MycN is a transcriptional regulator of livin in neuroblastoma

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Abstract. Our previous studies have suggested that MycN may have a role in regulating livin expression in neuroblastoma. Here, we show that siRNA-mediated repression of MycN in neuroblastoma cells with both elevated MycN and livin expression resulted in significant downregulation of livin. Conversely, induction of MycN in neuroblastoma cells with low endogenous levels of MycN and livin protein upregulated livin expression. MycN directly associated with its regulatory motif (E-box) within the putative livin promoter. Based on these results, we hypothesize that MycN is required for livin expression and that livin may counteract the proapoptotic effects of MycN.

Introduction

MYCN amplification occurs in about 25% of neuroblastoma and is associated with advanced stage, rapid tumor progression and poor prognosis (1-4). MYCN gene amplification typically results in high levels of MycN protein expression (5,6). MycN is a member of the MYC family of helix-loop-helix leucine-zipper transcription factors and transactivates genes containing a consensus MycN promoter binding site (E-box, CACGTG) (7,8). Asymmetric sequence variants such as CATGTG also bind MycN (8). MycN acts primarily as a transcriptional activator, although it also downregulates some genes (9,10).

Paradoxically, MycN not only induces cell proliferation but also has proapoptotic effects. Enforced expression of MycN in neuroblastoma cells can accelerate cell cycle progression but can also destabilize the cells and enhance their sensitivity to some chemotherapeutic agents (11,12). Furthermore, it has been suggested that neuroblastoma cells can circumvent MycN-induced apoptosis by either deactivating pro-apoptotic pathways or activating pro-survival proteins (13). For example, Bcl-2 is expressed at high levels in some neuroblastoma tumors and is correlated with MYCN amplification (14). Additionally, constitutive expression of neurotrophins and their receptors in neuroblastoma cells may provide survival signals to counteract MYCN-induced apoptosis (15).

Livin (BIRC7) is a recently identified member of the Inhibitor-of-Apoptosis Protein (IAP) family which has an important role in regulating apoptosis (16,17). Livin is undetectable in most normal differentiated tissues, but is expressed at high levels in a variety of human malignancies including neuroblastoma (18-23). It has been suggested that high expression of livin in different tumor types is correlated with poor prognosis (20,24). Previously, we reported that high expression of livin in MYCN amplified neuroblastoma tumors was associated with exceptionally poor prognosis (18, and unpublished data). To further evaluate the relationship between MycN and livin, we investigated the role of MycN as a potential regulator of livin.

Materials and methods

Cell lines. Neuroblastoma cell lines were cultured in RPMI-1640 media (Invitrogen, Carlsbad, CA) supplemented with 10%, fetal bovine serum and 1% penicillin/streptomycin. The lines LA155N, IMR32 and LA15S are MYCN-amplified while SK-N-AS, SK-N-F1, SK-N-SH, SH-SY5Y and SH-EP are MYCN non-amplified lines. The Tet21N, MYCN-inducible SH-EP cells (gift from Manfred Schwab and Jason M. Shohet) were grown as described above but in the presence of tet-system approved FBS (Clontech, Palo Alto, CA) and supplemented with 2 mM L-glutamine. Tetracycline was added to a final concentration of 1 μg/ml to repress MYCN transcription.

Gene knockdown assay. MYCN expression in LA155N cells was blocked by siRNA (25-mer Stealth RNAi duplexes, Invitrogen Corporation). A set of three non-overlapping siRNA oligonucleotides were individually tested for maximal knockdown of gene expression. The sequence of the selected oligonucletide is: sense 5'-GAGAUGCUGCUUGAGAAC GAGCUGU-3'. Appropriate control siRNA (with similar G:C content) was used per manufacturer's recommendations. Cells cultured in 6-well plates were transfected with 100 nM of each of the siRNA duplexes using Lipofectamine 2000 reagent (Invitrogen) per manufacturer's recommendation. After 24-48 h, cells were harvested and analyzed by quantitative RT-PCR and immunoblotting.

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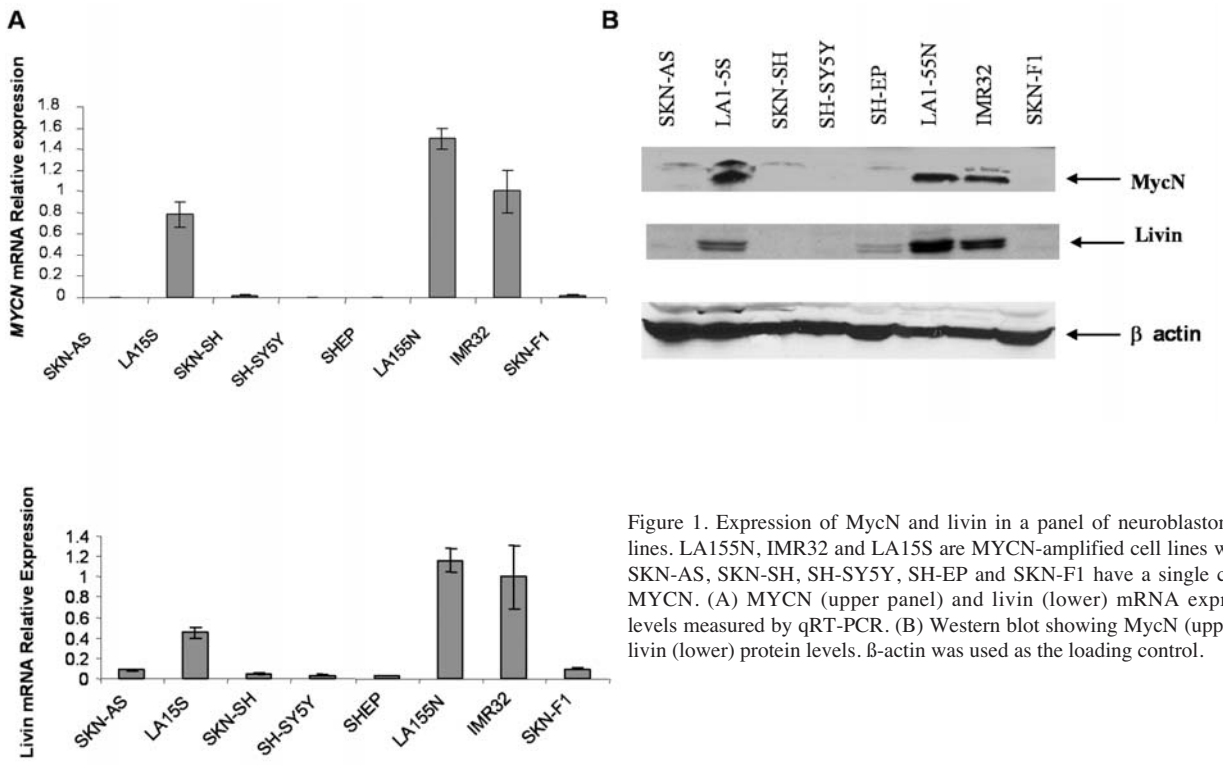


Figure 1. Expression of MycN and livin in a panel of neuroblastoma cell lines. LA155N, IMR32 and LA15S are MYCN-amplified cell lines whereas SKN-AS, SKN-SH, SH-SY5Y, SH-EP and SKN-F1 have a single copy of MYCN. (A) MYCN (upper panel) and livin (lower) mRNA expression levels measured by qRT-PCR. (B) Western blot showing MycN (upper) and livin (lower) protein levels. ß-actin was used as the loading control.

RNA extraction and quantitative-reverse transcription-polymerase chain reaction (qRT-PCR). RNA was extracted using RNeasy kit (Qiagen, Valencia, CA). Reverse transcription was performed using the Quantitect cDNA synthesis system (Qiagen) according to the manufacturer's recommendations. Quantitative RT-PCR for livin and MYCN mRNA was performed using validated primer sets (Qiagen) and SYBR Green PCR kit (Qiagen) on a 7500 real-time PCR instrument (Applied Biosystems. Foster City, CA). The livin primers did not discriminate between the two isoforms of livin. Results were normalized using GAPDH as an internal control. PCR amplification reactions included the following steps: enzyme activation at 95˚C for 15 min, followed by 40 cycles at 94˚C for 15 sec, 55˚C for 30 sec with the final extension at 72˚C for 34 sec. Gene expression was analyzed by relative-quantitation methods.

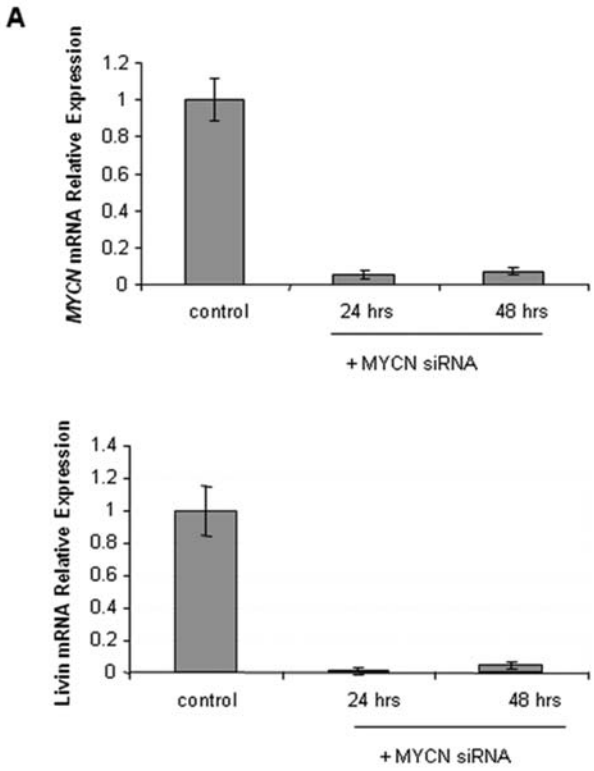
Western blot analysis. Cultured cells were homogenized in lysis buffer [20 mM Tris-HCl (pH 7.4), 200 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulphonyl fluoride, 1 μg/ml of leupeptin, 1 μg/ml aprotinin and 1 μg/ml pepstatin] for 1 h on ice. Protein concentrations were determined by Bradford Assay. Forty micrograms of total protein extracts were loaded per lane for SDS-PAGE and transferred to nitro-cellulose membranes. Immunoblots were probed with the following antibodies diluted at 1/500 for livin (IMG-4082, Imgenex, San Diego, CA), and 1/200 for MYCN (BD Biosciences, San Diego, CA). Mouse ß-actin (1:5000 dilutions, Sigma, MO) antibody was used as control. The primary antibody was detected with a 1:2000 dilution of either secondary anti-mouse or anti-rabbit IgG conjugated with horseradish peroxidase, and chemiluminescence was detected by the ECL reagent (Amersham Pharmacia Biotech, Piscataway, NJ).

Chromatin-immunoprecipitation (CHIP) assay. LA155N cells were grown in RPMI with 1% penicillin/streptomycin in the presence of 10% fetal bovine serum. The CHIP assay was performed following the EZ Chip Chromatin Immuno-precipitation protocol (Upstate, Temecula, CA). Immuno-precipitation was carried out with anti-MycN mouse (OP13) antibody (Calbiochem, Gibbstown, NJ), anti-RNA polymerase antibody (positive control, Upstate) and normal mouse antibody (negative control, Upstate). E-Box specific primers were forward 5'-CACATGTCCTTCCTTGAGCCTTCT-3' and reverse 5'-TTGCGGATGCCGCTGACT-3' (amplicon size 201 bp). The amplified products were analyzed by polyacrylamide gel electrophoresis.

Results

Livin expression positively correlates with MycN protein levels in neuroblastoma cell lines. In order to evaluate livin expression in neuroblastoma lines with different levels of MycN, we measured livin and MYCN mRNA and protein levels in eight lines by quantitative RT-PCR and immunoblot, respectively (Fig. 1A and B). Three cell lines (LA155N, LA15S and IMR32) expressing high MycN showed elevated livin mRNA as well as protein levels. In contrast, cell lines with either low or undetectable MycN expression showed low levels of livin expression.

Inhibition of MYCN downregulates livin. To evaluate a possible role for MycN in regulating livin expression, we performed a knock-down experiment using a neuroblastoma cell line (LA155N) expressing high levels of MycN and livin protein. MYCN siRNA significantly downregulated both MYCN and livin mRNA expression for up to 48 h post-transfection (Fig. 2A). Similar effects on MycN and livin



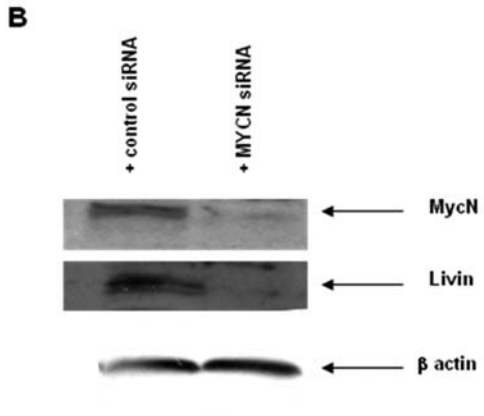
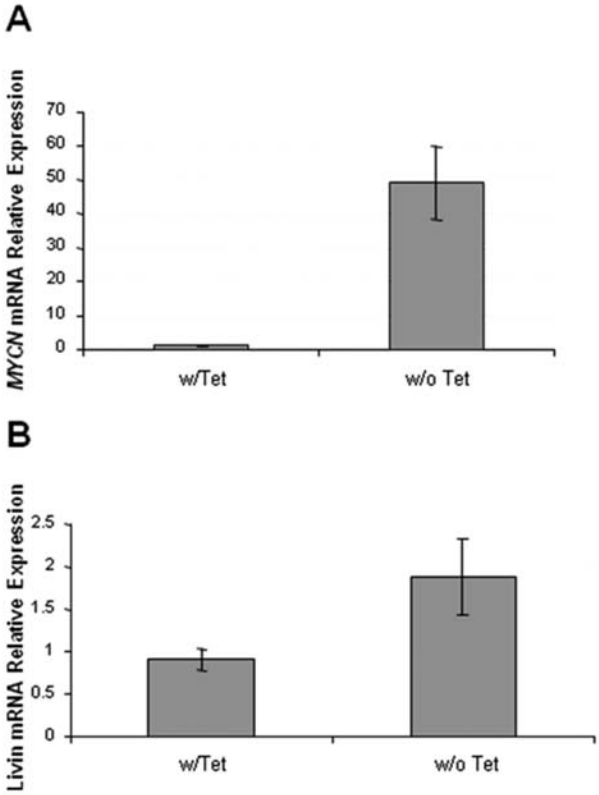


Figure 2. siRNA-mediated knockdown of MYCN repressed livin expression in LA155N, a MYCN-amplified neuroblastoma cell line expressing high levels of both MycN and livin proteins. (A) qRT-PCR to determine MYCN (upper panel) and livin (lower) mRNA expression measured at 24 and 48 h post-transfection with MYCN siRNA. (B) Immunoblots depicting MycN and livin protein levels following MYCN siRNA treatment for 48 h. ß-actin was used as the loading control.



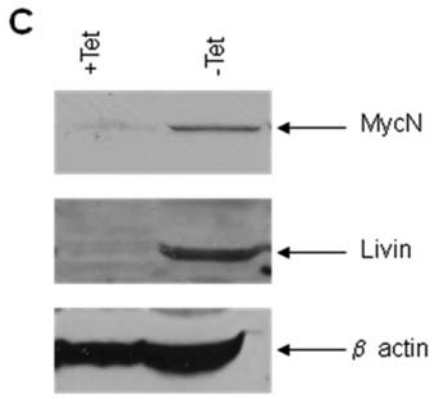


Figure 3. Effect of MycN induction on endogenous livin expression. Tet21N is a tetracycline-dependent, MYCN-inducible SH-EP line where removal of tetracycline upregulates MYCN. (A) Quantitation of MYCN mRNA by qRT-PCR at 48 h after tetracycline removal. (B) MycN-dependent increase in livin mRNA expression after 48 h of MycN induction as measured by qRT-PCR. (C) Representative immunoblots for MycN and livin proteins levels measured at 48 h after MycN upregulation. ß-actin was used as the loading control.

protein were also observed (Fig. 2B). This inhibitory effect was specific, since transfection with control siRNA did not alter either MycN or livin expression.

Induction of MYCN upregulates livin. To further investigate MycN-mediated regulation of livin in NB, we used a tetracycline-controlled, MYCN-inducible SH-EP cell line (Tet21N). Tet21N is a Tet-off system where removal of tetracycline from the growth media upregulates MycN expression (Fig. 3A and C). We observed a significant upregulation of livin mRNA and protein expression following MycN induction in Tet21N cells (Fig. 3B and C).

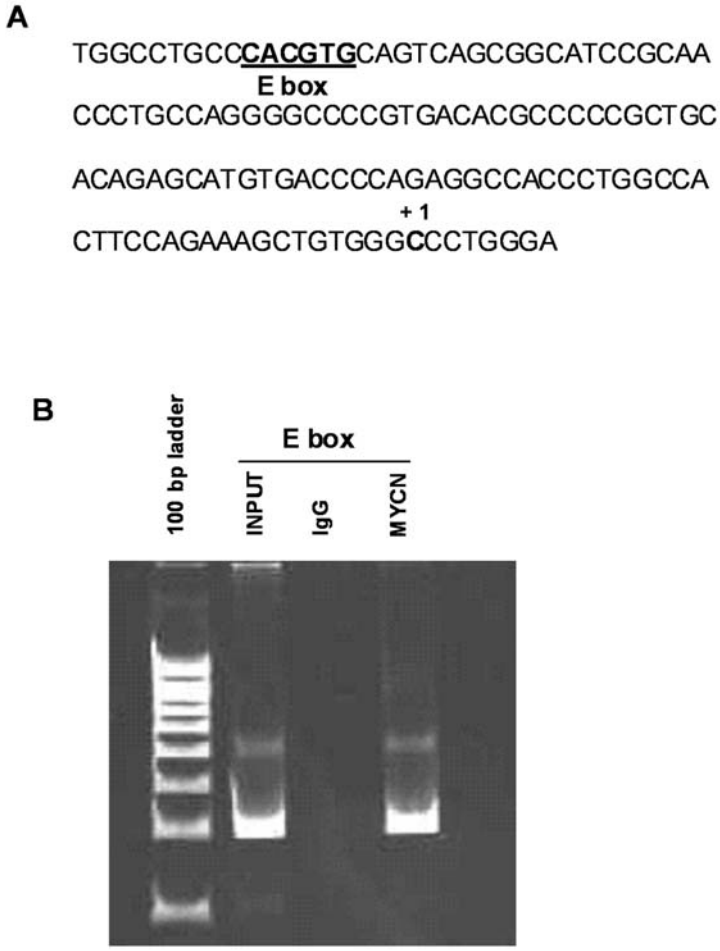


Figure 4. Binding of MycN to the E-box in the putative livin promoter is shown by chromatin immunoprecipitation assay. (A) DNA sequence immediately upstream (-121 bp) of the transcriptional start site of livin gene is shown. The location of the putative MycN-binding motif, E-box, is underlined. (B) CHIP assay: Chromatin from LA155N cells were immunoprecipitated with MycN antibody. IgG antibody was used as the control. DNA recovered from the immunoprecipitates served as the template for PCR amplification by E-box specific primers. The PCR products were separated on a 6% polyacrylamide gel. E-box was found to be enriched in anti-MycN immunopecipitation but not by the control IgG antibody immunoprecipitation, indicating the specificity of MycN interaction within the putative livin promoter.

MycN binds to target sites within the putative livin promoter. We identified a consensus MycN-binding site within the region proximal (-112) to the transcriptional start-site of the livin promoter (Fig. 4A). To test if MycN binds to this E-box, we performed a chromatin immunoprecipitation assay. To evaluate MycN binding to the E-box, primers were designed to include this binding site. As shown in Fig. 4B, the E-box was found to be occupied by MycN.

Discussion

MycN is involved in regulating a diverse set of genes involved in cell-cycle progression and cell survival (25-28). Prior to the present study, a role for MycN in regulating members of the IAP family of anti-apoptotic proteins has not been reported. We were prompted to study a possible MycN-mediated regulatory mechanism for livin based on our previous finding that MYCN amplification in combination with elevated livin is a poor-prognosis marker for neuroblastoma (18, and unpublished data).

We initially evaluated MYCN and livin mRNA and protein levels in a panel of neuroblastoma cell lines. Our findings suggested a positive correlation between MycN and livin expression in neuroblastoma. Additional studies of MycN and livin protein expression will be required to further evaluate this relationship in primary tumors. In our previous studies of livin in primary tumors, we did not find elevated livin in all MYCN-amplified cases, suggesting that factors in addition to MycN protein levels may be required to induce high-level expression of this IAP.

We next investigated whether MYCN knockdown resulted in suppression of livin. We found that siRNA-mediated inhibition of MYCN effectively abrogated livin expression, suggesting MycN is required for livin expression. Previous studies have shown that siRNA-mediated repression of MYCN in neuroblastoma cells resulted in induction of apoptosis (29-31). Our results suggest that the observed apoptosis may be partly due downregulation of livin following MYCN knockdown.

Furthermore, we evaluated the role of MycN protein in regulating livin in a tetracycline-inducible MYCN system. We found that MycN induction rapidly upregulated livin expression. This system has been widely used to investigate MycN regulation of a number of target genes in neuroblas-

toma (25,27,28). Our results with the MYCN-inducible system complement and reinforce our data from the MYCN-knockdown experiments.

To evaluate whether MycN upregulates livin directly, we performed promoter-binding studies. We detected a consensus MycN binding domain within the 5' proximal sequence of the putative livin promoter. Similar direct binding of MycN to E-boxes within the target promoters of MDM2, MRP1 and CRABII in neuroblastoma cells have been reported as evidence that MycN positively regulates expression of these genes (25,27,28). We are currently characterizing the role of this MycN binding site in the livin promoter by mutation/deletion experiments.

Taken together, our results suggest that MycN is required for livin expression in neuroblastoma. Since elevated MycN expression has been reported to sensitize neuroblastoma cells to some chemotherapeutic agents, it is possible that livin upregulation counteracts this pro-apoptotic effect and protects MYCN-amplified neuroblastomas from chemotherapy. This may partly explain our previous observation that neuroblastoma tumors expressing high levels of livin in the context of amplified MYCN are associated with a worse prognosis than are tumors with either marker alone.

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