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Intersectin 1 is required for neuroblastoma tumorigenesis

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Abstract

Intersectin 1 (ITSN1) is a scaffold protein that regulates diverse cellular pathways including endocytosis and several signal transduction pathways including phosphotidylinositol 3-kinase, Class IIβ (PI3K-C2β). ITSN1's transforming potential *in vitro* suggests that this scaffold protein may be involved in human tumorigenesis. Herein, we demonstrate that ITSN1 is expressed in primary human neuroblastoma tumors and tumor cell lines and is necessary for their *in vitro* and *in vivo* tumorigenic properties. Silencing ITSN1 dramatically inhibits the anchorage independent growth of tumor cells *in vitro* and tumor formation in xenograft assays independent of MYCN status. Overexpression of the ITSN1 target, PI3K-C2β, rescues the soft agar growth of ITSN1-silenced cells demonstrating the importance of the ITSN1-PI3K-C2β pathway in NB tumorigenesis. These findings represent the first demonstration that the ITSN1-PI3K-C2β pathway plays a requisite role in human cancer, specifically neuroblastomas.

Keywords

Scaffold protein; SH3 domains; EH domains; endocytosis; transformation; oncogenesis

Introduction

Neuroblastoma (NB) is a malignant tumor that derives from the neural crest and is responsible for 13% of cancer related deaths in children (1). Although the underlying molecular causes for NB development remains unresolved, a number of molecular changes are associated with tumor progression and disease prognosis including amplification and overexpression of the MYCN oncogene, increased activation of receptor tyrosine kinases such as ALK and TRKB, chromosome deletion, oncogene activation and tumor suppressor loss [reviewed in (2)]. The phosphoinositide 3-kinase (PI3K) family of lipid kinases has been identified as a potential target for NB tumor treatment (3, 4). Inhibition of PI3Ks by pan-inhibitors block malignant progression *in vivo* (3); however, these inhibitors blocks all PI3Ks causing serious toxic effects, thereby limiting their promise for use in NB treatment. The role of PI3Ks in NB tumorigenesis has been associated with their ability to stabilize the MYCN oncogenic transcription factor that is amplified in 25% of NB patients (5). Emerging

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data implicate different PI3K isoforms in neuroblastomas through stabilization of MYCN oncoprotein (3, 6, 7).

PI3Ks represent a family of lipid kinases that phosphorylate the 3′ position of phosphatidylinositol. This family consists of three distinct classes based on sequence homology. Class I PI3Ks have garnered significant attention due to the presence of specific activating mutations in human cancers resulting in the effort to develop specific Class 1A isoform-specific inhibitors as therapeutics (8). The role of Class II PI3Ks in human cancers is less clear due to the lack of identified mutations in this family of enzymes. However, PI3K Class IIβ (PI3K-C2β) expression is upregulated in a number of cancers (9-11) and overexpression of PI3K-C2β leads to *in vitro* transformation of colonic epithelia cells (12). Furthermore, PI3K-C2β regulates cell survival signaling both in adherent cells (13, 14) and in suspension (15) which may aid in the survival of metastatic cells once they migrate from the primary tumor site.

Intersectin 1 (ITSN1) is a multi-domain scaffold protein encoded by two major splice products. Both ITSN1-short (ITSN1-S) and ITSN1-long (ITSN1-L) possess two NH_2-terminal Eps15 homology (EH) domains followed by a coiled-coil (CC) domain and five Src homology 3 (SH3) domains. These domains allow ITSN1 to interact with a multitude of partners to regulate endocytosis as well as a variety of signal transduction pathways [reviewed in (16)]. ITSN1-L possesses an extended COOH-terminus encoding a guanine nucleotide exchange factor domain specific for Cdc42, a member of the Rho subfamily of Ras-like GTP-ases (17, 18). ITSN-L is specifically expressed in neurons whereas ITSN1-S is more widely expressed in multiple cell types. Overexpression of ITSN1 promotes transformation of rodent fibroblasts suggesting that this scaffold may play a role in cancer development (19, 20). Furthermore, ITSN1 regulates the compartmentalized activation of the Ras protooncogene further supporting a potential role for this scaffold in human tumorigenesis (21). We discovered that ITSN1 interacts with PI3K-C2β and regulates its activation in the N1E-115 mouse NB cell line (13). However, the role of this ITSN1-PI3K-C2β pathway in cancer has not been explored.

We previously demonstrated that ITSN1 silencing reduced survival of N1E-115 cells during differentiation and that overexpression of PI3K-C2β or AKT was sufficient to rescue survival of these ITSN1-silenced cells suggesting that the ITSN1-PI3K-C2β pathway plays an important role in cell survival during differentiation (13). However, the involvement of ITSN1 in NB tumorigenesis was not explored. Given ITSN's ability to induce transformation of rodent fibroblasts, we have examined whether the ITSN1-PI3K-C2β pathway regulates the tumorigenic properties of human NBs. Herein, we demonstrate that ITSN1-S and PI3K-C2β are expressed in primary human NB tumors as well as NB tumor cell lines and that silencing of ITSN1 in NB cell lines reduces their anchorage independent growth *in vitro* as well as tumor growth in a xenograft model *in vivo*. Finally, we demonstrate that overexpression of PI3K-C2β rescues the soft agar growth of ITSN1-silenced NB cells suggesting that PI3K-C2β functions downstream of ITSN1 to regulate the tumorigenic properties of NB cells.

Results

ITSN and PI3K-C2β are expressed in human NB cell lines and primary tumors

ITSN1 regulates survival during differentiation of mouse N1E-115 NB line through activation of PI3K-C2β (13). Although ITSN1 is expressed as both a long and short isoform (ITSN1-L and ITSN1-S, respectively) with the long isoform predominant in neurons (16, 22), ITSN1-S is sufficient to regulate this survival pathway in N1E-115 cells. Furthermore, ITSN1-S activates the Ras proto-oncogene in a compartmentalized specific manner (21). Given the importance of Ras in oncogenic transformation coupled with ITSN1's transforming activity in rodent fibroblasts *in vitro* (19, 20), we sought to examine whether ITSN1 might also play an important biological role in human NB tumors. Western blot analysis of human NB lines revealed significant expression of ITSN1-S in both MYCN-amplified (MYCN+; includes NLF, LAN-1, IMR-5, CHP-134) and non-amplified (MYCN-; includes NB-69, SH-SY5Y, SK-N-AS) cells (Fig. 1). Although ITSN1-S is the predominant isoform expressed in this panel of NB cells, ITSN1-L is expressed at significant levels in LAN2, LAN5, and IMR-32 cells (data not shown). Analysis of PI3K isoform expression revealed high levels of PI3K-C2β whereas the well-studied PI3K-C1α (p110) isoform was expressed at more varied levels (Fig. 1A). Since NB lines are invariably derived from high-grade tumors, we examined the expression of ITSN1 and PI3K-C2β in primary human NB tumor samples of differing clinical stages (Fig. 1B). Ten of the eleven tumors expressed levels of ITSN1-S equivalent to or higher than levels observed in CHP-134 NB cell line. The acute promyelocytic leukemia cell line HL-60 does not express ITSN1 and was included as a negative control for comparison.

ITSN1 regulates anchorage-independent growth of NB cells

Given ITSN1's expression in NB cells coupled with its transforming activity in rodent fibroblasts (19, 20), we examined the effect of ITSN1 silencing on the tumorigenic properties of both MYCN+ and MYCN- human NBs. Western blot analysis of polyclonal populations both CHP-134 (MYCN+) and SK-N-AS (MYCN-) lines stably infected with retrovirus encoding specific shRNAs revealed that two independent ITSN1-specific shRNAs (sh#1 and sh#2) reduced ITSN1 expression by >90% compared to control cell lines infected with virus generated from empty vector (pSR) or expressing a scrambled shRNA (pSCR) (Fig. 2A). Similar results were obtained with two additional NB lines, IMR-5 (MYCN+) and NLF (MYCN+) (Fig. S1A). ITSN1 silencing did not affect the proliferation or viability of these NB cells compared to controls (Fig. 2B and S1B). Although silencing ITSN1 in neurons and endothelial cells results in increased apoptosis (13, 23), we did not observe any increase in apoptosis ITSN1-silenced NB cell lines grown under anchorage-independent conditions (Fig. S1C). We next assessed whether ITSN1-silencing altered the anchorage-independent growth of the NB lines in soft agar assays. Strikingly, ITSN1-silencing reduced the anchorage-independent growth of NBs by >70% compared to control lines in three of the four lines examined (Fig. 3A&B and S2A&B). Furthermore, overexpression of ITSN1-S in parental NB cells enhanced soft agar growth by 2.5-fold compared to control cells (Fig. 3C-E). These data reveal that ITSN1 is an important component in the anchorage-independent growth of NB cells.

ITSN1-silencing did not alter the endocytic process

One function of ITSN1 is to regulate the process of endocytosis (24, 25). Given the link between endocytosis and oncogenesis (26, 27), we next tested whether the inhibition of soft agar growth of ITSN1-silenced NB cells was due to defects in endocytosis. Similar to our previous results in N1E-115 cells (13), ITSN1 depletion in human NBs did not alter transferrin internalization (Fig. 4).

ITSN-silencing reduced tumor growth in vivo

Based on the soft agar data, we next assessed whether ITSN1 was important for tumorigenic properties of NB cells *in vivo*. Although control lines (pSR and pSCR) for CHP-134, SK-N-AS, and IMR-5 readily formed tumors in a xenograft model, ITSN1-silenced subclones (sh#1 and sh#2) were significantly impaired in tumor growth (Figs. 5 and S3). However, ITSN1-silencing did not significantly impact the tumor growth of NLF cells (Fig. S3). To determine whether ITSN1 may become re-expressed in tumors that emerged in mice injected with ITSN1-silenced cells, we analyzed lysates of tumors from mice injected with the IMR-5 derived cells (Fig. S4). Interestingly, those tumors that emerged from injection of ITSN1-silenced cells appear to re-express ITSN1. These observations further support the notion that ITSN1 is indeed necessary for tumor growth *in vivo*. There did not appear to be any consistent changes in PI3K-C2β levels. In sum, ITSN1 silencing reduced the tumorigenic growth of 3 out of 4 human NB cell lines strongly suggesting that ITSN1 is important for the tumorigenic potential of NB cells.

PI3K-C2β overexpression rescues anchorage-independent growth of ITSN-silenced NB cells

PI3K-C2β binds ITSN1 and mediates survival elicited by ITSN1 during neuronal differentiation (13). Thus, we tested whether PI3K-C2β might also mediate ITSN1's role in human NB tumorigenesis. CFP-tagged PI3K-C2β was stably overexpressed in ITSN1-silenced cells (Fig. 6A) and the resulting stable cell line used in a soft agar assay to assess effects on anchorage-independent growth. Interestingly, CFP-PI3K-C2β overexpression rescued anchorage-independent growth of ITSN1-depleted NB cells (Fig. 6B). Consistent with the ITSN1 silencing results, overexpression of PI3K-C2β did not alter growth of adherent cells (Fig.6C).

Discussion

ITSN1 is highly expressed in the central nervous system with neurons expressing mainly ITSN1-L and little to no ITSN1-S. Interestingly, neuroblastoma tumors present the opposite pattern of expression where ITSN1-S predominates with ITSN1-L at much reduced levels. However, several of the tumors do express ITSN1-L with at least one of these tumors expressing similar levels of both isoforms (Fig. 1B). Given the ability of ITSN1 to induce oncogenic transformation (19, 20), these findings suggest that ITSN1 may play a role in NB tumorigenesis. Although oncogenic mutations have not been described for ITSN1, examination of gene expression databases reveals a potential involvement of ITSN1 in several cancers. The ONCOMINE™ database indicates that ITSN1 message is overexpressed in pancreatic, lung, liposarcomas, and Wilm's tumor. Several public databases

available through the Oncogenomic website (http://pob.abcc.ncifcrf.gov/cgi-bin/JK) indicate that ITSN1 message is overexpressed in NBs and that ITSN1 expression (either ITSN-S or ITSN-L) predicts for poor prognosis in high risk NB patients (28, 29). Indeed, our study demonstrates for the first time that ITSN1-S is required for anchorage-independent growth of NBs both *in vitro* in soft agar assays as well as *in vivo* in xenograft assays. Furthermore, recent reports demonstrate an important role for ITSN1 in the *in vivo* tumor growth of gliomas (30, 31). These findings coupled with our results suggest that ITSN1 may play a more general role in human tumorigenesis. Interestingly, neither ITSN1 silencing nor PI3K-C2β overexpression affects the growth of adherent cells or survival of cells in suspension indicating that the ITSN1-PI3K-C2β pathway may specifically regulate growth of tumor cells in suspension. These observations suggest that ITSN1 may be involved in the process of tumor colonization of distant organs.

Somatic mutations in Class 1 PI3K (PIK3CA) are found in a number of cancers while mutations in the remaining seven PI3K isoforms have not been identified thus far [reviewed in (32)]. PI3K-C2β overexpression, however, rescues anchorage-independent growth of ITSN1-depleted cells suggesting that PI3K-C2β may mediate ITSN1's role in tumorigenesis. Indeed, several lines of evidence suggest a role for PI3K-C2β in cancer. Single nucleotide polymorphisms in the promoter region of PI3K-C2β are associated with increased risk for prostate cancer suggesting enhanced expression of the protein in these cancers (33). Overexpression of PI3K-C2β in colonic epithelial cells results in oncogenic transformation (12). Furthermore, several microarray studies suggest that PI3K-C2β is upregulated in a variety of cancers including mixed lineage leukemias (MLL) (11), a subset of acute myeloid leukemias (9), and pancreatic cancers (10). As noted earlier, ITSN1 has also been reported to be overexpressed in pancreatic cancers suggesting that upregulation of the ITSN1-PI3K-C2β signaling pathway may play an important role in pancreatic cancers as well.

Although both ITSN1-S and ITSN1-L are expressed in primary NB tumors and NB cell lines, ITSN1-S is the predominant isoform in these samples. The reason for this difference in expression of the two isoforms is not clear. ITSN1-L possesses all the domains present in ITSN1-S in addition to an extended COOH-terminus encoding a GEF specific for Cdc42 resulting in unique functions for ITSN1-L vs ITSN1-S. Indeed, ITSN1-L is important in regulation of exocytosis through activation of Cdc42 (22, 34, 35). However, it is not clear that ITSN1-L possesses all the same activities as ITSN1-S [reviewed in (16)]. For example, the SH3 domains of ITSN1-L make contacts with the DH-PH region suggesting that these interaction may result in steric constraints that interfere with the SH3 domains binding to specific targets. We have not been successful in stably overexpressing ITSN1-L in human NB cells suggesting that elevated levels of ITSN1-L are not compatible with the growth of these cells. Given that ITSN1-L is highly expressed in neurons and poorly expressed in NBs, ITSN1-L may be more important for regulating neuronal differentiation versus oncogenic transformation.

ITSN1's role in NB tumorigenesis is linked to its ability to regulate signaling rather than due to its role in endocytosis. Although ITSN1 regulates clathrin- and caveolin-dependent internalization [reviewed in (16)], stable silencing of ITSN1 did not affect transferrin uptake consistent with previous findings (13). Furthermore, overexpression of PI3K-C2β was

sufficient to rescue anchorage-independent growth of ITSN1-silenced IMR-5 cells suggesting that the defect in tumor formation by ITSN1-silenced cells is due impairment in the PI3K-C2β signaling pathway. Indeed, PI3K activation in NBs enhances PKB/AKT activation leading to the stabilization of MYCN levels as a result of decreased GSK-3β phosphorylation of MYCN which leads to degradation of the oncoprotein (3). ITSN1 depletion results in reduced basal phosphorylation of AKT in neurons and in NB cells (13) (data not shown) suggesting a defect in PI3K pathway activation in these cells. ITSN1 also regulates the compartmentalized activation of Ras (21); however, the effects of ITSN1-Ras activation in human NB is not known. Finally, a number of RTKs such as TrkB and Alk play important roles in NB tumorigenesis (36-40). ITSN1 regulates RTK trafficking and PI3K-C2β associates with multiple RTKs (41, 42). Thus, future studies on the role of ITSN1-PI3K-C2β in RTK signaling in NBs may reveal new signaling pathways important for NB tumorigenesis. In conclusion, our findings reveal a novel role for both ITSN1 and PI3K-C2β in NB tumorigenesis providing new insight in the molecular signaling pathways underlying NB tumorigenesis.

Materials and Methods

Reagents and cell culture

The polyclonal rabbit anti-ITSN1 antibody has been previously described (42). Monoclonal antibody against human PI3K-C2β was purchased from BD Transduction Laboratories. Antibody to the p110 subunit of Class 1 PI3K (PI3K-C1α) was purchased from Cell Signaling Technologies. Antibody to β-actin were purchased from Sigma. All NB cell lines used in this study were maintained in RPMI with 10% fetal bovine serum at 37°C in 5% CO_2. and were the kind gifts of Drs. Bernard Weissman (University of North Carolina at Chapel Hill) and Naohiko Ikegaki (University of Illinois at Chicago). Puromycin (GIBCO) was used at 1μg/ml and G418 (GIBCO) was used at 200 μg/ml.

Stable silencing of ITSN1

Phoenix-GP cells were kindly provided by Dr. Gary Nolan (Stanford University). These packaging cell were transiently transfected using calcium phosphate method with 20 μg of vector alone (pSUPER.retro.puro; pSR) or pSR expressing shRNAs to ITSN1 (sh#1 or sh#2) along with a plasmid encoding the VSV-G envelope to generate viral particles. On the following day, the media was replaced with fresh media, and NB cells seeded for infection. On day 2 post-transfection, conditioned media from the Phoenix-GP cells was collected, filtered, and used to infect NB cells followed by selection in puromycin. Following selection, colonies were pooled to generate a polyclonal cell line which was used for all subsequent analyses. Western Blot analyses of polyclonal cell lines were performed as previously described (19). The sequences of oligonucleotides use to construct these vectors are as follows: sh#1A: 5′-gatccccggatatcagctaccctc tgcacttccttcaagagaggaagtgcagagggta gctgatatccttttt-3′ and sh#1B 5′-TCGATTTTTCCTATAGTCGATGGGAGACGTGAAGGAGAGAACTTCCTTCACGTC T CCCATCGACTATAGGCCC-3′; sh#2A 5′-gatccccgaaggcaagaactacta aatcaaagattcaagagatctttgatttagtagttcttgccttcttttt-3′ and sh#2B 5′-AAAAAGAAGGCAAGAACTACTAAATCAAAGATCTCTTG

AATCTTTGATTTAGTAGTTCTTGCCTTCGGGGATC-3′; pSCR-A-5′-gatccccgg tactaaagcgaatattattcaagagataatattcgctttagtacctttttt and pSCR-B 5′-AAAAAGGTACTAAAGCGAATATTATCTCTTGAATAATATTCG CTTTAGTACCGGGGATC.

Proliferation assay

NB cells (700 per well) were plated on 24-well plates in complete media (RPMI +10%FBS plus puromycin) for the indicated number of days. On the indicated day, media was removed and replaced with 100μl of complete media to which 100μl of CellTiter Glow (Promega) was added to the cells. Luminescence was quantified on a Dynex 96-well microtiter plate luminometer according to the manufacture's instructions.

Soft agar assay

Assays were performed essentially as described (43). Briefly, 5% (w/v) agar (DIFCO, Detroit MI) was prepared in distilled water then diluted to 0.5% final concentration with complete media and kept @ 60°C in water bath. A bottom layer of 0.5% agar was plated in each well of six-well plates to which cells were subsequently plated in a top layer of 0.37% agar cooled at 37°C. Samples were placed in humidified cell culture incubators at 37°C for 2-3 weeks after which colonies were stained using 100 μl solution of (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 2 mg/ml in H_2O).

Transferrin internalization assay

Cells (2×10^5 per well) were plated in 6-well plates. The following the day, cells were equilibrated in the presence of 2% BSA for 1h @ 37°C then incubated in the presence of Biotinylated transferrin for 15 min @ 37°C. Cells were washed with ice cold PBS, followed by an acid washed (0.2M NaCl/0.2M acetic acid) on ice for 8 min then lysed and analyzed by Western blot using horseradish peroxidase linked to streptavidin as previously described (13).

Apoptosis assays

Cells were seeded on Ultra-Low attachment plates (Corning) at a density of 0.5×10^6 cells per well. After 24 hrs, cells were collected, washed once with PBS and trypsinized (0.25%) for 10 min @ 37°C, washed again with PBS, centrifuged and resuspended in 1× Binding Buffer at a concentration of 1×10^6 cells/ml. 100 μl of the cell suspension (1×10^5 cells) were transferred into a 5 ml culture tube. 5 μl of FITC-conjugated-Annexin V and/or 1 μl of PI were added to the cells. The cells were then incubated for 15 min at RT (25 °C) in the dark followed by addition of 400 μl of 1× binding buffer to each tube. Apoptosis was then quantified by flow cytometry within one hour.

Xenograft tumor assays

Approximately 10×10^6 cells for each stable NB line were collected in resuspended in Matrigel (BD bioscience) at a 1:1 volume ratio in a total volume of 200 μl. This cell-Matrigel mixture was kept on ice then injected subcutaneously into the mouse flanks using a 25G syringe. Tumor growth was monitored by caliper every other day for 4 weeks or until

the tumors reached a diameter of 1 cm. Tumor volume was calculated using the formula ($d_1 \times d_2 \times d_3$) × π/6. Animals were then sacrificed and tumors extracted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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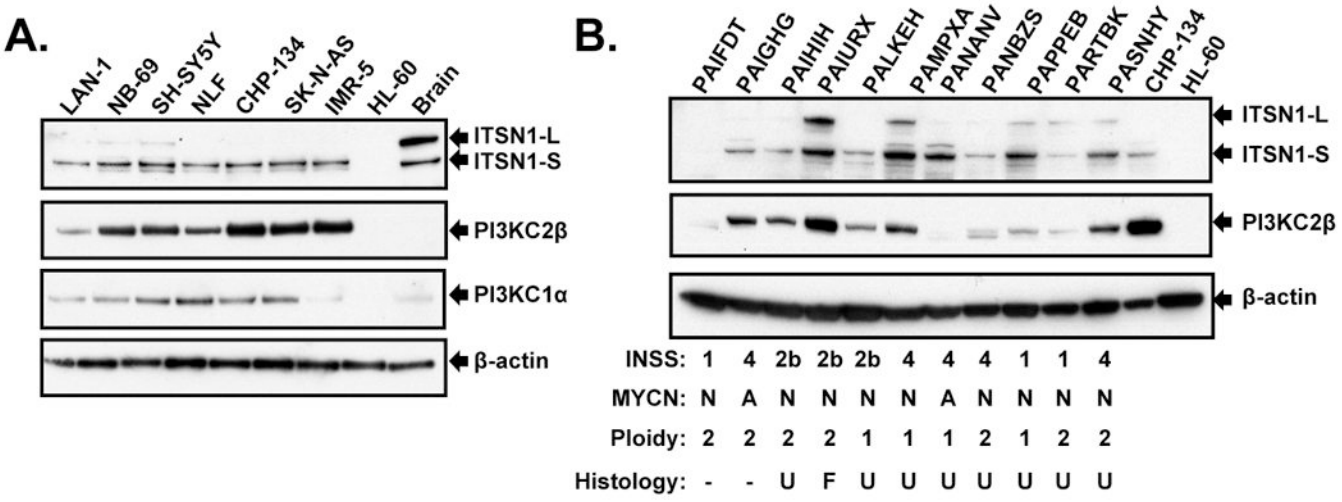

Figure 1. Expression of ITSN1 and PI3K isoforms in NB cells

A) Protein expression levels of ITSN1, PI3K-C2β and PI3K-C1α (p110) were assessed by Western blot analysis of the indicated NB cell lines. β-actin was used as loading control. ITSN1 was expressed in both MYCN+ (NLF, LAN-1, IMR-5, CHP-134) and MYCN- (NB60, SH-SY5Y, SK-N-AS [Fig. 2]) NB lines. B) Expression of ITSN1 and PI3K-C2β in primary human NB tumor samples was determined by Western blot. β-actin was used as loading control. Clinical information of each tumor is indicated below the panels. INSS, International neuroblastoma staging system; MYCN, denotes whether tumors have amplified (A) or non-amplified (N) the MYCN locus; Ploidy, ploidy status of tumor - 1, diploid; 2, hyperdiploid; Histology, Shimada histopathologic classification of tumor - U, unfavorable; -, missing.

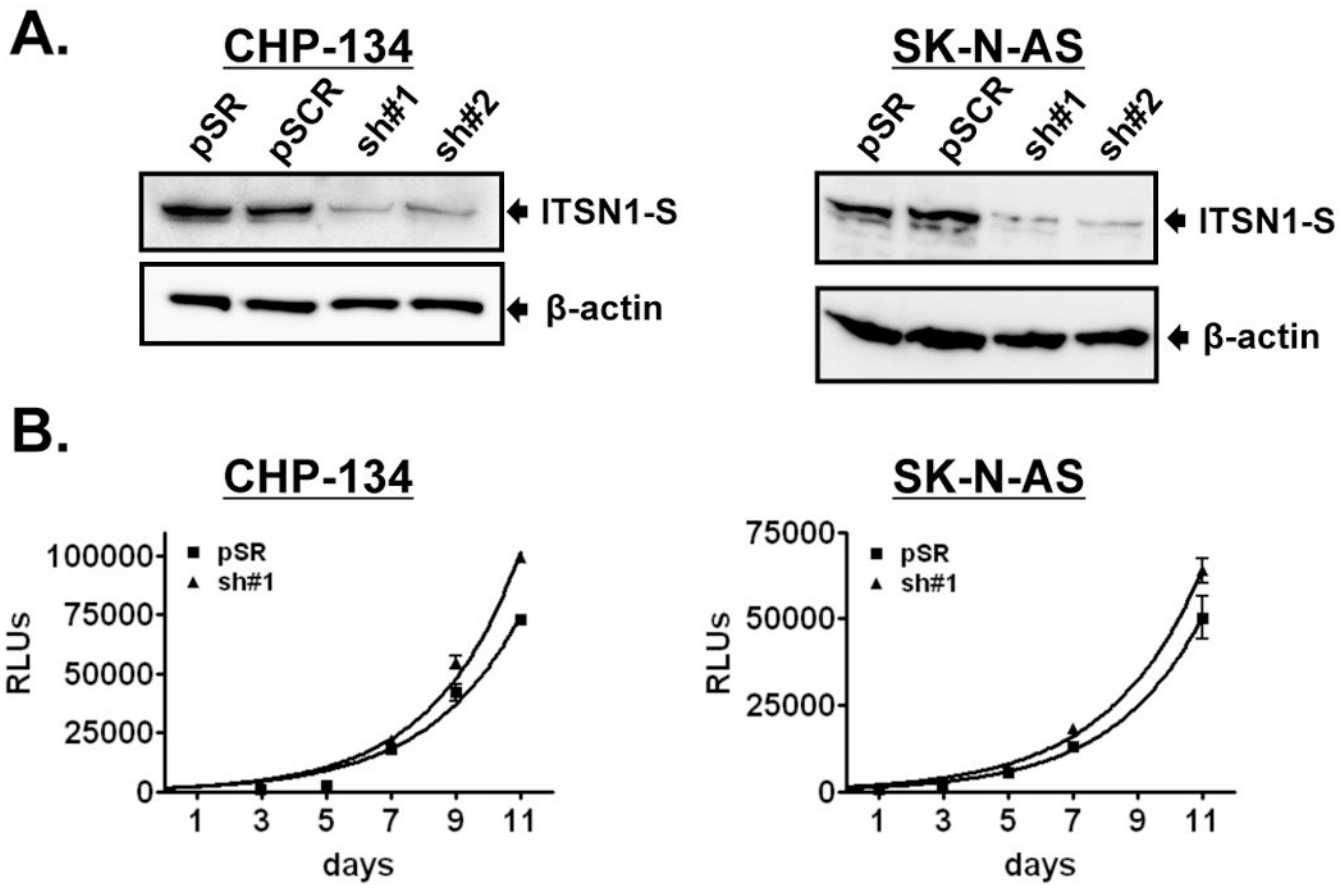

Figure 2. ITSN silencing does not alter adherent growth of NB cells

A) CHP-134 and SK-N-AS NB cells stably transfected with the indicated shRNAs were lysed and analyzed by Western blot to assess ITSN1 levels. B) Growth curves of ITSN1-depleted (sh#1) as well as control (pSR) CHP-134 and SK-N-AS cells were determined using Cell TiterGlo (Promega). Each point is the average of three independent wells of cells +/-standard deviation.

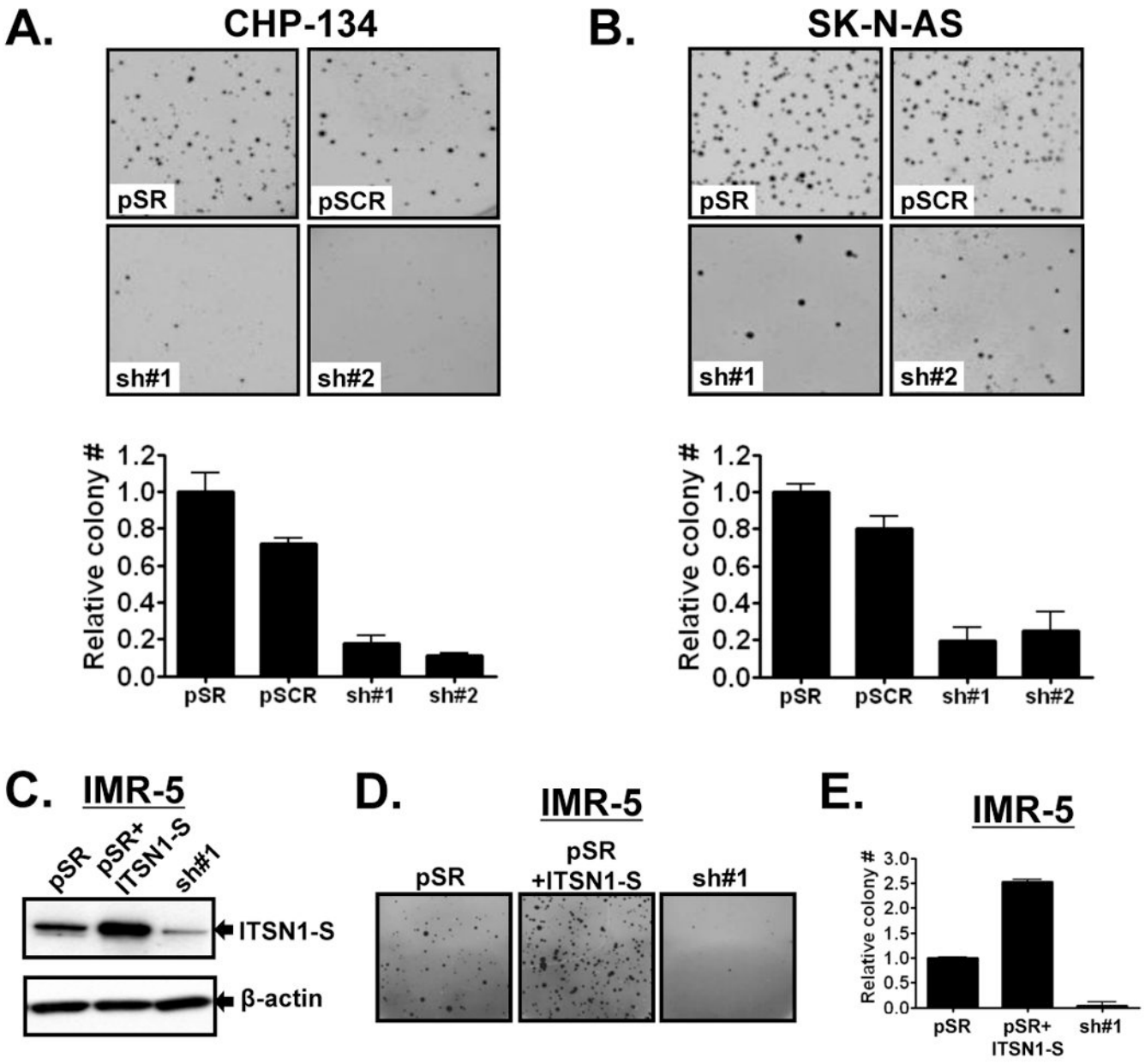

Figure 3. ITSN-silencing decreases anchorage-independent growth of NB cells

A-B) CHP-134 and SK-N-AS NB cells infected with indicated shRNAs were plated in triplicate in soft agar. Colonies were stained with MTT after 3 weeks of growth and then photographed using a digital scanner. B) Quantification of soft agar assays. Colonies were counted using the Image Quant LAS4010 (sensitivity set at 9498; size operator at 17; noise at 1) (GE Healthcare). Data represent the mean +/- standard deviation from the three repetitions. These experiments were repeated in different conditions and gave the same results. C) Western blot of ITSN1 levels in control (pSR), pSR and ITSN1 transfected (pSR +ITSN1-S), and ITSN1-silenced (sh#1) IMR-5 cells. D) Soft agar assay of cells from (C). E) Quantification of soft agar assay from (D). Results represent the average of three independent wells of cells +/- standard deviation.

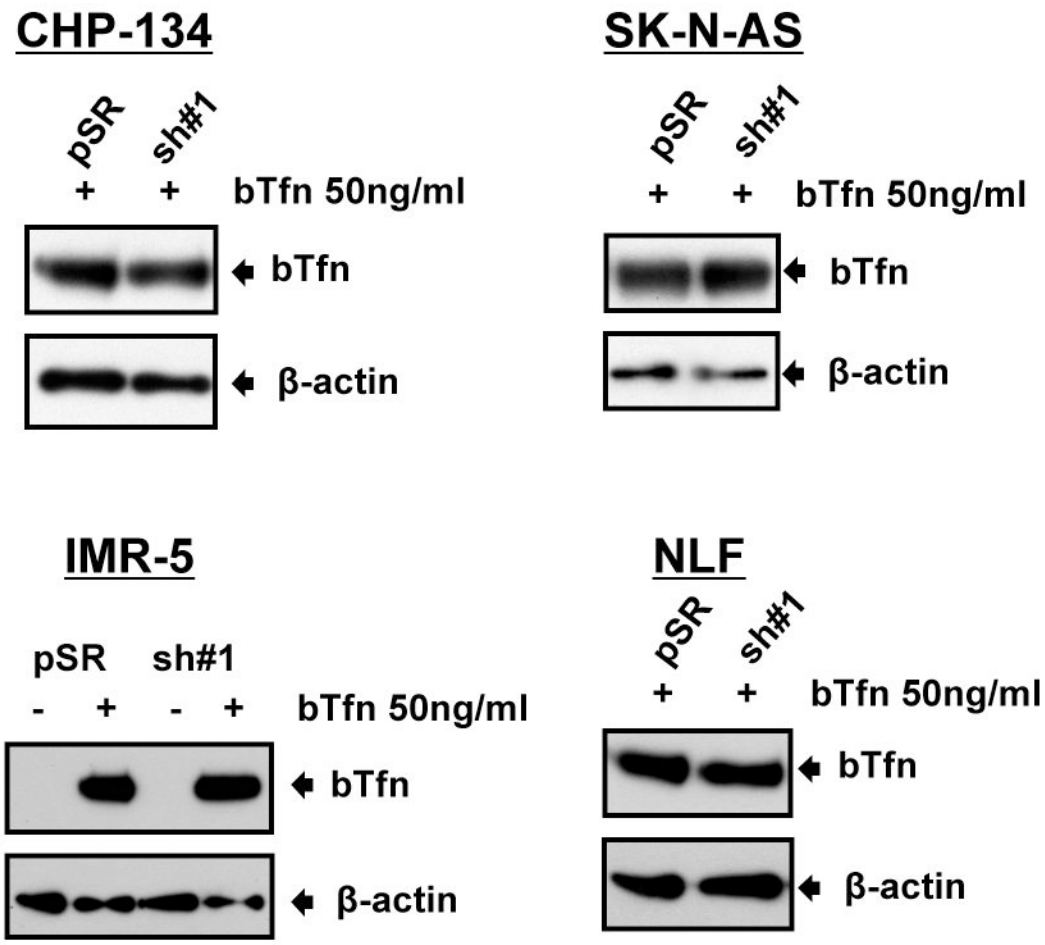

Figure 4. Stable ITSN1 silencing does not alter transferrin endocytosis in NB cells

Internalization of biotinylated transferrin (bTfn) was determined using four independent NB lines by Western blot of cell lysates using HRP-conjugated streptavidin. Actin is shown as a normalization control for loading.

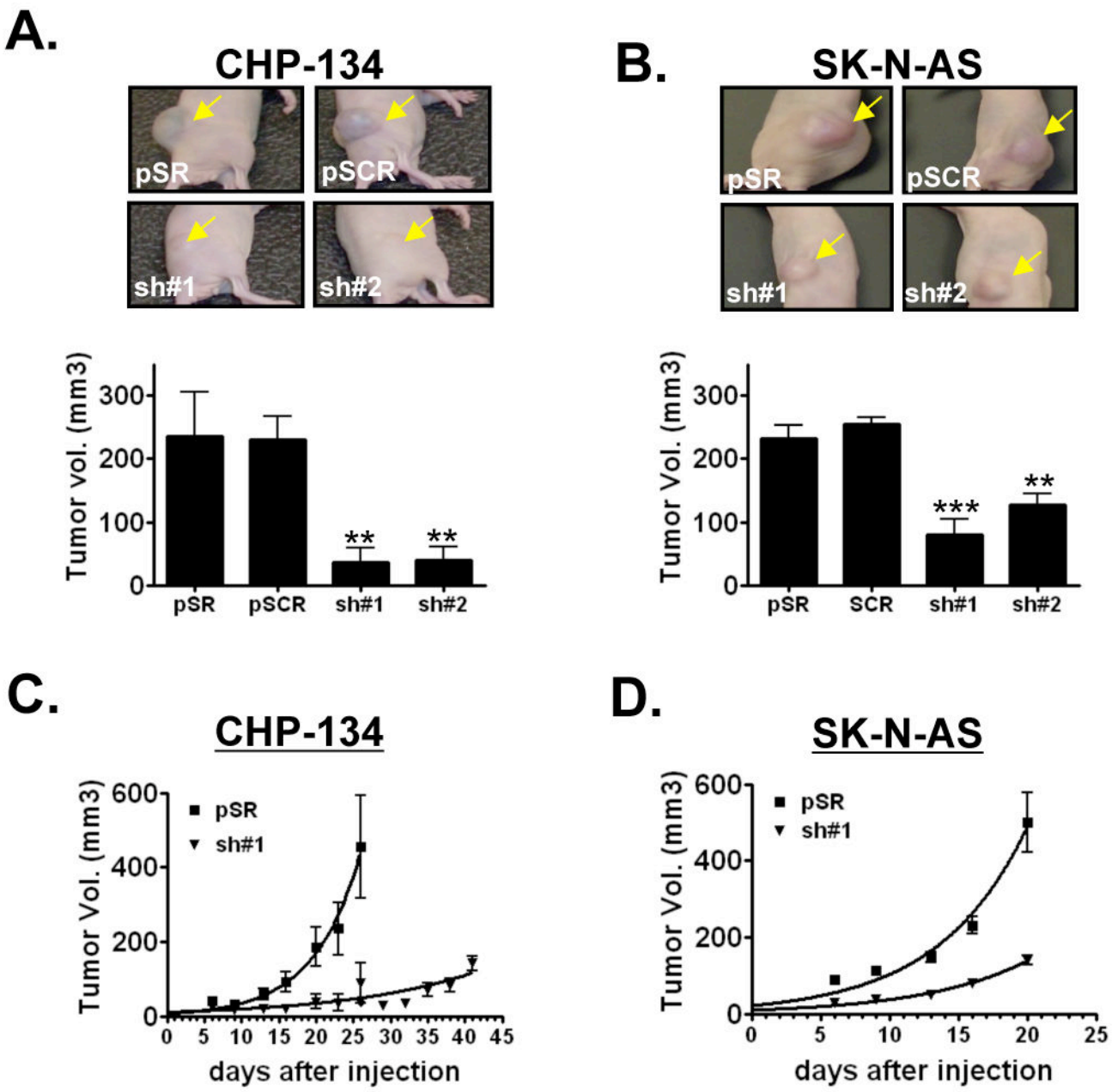

Figure 5. ITSN depletion results in reduced tumor growth *in vivo*

A-B) Xenograft assays. CHP-134 cells (A) or SK-N-AS cells (B) were injected into nude mice. Shown are tumors that formed in mice at 15 days post injection. a, pSR; b, pSCR; c, sh#1; d, sh#2. Grafts below pictures represent the mean tumor volume +/- standard error at day 15 post-injection. A two tailed Student's T test was applied to determine significance compared to pSR. For CHP-134 sh#1 p=0.004** and sh#2 p=0.004**; for SK-N-AS sh#1 p=0.0001*** and sh#2 p=0.004**. Differences between pSR and pSCR were not significant. C-D) Tumor growth for CHP-134 cells (C) and SK-N-AS cells (D) were monitored for the indicated times. Control mice were sacrificed at earlier times than ITSN1-depleted lines due to tumor volumes.

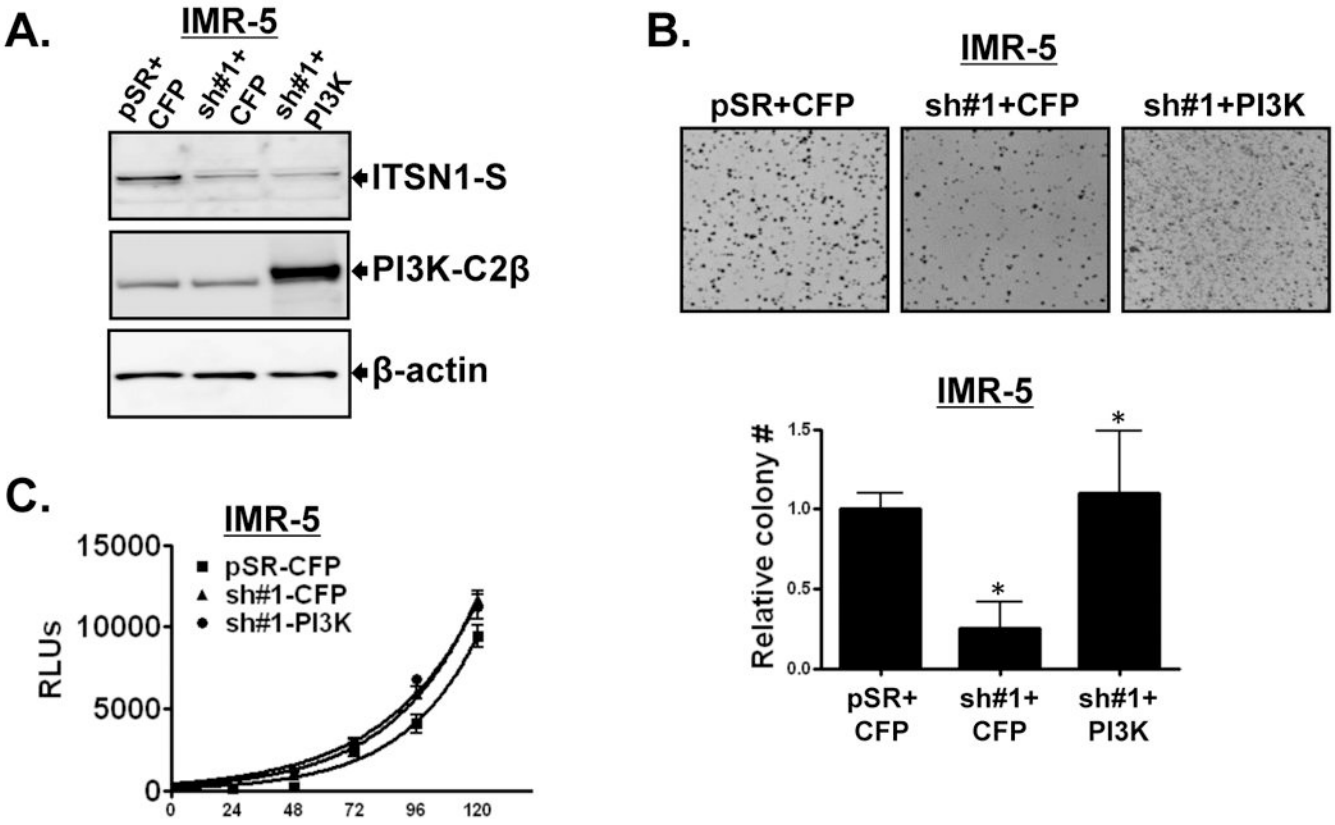

Figure 6. PI3K-C2β rescues anchorage-independent growth of ITSN1-silenced cells

A) Western blot analysis of IMR-5 cells transfected with the indicated plasmids was performed to determine expression of ITSN1 and PI3K-C2β. Actin was used as a loading control. pSR, empty vector; sh#2, ITSN1 silenced, PI3K, CFP-tagged PI3KC2β. B) Soft agar growth of cells from (A). Assays were performed as in Fig. 3. Quantification of the relative soft agar colonies is shown in the graph below. C) Growth curves of cell lines from (A). Each point is the average of three independent wells +/- standard deviation.