the expression of *CXCR4, c-MET, c-MYC, LIN28B* and *XAF1*, all of which are important for melanoma and/or tumor malignancy. For instance, high CXCR4 expression predicts poor prognosis in malignant melanoma (Scala et al., 2005). The c-MET tyrosine kinase receptor and the c-MYC transcription factor stimulate survival and proliferation (Lai et al., 2009; Ruggero, 2009). It was reported that both of these genes are amplified in metastatic melanoma patient tumors (Moore et al., 2008). Specifically, both c-MET and c-MYC are proposed to be targets for melanoma therapy (Pastorino et al., 2003; Puri et al., 2007). Depletion of NIK significantly diminishes the expression of *CXCR4, c-MET* and *c-MYC* mRNA. In addition XAF1, a negative regulator of cell survival that counteracts the function of the survival factor XIAP (Liston et al., 2001), is significantly decreased in melanoma (Ng et al., 2004), but is increased at the mRNA level in NIK depleted WM115 cells. Remarkably, a potent tumor promoter, LIN28B, is down-regulated in NIK knock-down WM115 cells. LIN28B de-represses oncogenes such as K-RAS and c-MYC by regulating the let-7 miRNA family (Viswanathan et al., 2009). Given the important role of the RAS/RAF/MAPK pathway in melanoma, suppression of LIN28B in NIK knock-down tumor cells is likely biologically relevant. Since changes in *CXCR4, c-MET, c-MYC, LIN28B* and *XAF1* observed in cultured NIK knock-down melanoma cells were retained in NIK knock-down tumors, our data indicate that these genes contribute to tumorigenicity. Based on its ability to regulate the expression of genes important for melanoma progression, we propose that NIK is a promising target for melanoma therapy.

Up-regulation of survival factors is one of the mechanisms by which cancer cells resist apoptosis (Baldwin, 2001). Previous studies suggest that pro-survival proteins such as BCL2, cIAP1 and survivin contribute to tumorigenesis of melanoma. As a result, functional disruption of these proteins impairs melanoma tumor cell growth or survival (Grossman et al., 2001; Hilmi et al., 2008; Lecis et al.). Consistent with these findings, BCL2, cIAP1 and survivin levels were markedly decreased in NIK deficient melanoma cells. Yet, the canonical NF-κB transcriptional activity, which typically regulates the expression of survival proteins (Baldwin, 2001), is not diminished in NIK deficient melanoma cells, in agreement with the genetic data (Yin et al., 2001). The activity of the non-canonical pathway is down-regulated in NIK deficient melanoma cells, possibly contributing to downstream effects of NIK on melanoma growth. However, the non-canonical NF-κB pathway has not been directly linked to the expression of genes such as survivin. Interestingly, *Nik*$^{-/-}$ MEFs which also do not exhibit a defect in the canonical NF-κB activation (Yin et al., 2001), have decreased survivin expression. Thus, we propose that decreased pro-survival factor expression in NIK depleted cells is attributed to a non-NF-κB function of NIK. Thus NIK, like IKKα and IKKβ, appears to have NF-κB-independent functions (Chariot, 2009).

Here our data suggest that NIK regulates β-catenin activity, a novel mechanism by which NIK mediates cancer progression. Down-regulation of β-catenin transcriptional targets (*c-MYC, CCND2, c-MET, AXIN2, TCF7, and survivin*) in NIK deficient cells supports the notion that NIK mediates β-catenin transcriptional activity. Specifically, NIK depletion reduces β-catenin occupancy at the promoter of survivin, diminishing the expression of survivin. β-catenin is possibly recruited to the survivin promoter by TCF4 (Kim et al., 2003). Interestingly, several studies have reported that β-catenin activity contributes to melanoma tumor growth (Delmas et al., 2007; Rubinfeld et al., 1997; Sinnberg et al., 2010).

Although NIK knock down cells exhibited a reduction in β-catenin mediated transcription of genes important for melanoma tumorigenesis, it is important to note that NIK depletion does not affect all of the transcriptional targets of β-catenin. For example, *Mitf*, a transcriptional target of β-catenin, plays an important role in melanoma development by regulating genes such as BCL2 (Cheli et al., 2010). However, a difference in *Mitf* gene expression was not detected in our microarray analysis, suggesting that decreased BCL2 expression detected in NIK deficient cells may be regulated by transcription factors other than *Mitf*.

The specific mechanisms by which NIK activates β-catenin are yet to be determined. One possible mechanism is that NIK modulates the phosphorylation of regulators of β-catenin, such as GSK3β. Alternatively, NIK may directly or indirectly regulate the nuclear transport of β-catenin, based upon the finding that NIK depleted cells exhibited reduced nuclear β-catenin. Although we did not examine in detail the plasma membrane localization of β-catenin in this study, it is possible that the localization is altered in NIK deficient cells and this change may contribute to NIK-mediated tumorigenesis. The membrane cadherins/β-catenin complex plays a crucial role in invasiveness of melanoma by mediating homotypic or heterotypic cell adhesions (McGary et al., 2002).

NIK may also affect other signaling pathways that mediate melanoma gene expression and growth. Our group has demonstrated that ERK phosphorylation was reduced by over-expression of dominant negative NIK in melanoma cells (Dhawan & Richmond, 2002) suggesting that this MAP3K (NIK) can alter the MAPK pathway, which is essential for melanoma growth and survival. Therefore, NIK may modulate melanoma progression by influencing other signaling pathways in addition to the ones mentioned above.

Recently, significant progress has been made in melanoma therapy for those patients that exhibit BRAF mutation (Dhomen & Marais, 2009). However, it is imperative to also increase our understanding of other pathways that are involved in melanoma growth so that therapies for patients that cannot be effectively treated with $BRAF^{V600E}$ inhibitors can be developed. Due to its non-essential role in the canonical NF-κB activation and its ability to regulate survival factors, NIK is an attractive target to induce melanoma cell death without substantially reducing NF-κB activity, which is important for normal cellular processes. Moreover, since both NIK and β-catenin are involved in other cancers such as multiple myeloma and colon cancer, it may be a good therapeutic target for a number of malignancies (Annunziata et al., 2007; Keats et al., 2007; Malbon, 2005). However, since NIK depleted melanoma cells may eventually escape apoptosis as observed in our studies, combination of NIK inhibitors with other therapies should be carefully considered when NIK is used as a targeted therapy in cancer patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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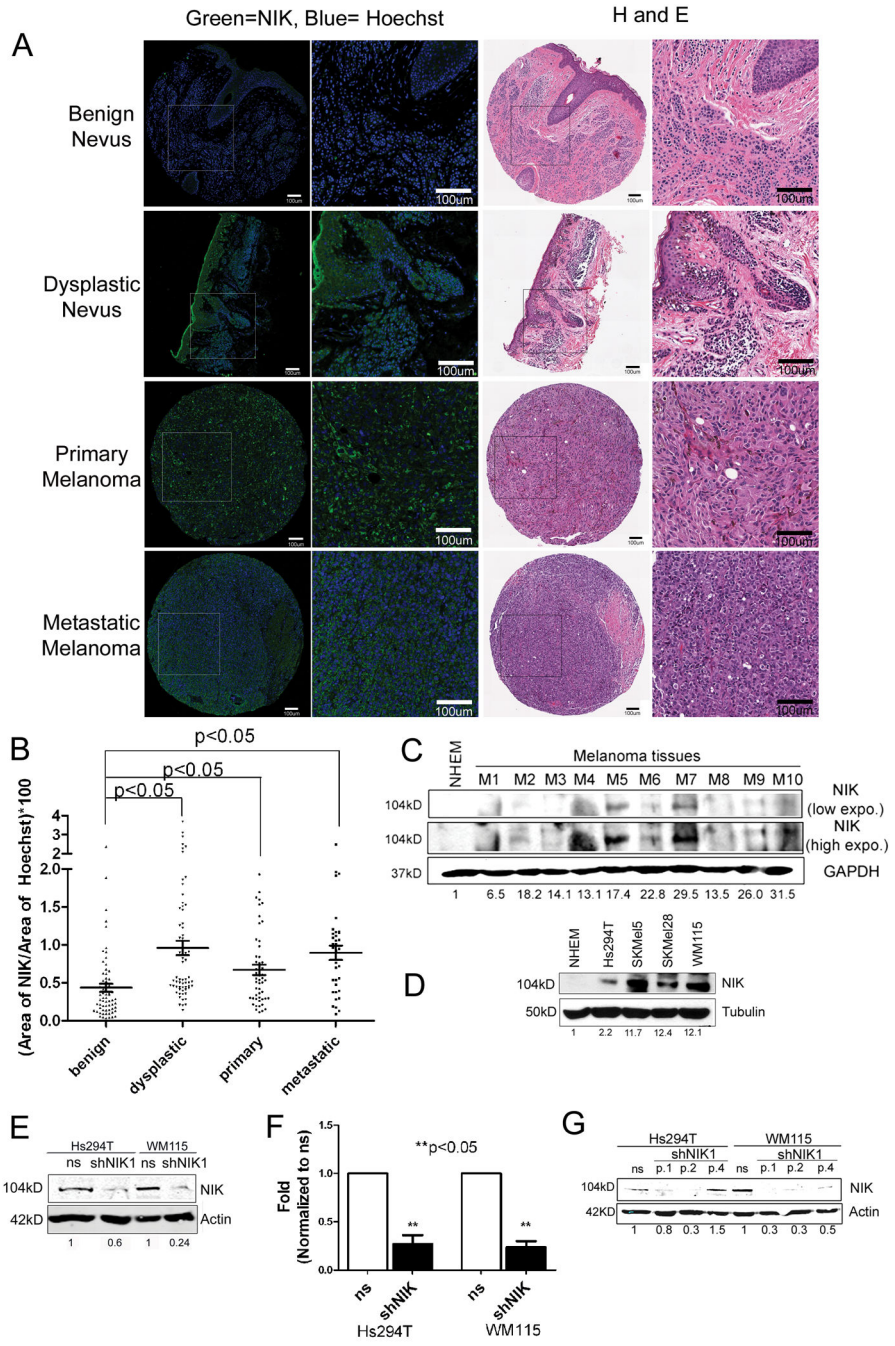

Figure 1.

NIK expression is up-regulated in melanoma tissues and cells. **A.** Left to right: first column: Immunofluorescent analysis of NIK expression in benign (n=30), and dysplastic (n=22) nevi, primary (n=15) and metastatic (n=13) melanoma tissue samples from tissue microarray (TMA). NIK positive cells are green, based upon Alexa 488 anti-rabbit antibody recognition of NIK antibody. Nuclei are blue based upon Hoechst stain. Second column: Enlargement of area denoted by box in the adjacent left panel showing fine cellular details of the histology of the lesions. Third column: H&E stain of the tissues shown in the first column. Fourth

column: Enlargement of area denoted by box in the adjacent left panel showing fine cellular details of the histology of the lesions. [Scale bar =100μm] **B.** Quantitation of NIK staining from TMA, based upon scans of images from duplicate or triplicate core samples of each lesion using the Ariol SL-50 imaging system (Genetix) calculated as described in Supplementary Materials and Methods: (area of NIK staining/area of Hoechst) ×100. Statistical significance was determined by ANOVA, where $p<0.05$ indicates significance. **C and D.** Western blot analysis of NIK expression in melanoma tissues (**C**) and cell lines (Hs294T, SKMel5, SKMel28, WM115) (**D**) compared to normal human epidermal melanocytes (NHEM). Densitometric scans from triplicate assays were quantitated, normalized to the loading control and calculated as fold difference from NHEM cells. **E.** Western blot and **F.** qRT-PCR analysis of NIK expression in NIK knock-down cells compared to the non-silencing (ns) control in Hs294T and WM115. Ns shRNA is a mock shRNA with the same vector backbone as the NIK shRNA. **G.** Western blot analysis of NIK in different passages of Hs294T_shNIK1 and WM115_shNIK1 cells (p=passage). Densitometric scans from triplicate assays were quantitated, normalized to the loading control and calculated as fold difference from ns.

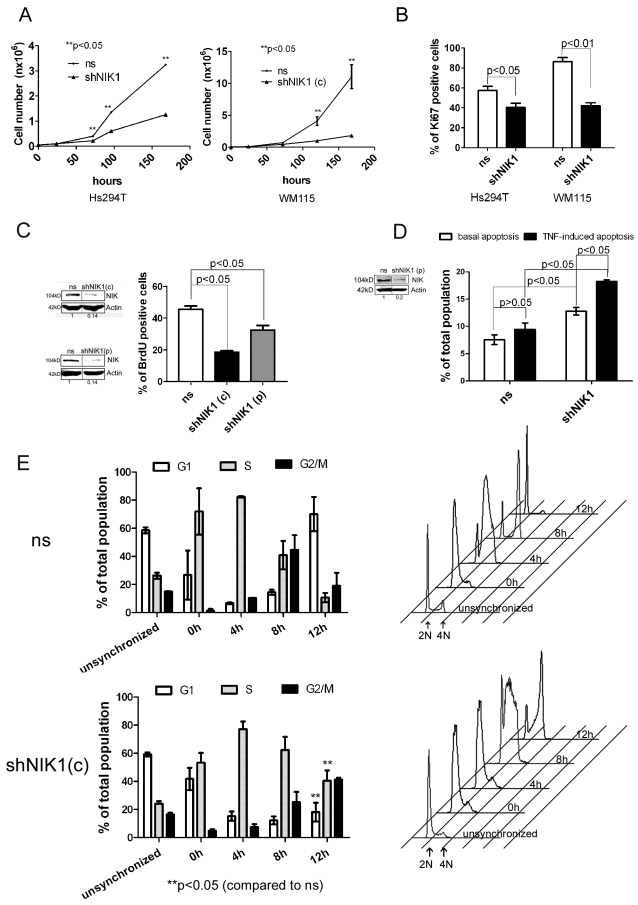

Figure 2.

Depletion of NIK decreases growth and survival of melanoma cells. **A.** Growth curves of NIK knock-down and non-silencing Hs294T and WM115 cells. Error bars are ± S.E.M. In Hs294T graph, S.E.M.<0.02. (n=9) **B.** Quantification of Ki67 staining in NIK deficient Hs294T or WM115 cells compared to the non-silencing control. Error bars are ± S.E.M. (n=10) **C.** BrdU incorporation analysis of NIK knock-down WM115 cells compared to WM115 with non-silencing control. Error bars are ± S.E.M. (n=7) **D.** Apoptosis analysis of basal and TNFα-treated NIK knock-down WM115 compared to the control. Percentage of apoptosis is defined by % of cells that are Annexin V^+ and Annexin V^+ PI^+. Error bars are ± S.E.M. (n=9) In Figures C and D, insets are Western blot showing the efficiency of NIK knock-down. **E.** Cell cycle progression of non-silencing shRNA control and NIK knock-down WM115 cells. Left panel: Percentage of unsynchronized and synchronized (thymidine block, 16h) cells in each phase of cell cycle. Error bars are ± S.E.M. (n=3) Right panel: Time course graphs showing the progression of cell cycle in ns and NIK depleted WM115 populations. Times indicated (0h, 4h, 8h, 12h) were hours after the release from the thymidine block of 16h. Scales for number of non-silencing control WM115 cells: unsynchronized: 0-4500, 0h: 0-1600, 4h: 0-1000, 8h: 0-2500, 12h: 0-5000. Scales for number of clonal shNIK1(c) WM115 cells: unsynchronized: 0-4000, 0h: 0-2200, 4h: 0-1200, 8h: 0-600, 12h: 0-1500.

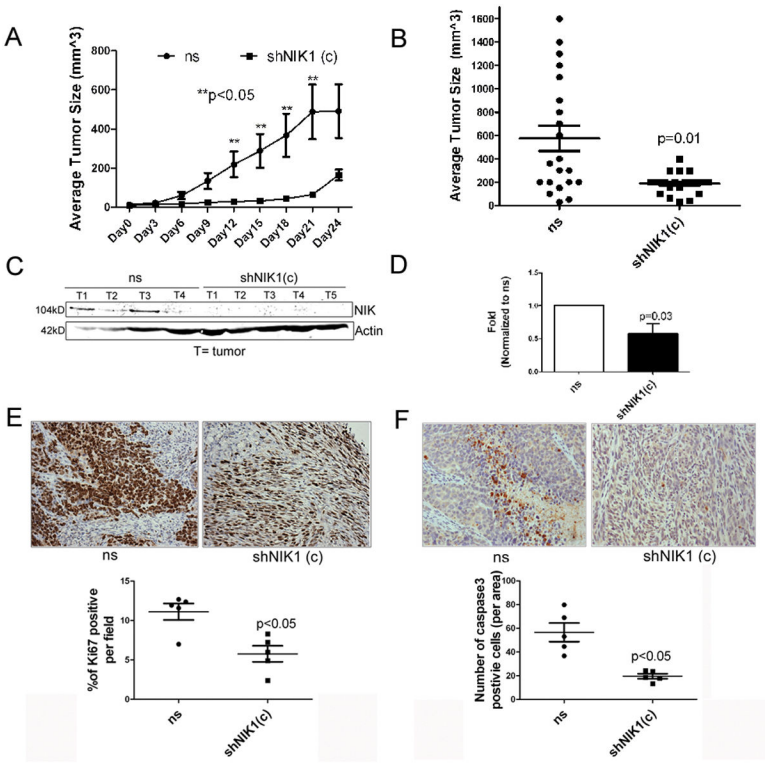

Figure 3.

NIK depletion significantly reduces tumor burden in a melanoma xenograft model of NIK knock-down WM115 [shNIK1 (c)] and non-silencing WM115. **A.** Tumor growth over 24 days. **B.** size of tumors at the 24 day end point. Error bars are ± S.E.M. (ns n=21 and shNIK1(c) n=22). **C.** Western blot and **D.** qRT-PCR analyses of NIK in tumor samples. Fold changes in Figure D were calculated by normalizing to the loading control and comparing it to ns. **E.** Ki67 (proliferation marker) and **F.** cleaved caspase-3 (apoptosis marker) staining of non-silencing control WM115 tumors compared to WM115 with NIK knock-down. Error bars are ± S.E.M. (n=5). Statistical significance was determined by Mann-Whitney test, where $p<0.05$ indicates significance.

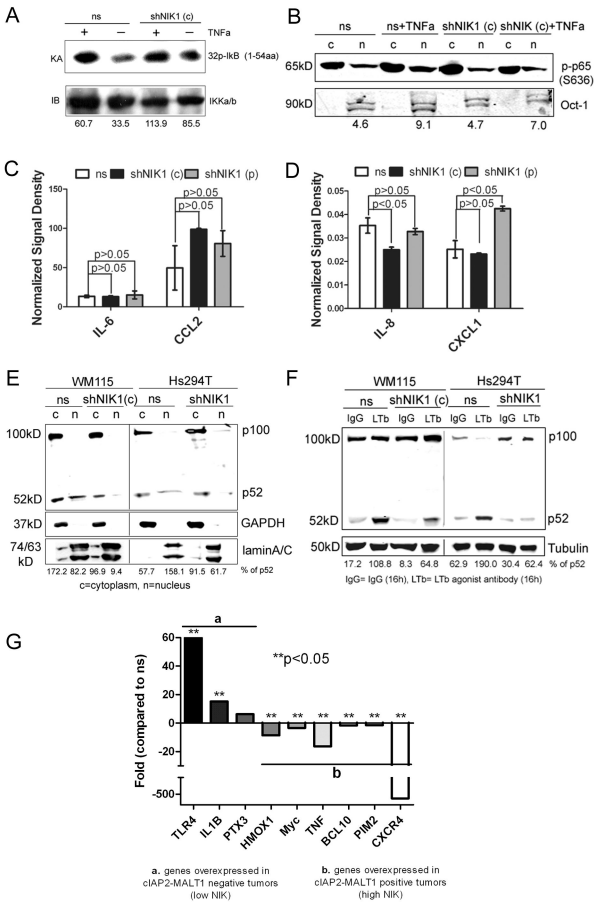

Figure 4.

Knocking-down NIK in WM115 melanoma reduces the non-canonical but not the canonical NF-κB activation. **A.** Basal IKK activity in NIK knock-down WM115 compared to WM115 non-silencing control in an *in vitro* kinase assay. Densitometric scans from duplicate assays were quantitated and normalized to the total immunoprecipitated IKKs. **B.** Nuclear p-p65 in NIK knock-down WM115 compared to the non-silencing control (c=cytoplasm, n=nucleus). Densitometric scans from four assays were quantitated and normalized to the loading control. TNFα-treatment was used as a positive control in both A and B. **C** and **D.** Cytokine array data from conditioned media of NIK knock-down cells (clonal and polyclonal) compared to the non-silencing control. Signal intensity for each cytokine was normalized to the internal positive control. Error bars are ± S.E.M. (n=4) Statistical significance was determined by Mann-Whitney test, where $p<0.05$ indicates significance. **E.** Nuclear and cytoplasmic localization of p52 in non-silencing and NIK knock-down WM115 and Hs294T (c=cytoplasm, n=nucleus). Densitometric scans from triplicate assays were quantitated, normalized to the loading control, and calculated as fold difference from ns. **F.** The proteolytic processing of p100 to p52 in non-silencing and NIK knock-down WM115 and Hs294T. Cells were treated with LTβR agonist antibody or IgG (2μg/ml) for 16h. Densitometric scans from triplicate assays were quantitated, normalized to the loading control, and calculated as fold difference from ns. **G.** qRT-PCR analysis of gene expression

in NIK deficient WM115 cells normalized to the control, compared to study by Rosebeck et al., 2011.

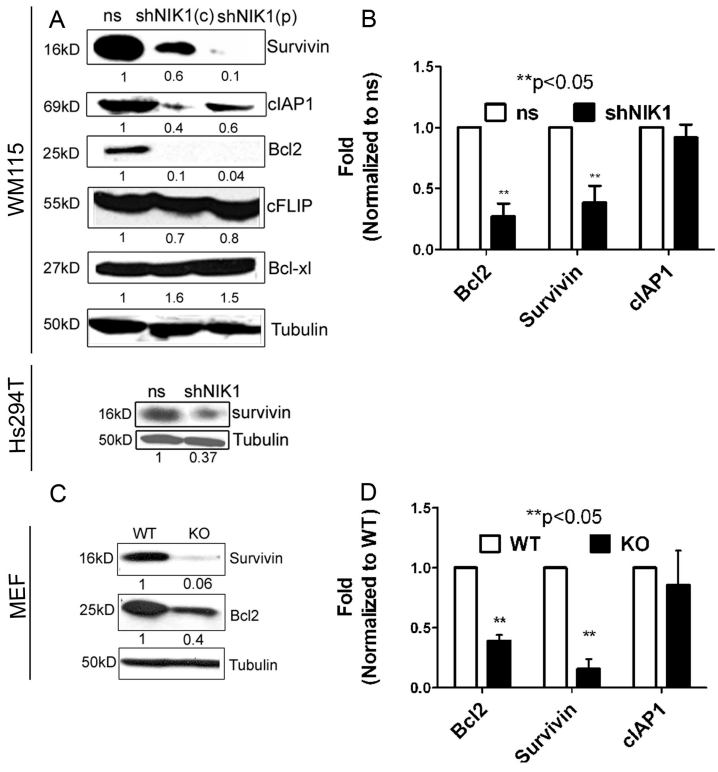

Figure 5.

NIK modulates the expression of pro-survival factors. **A.** Protein expression of survival factors such as BCL2, cIAP1 and survivin in NIK knock-down WM115 (both clonal and polyclonal) compared to the non-silencing control. Similar to WM115, Hs294T cells with NIK knock-down exhibited decreased protein expression of survivin. Densitometric scans from triplicate assays were quantitated, normalized to the loading control, and calculated as fold difference from ns. **B.** mRNA levels (qRT-PCR) of BCL2, survivin and cIAP1 in NIK depleted and non-silencing WM115 cells. Fold changes were calculated by normalizing to the loading control and comparing it to ns. **C.** Western blot and **D.** qRT-PCR of BCL2 and survivin in *Nik*$^{+/+}$ and *Nik*$^{-/-}$ MEFs. In Figure C, densitometric scans from triplicate assays were quantitated, normalized to the loading control, and calculated as fold difference from WT. In Figure D, fold changes were calculated by normalizing to the loading control and comparing it to WT.

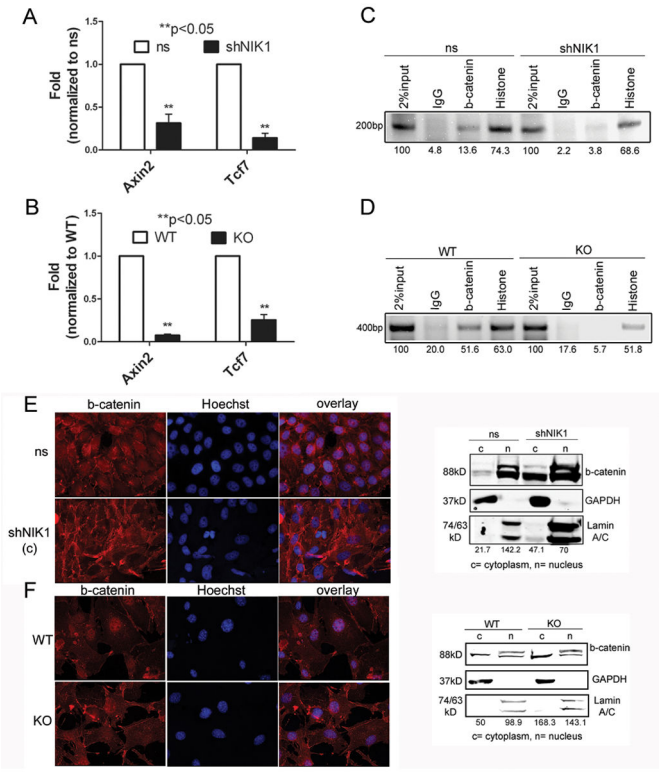

Figure 6.

NIK regulates survivin expression through β-catenin activity. **A and B.** Expression of traditional β-catenin transcriptional targets such as *AXIN2* and *TCF7* in NIK knock-down WM115 (**A**) and *Nik*$^{-/-}$ MEFs (**B**). Fold changes were calculated by normalizing to the loading control and comparing it to ns or WT. **C and D.** ChIP analysis using β-catenin antibody and primers flanking the survivin promoter exhibited decreased β-catenin occupancy at the survivin promoter in NIK knock-down WM115 (**C**) and *Nik*$^{-/-}$ MEFs (**D**). Amount of DNA bound to IgG, β-catenin or Histone was calculated as percentage of the 2% input. **E and F.** Immunofluorescent staining and Western blot analyses showing sub-cellular localization of β-catenin in NIK knock-down WM115 (**E**) and *Nik*$^{-/-}$ MEFs (**F**). Images for immunofluorescence staining were taken using a 63× oil lens and pseudo-colored. Densitometric scans of Western blot from triplicate assays were quantitated and normalized to the loading control.

Table 1

Microarray data from NIK depleted cells and tumors confirmed by qRT-PCR (normalized to non-silencing control) to examine gene expression changes which are indicative of less tumorigenecity compared to the control

	Microarray (cells)	p-value	Microarray (tumors)	p-value	qRT-PCR	p-value
c-Myc	-3.90	0.14	-2.20	0.06	-3.39	p<0.05
c-Met	-3.72	0.12	-6.80	0.002	-4.30	p<0.05
CXCR4	-14.50	0.08	-5.80	0.01	-620.35	p<0.05
CCND2	-9.60	0.13	-1.04	0.92	-58.08	p<0.05
Xaf1	3.78	0.17	3.65	0.01	5.78	p<0.05
CD36	7.80	0.07	1.70	0.003	5.11	p<0.05
TSC22D3	-10.40	0.04	-7.30	0.02	-33.05	p<0.05
TRIM22	5.30	0.15	1.03	0.73	5.26	p<0.05
ZNF711	-11.20	0.06	-7.10	0.003	-28.57	p<0.05
LIN28B	-18.02	0.09	-5.20	0.02	-32436417	p<0.05