

EWS/FLI and Its Downstream Target NR0B1 Interact Directly to Modulate Transcription and Oncogenesis in Ewing's Sarcoma

Michelle Kinsey,¹ Richard Smith,² Anita K. Iyer,⁴ Edward R.B. McCabe,^{4,5} and Stephen L. Lessnick^{1,2,3}

¹Department of Oncological Sciences, University of Utah School of Medicine; ²Center for Children's Cancer Research, Huntsman Cancer Institute and ³Division of Pediatric Hematology/Oncology, University of Utah, Salt Lake City, Utah; Departments of ⁴Human Genetics and ⁵Pediatrics, David Geffen School of Medicine at University of California at Los Angeles, Los Angeles, California

Abstract

Most Ewing's sarcomas harbor chromosomal translocations that encode fusions between EWS and ETS family members. The most common fusion, EWS/FLI, consists of an EWSR1-derived strong transcriptional activation domain fused, in-frame, to the DNA-binding domain-containing portion of FLI1. EWS/FLI functions as an aberrant transcription factor to regulate genes that mediate the oncogenic phenotype of Ewing's sarcoma. One of these regulated genes, NR0B1, encodes a corepressor protein, and likely plays a transcriptional role in tumorigenesis. However, the genes that NR0B1 regulates and the transcription factors it interacts with in Ewing's sarcoma are largely unknown. We used transcriptional profiling and chromatin immunoprecipitation to identify genes that are regulated by NR0B1, and compared these data to similar data for EWS/FLI. Although the transcriptional profile overlapped as expected, we also found that the genome-wide localization of NR0B1 and EWS/FLI overlapped as well, suggesting that they regulate some genes coordinately. Further analysis revealed that NR0B1 and EWS/FLI physically interact. This protein-protein interaction is likely to be relevant for the development of Ewing's sarcoma because mutations in NR0B1 that disrupt the interaction have transcriptional consequences and also abrogate oncogenic transformation. Taken together, these data suggest that EWS/FLI and NR0B1 physically interact, coordinately modulate gene expression, and mediate the transformed phenotype of Ewing's sarcoma. [Cancer Res 2009;69(23):9047–55]

Introduction

Ewing's sarcoma is an aggressive bone-associated tumor that affects the pediatric population. The majority of Ewing's sarcomas harbor a reciprocal translocation, t(11;22) (q24;q12), which links a strong transcriptional activation domain from EWSR1 to the ETS DNA-binding portion of FLI1 (1). The EWS/FLI fusion is required for Ewing's sarcoma oncogenesis, as inhibition of fusion function or expression results in the loss of transformation of Ewing's sarcoma cells (2–4). Thus, understanding the function of EWS/FLI is critical in understanding the development of Ewing's sarcoma.

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Current address for A.K. Iyer: Department of Reproductive Medicine, University of California, San Diego, La Jolla, CA.

Requests for reprints: Stephen L. Lessnick, Huntsman Cancer Institute, 2000 Circle of Hope, Salt Lake City, UT 84112. Phone: 801-585-9268; Fax: 801-585-5357; E-mail: stephen.lessnick@hci.utah.edu.

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EWS/FLI is thought to function as a transcriptional activator (5–7). However, in Ewing's sarcoma cells, thrice as many genes are downregulated by EWS/FLI than are upregulated (4, 8, 9). One hypothesis for this observation is that some EWS/FLI-upregulated gene targets function as transcriptional repressors. Indeed, this was supported by the demonstration that one EWS/FLI-upregulated gene product, NKX2.2, functions as a transcriptional repressor in Ewing's sarcoma. However, NKX2.2-mediated gene repression accounts for a small portion of the EWS/FLI-downregulated gene expression signature, suggesting that other targets may also function as repressors (10).

A second critical target, NR0B1 (DAX1), is an attractive candidate to mediate gene repression downstream of EWS/FLI. We recently showed that NR0B1 is directly regulated by EWS/FLI and that it is required for the transformed phenotype of Ewing's sarcoma cells (8, 11). NR0B1 is an orphan member of the nuclear hormone receptor superfamily. NR0B1 is unusual because it lacks a conventional DNA-binding domain; hence, it is not thought to directly interact with DNA like other family members (12). Although the molecular function of NR0B1 in Ewing's sarcoma is unknown, it seems to function primarily as a transcriptional corepressor during the development and function of the hypothalamic-pituitary-adrenal-gonadal axis (13). To better understand the role of NR0B1 in Ewing's sarcoma, we tested the hypothesis that it functions as a transcriptional coregulator during oncogenesis.

Materials and Methods

Constructs and retroviruses. For “knockdown” experiments, previously described NR0B1-RNAi, luc-RNAi, and EF-2-RNAi constructs were used (4, 8). For overexpression experiments, a 3× FLAG-tag was introduced onto the NH₂ terminus of NR0B1 and its mutants, EWS/FLI and its mutants, and wild-type FLI1 in the pMSCV-Neo retroviral vector (Clontech). For yeast two-hybrid experiments, wild-type NR0B1 (14), NR0B1 mutants, wild-type EWS/FLI, and EWS/FLI mutants were cloned into pGBKT7 and pGADT7 (Clontech). For luciferase assays, ~700 bp of the NR0B1 intron (Supplementary Data 1) was cloned upstream of the SV40 promoter in the pGL3-Promoter luciferase reporter vector (Promega).

Cell culture. Ewing's sarcoma cell lines A673, TC71, and EWS502, and the human embryonic kidney cell line 293EBNA, were grown as previously described (8, 15). Following retroviral infection, polyclonal cell populations were prepared by growth in selection media (2 mg/mL puromycin and 300 mg/mL G418). Soft agar transformation assays and 3T5 growth assays were performed as previously described (8).

Microarray analysis. RNA preparation, microarray hybridization, normalization, and analysis were performed as previously described (4, 10). Expression data were filtered for a 5-fold change across samples, with a minimal “δ” value of 50. Overlaps between different gene sets were performed using the VennMaster program and χ^2 analysis (4, 10).

Chromatin immunoprecipitation, sequential ChIP, and whole genome localization studies (ChIP-chip). Chromatin immunoprecipitation (ChIP) and

promoter microarray analysis (ChIP-chip) were performed as previously described (10, 11), by using M2 anti-FLAG (F1804; Sigma), anti-FLI-1-X, anti-cMyc (sc-356x or sc-764, respectively; Santa Cruz Biotechnology), or anti- α -tubulin (CP06; Calbiochem). Quantitative PCR was performed with *NROB1* or *ALB* primers (Supplementary Table S1). Sequential ChIP was performed with the Re-ChIP-IT kit (Active Motif) and the above listed antibodies.

Luciferase assays. cDNA constructs (described in Constructs and Retroviruses above) and 293EBNA were used as previously described (11). Two-tailed Student's *t* tests were used for statistical comparisons.

Yeast two-hybrid assays. AH109 yeast cells were transformed with the indicated combinations of plasmids using the Matchmaker kit (Clontech). Yeast was then plated on SD/Leu/Trp plates or SD/Leu/Trp/His plates. Plates were incubated for 4 d and colony growth was counted. Three-aminotriazole (Sigma) was included in some instances to minimize autoactivation effects.

Coimmunoprecipitation assays. 293EBNA were cotransfected with the indicated constructs, protein was extracted using NP40 lysis buffer [0.05 mol/L Tris-HCl (pH 7.4), 0.15 mol/L NaCl, 1% NP40, 1 mmol/L EDTA, and protease inhibitor (Roche)]. Coimmunoprecipitation experiments were conducted using FLAG-M2-Agarose beads (Sigma) or Dynabeads M-280 (Dyna) according to the directions of the manufacturer.

Immunodetection. Western blots were performed with the indicated antibodies: M2 anti-FLAG, anti-FLI-1-X, anti- α -tubulin, or anti-mSin3A (a gift from D. Ayer; ref. 16). Immunofluorescence experiments were performed using M2 anti-FLAG and anti-FLI-1-X primary antibodies according to the instructions of the manufacturer (Sigma). Alexafluor 488 goat anti-mouse and Alexafluor 568 goat anti-rabbit (Invitrogen) were used as secondary antibodies.

Subcellular fractionation. Transfected 293EBNA cells were collected and resuspended in hypotonic buffer [20 mmol/L Hepes (pH 7.5), 5 mmol/L NaF, 0.1 mmol/L EDTA, 10 μ mol/L Na_2MoO_4] and incubated on ice. NP40 (0.5%) was then added and the homogenate was centrifuged. The cytoplasmic fraction was collected and the nuclear pellet was resuspended in complete lysis buffer [400 mmol/L NaCl, 20 mmol/L Hepes (pH 7.5), 10 mmol/L NaF, 10 mmol/L *p*-nitrophenyl phosphate, 1 mmol/L NaVO_3 , 0.1 mmol/L EDTA, 10 μ mol/L Na_2MoO_4 , 10 mmol/L β -glycerophosphate, 20% glycerol, 0.1 mmol/L DTT, and protease inhibitor (Roche)] and incubated with rocking at 4°C. After centrifugation, the nuclear cell extract was then collected.

Results and Discussion

NROB1 both upregulates and downregulates gene expression in Ewing's Sarcoma. To determine the effect of NROB1 on gene expression in Ewing's sarcoma, we used our previously described RNAi-based loss-of-function approach in the patient-derived Ewing's sarcoma TC71 cell line (8, 10). We chose TC71 cells for this assay because "knockdown" of NROB1 in the TC71 cell line results in a loss of transformation without affecting growth in tissue culture, thus the confounding transcriptional effects seen with other cell lines (e.g., A673 and EWS502) could be avoided (8). The transcriptional signature was determined using Affymetrix U133 Plus 2.0 microarrays. The signal-to-noise metric was used to rank-order genes, the expression of which correlated with NROB1 expression, and permutation testing at the 99% confidence level defined the cohort of NROB1-regulated genes. As would be expected, based on prior assertions that NROB1 functions as a transcriptional corepressor, we found 846 genes that were downregulated by the protein. Surprisingly, we found that NROB1 also upregulated 1,131 genes (Supplementary Table S2).

To define the contribution of NROB1 to the EWS/FLI transcriptional profile, we compared the gene signatures of NROB1 to that previously reported for EWS/FLI (10). We found that 300 NROB1-regulated genes overlapped with the EWS/FLI signature ($P = 1.05 \times 10^{-57}$; Fig. 1A). When the data were segregated into upregulated and downregulated gene sets, we found 159 of the NROB1-downregulated genes were also downregulated by EWS/FLI ($P = 1.05 \times$

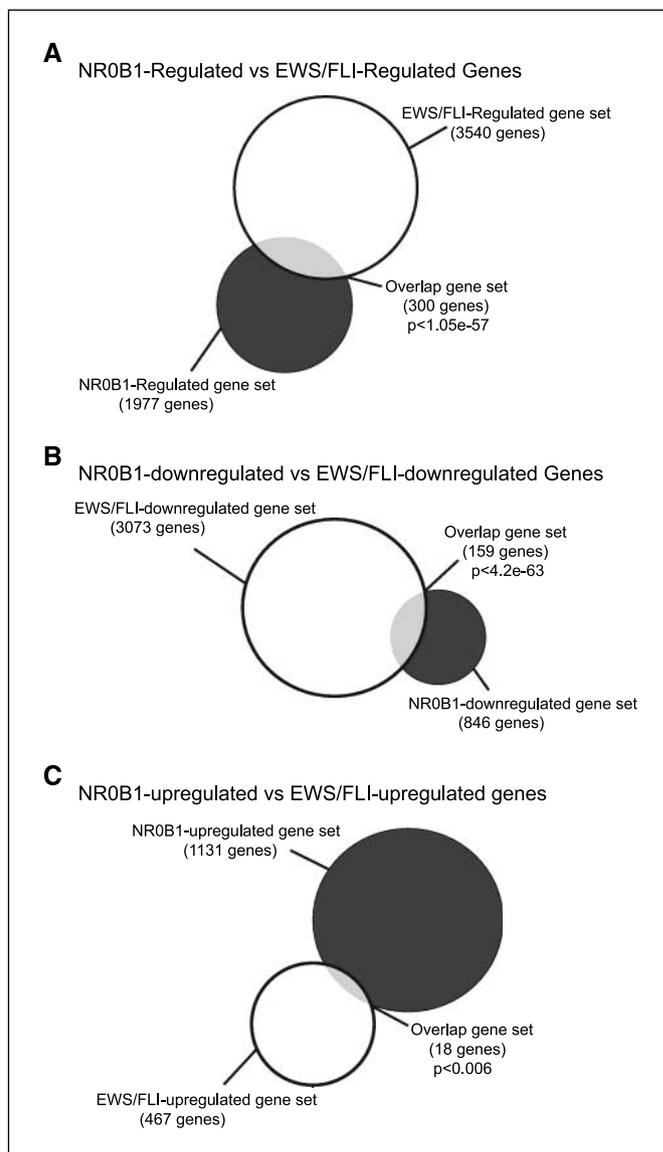


Figure 1. NROB1 target genes contribute to the EWS/FLI gene expression signature. Venn diagrams representing the overlap between the overall NROB1 and EWS/FLI gene expression signatures (A), downregulated genes (B), and upregulated genes (C), respectively. The χ^2 -determined *P* value is indicated.

10^{-63} ; Fig. 1B), and that 18 of the NROB1-upregulated genes were also upregulated by EWS/FLI ($P = 0.006$; Fig. 1C). These data confirm that NROB1 contributes to the transcriptional signature of EWS/FLI, and support a hierarchical relationship in which EWS/FLI regulates NROB1 (among other genes), which then transcriptionally influences other downstream targets. Given the higher level of statistical significance of the overlap between downregulated genes compared with upregulated genes, we speculate that NROB1 has a more prominent function as a transcriptional repressor in Ewing's sarcoma.

Identification of direct NROB1 targets. To define direct NROB1 targets, we used our previously described "knockdown/rescue" approach to replace endogenous NROB1 with a 3 \times FLAG-tagged version (8). We next performed genomewide localization studies, using a ChIP approach followed by ChIP-chip analysis. These microarrays interrogate $\sim 17,000$ human promoters, and

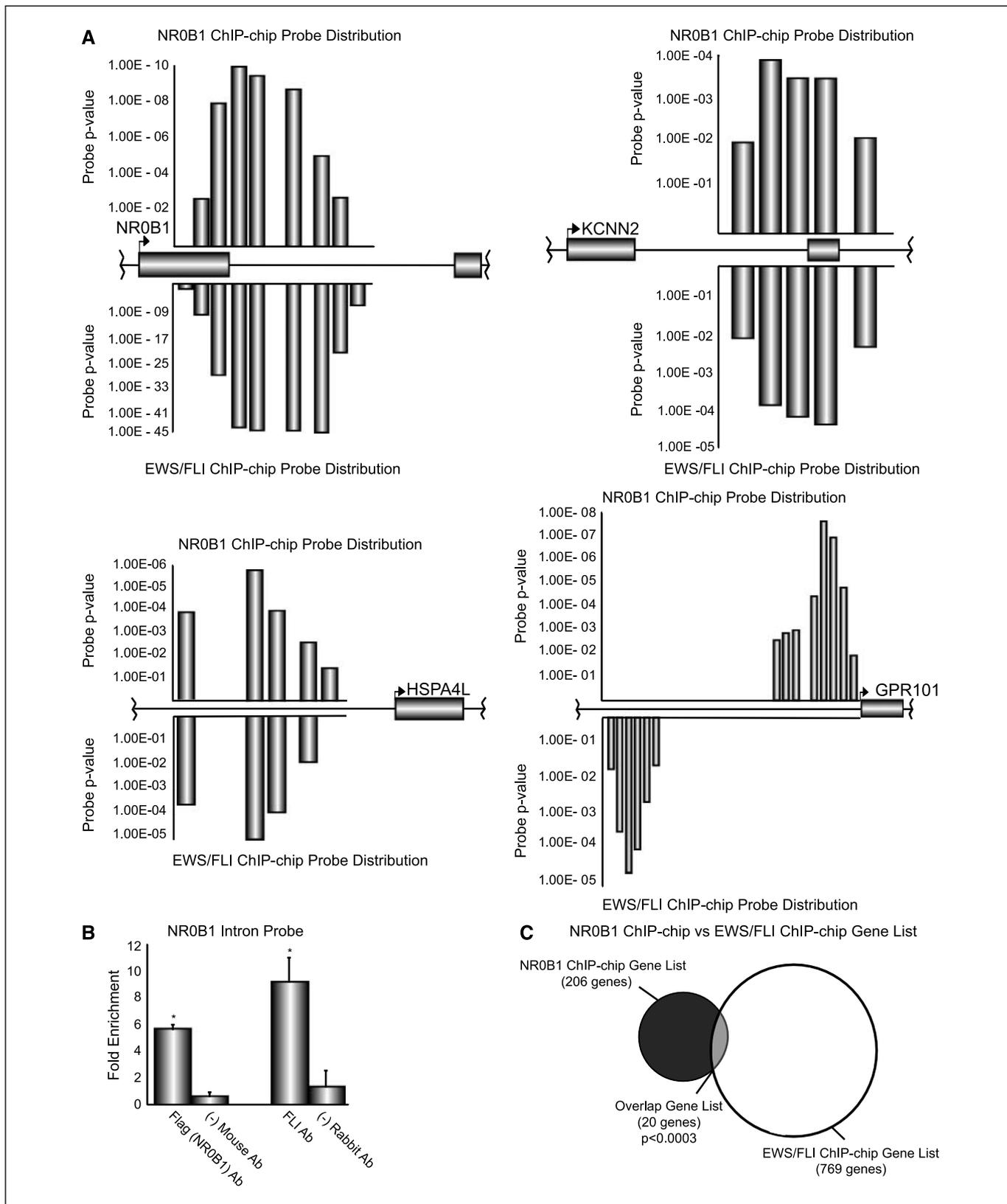


Figure 2. Coordinate occupancy of NR0B1 and EWS/FLI at specific genomic loci. **A**, probe enrichment pattern for NR0B1 and EWS/FLI ChIP-chip derived from two separate biological samples per experimental condition. Relative genomic positioning of probes was determined by the Integrated Genome Browser software program (Affymetrix). Probe *P* values were determined by the Agilent ChIP Analytics program. Examples of overlapping probe patterns (*NR0B1*, *KCNN2*, and *HSPA4L*) and distinct probe distribution (*GPR101*). **B**, ChIP of the *NR0B1* intronic region using the indicated antibodies. Columns, mean fold enrichment of the intron compared with the enrichment of a negative control gene (*ALB*); bars, SD of three independent experiments (*, $P < 0.05$). **C**, Venn diagram representing the overlap between ChIP-chip identified bound NR0B1 and EWS/FLI gene targets. The χ^2 -determined *P* value is indicated.

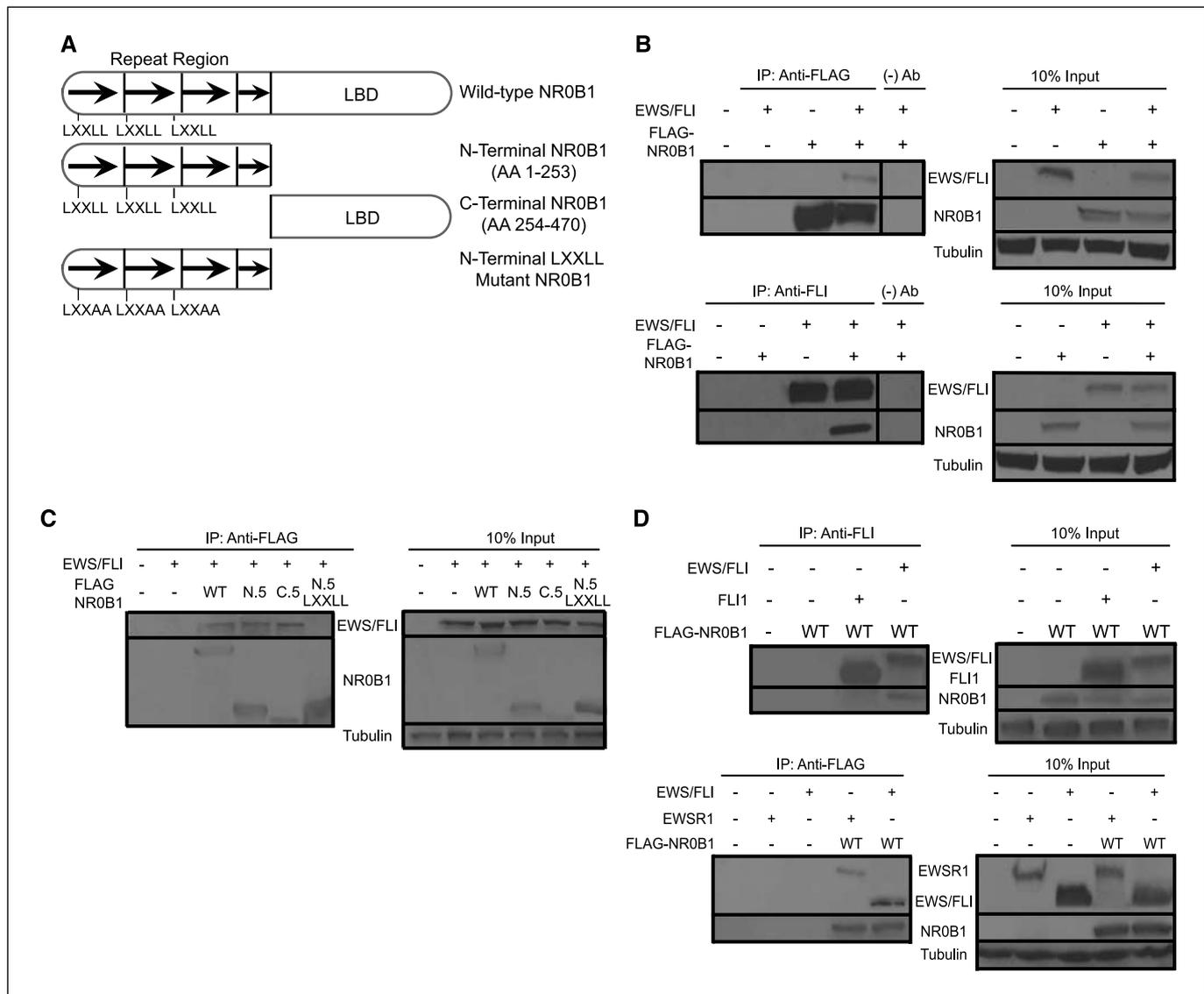


Figure 3. NR0B1 and EWS/FLI interact directly. *A*, schematic of wild-type and mutant NR0B1 constructs. NR0B1 consists of an amino-terminal domain (amino acids 1–253) that contains 3-1/2 alanine-glycine-rich repeating units harboring three LXXLL motifs, and a carboxyl-terminal domain (amino acids 254–470) that is homologous to other nuclear hormone receptors' ligand-binding domains. *B*, Western blot analysis of input and coimmunoprecipitation samples of 293EBNA cells transfected with wild-type EWS/FLI and NR0B1. Immunoprecipitation experiments and Western blots were performed with the indicated antibodies. *C*, coimmunoprecipitation of 293EBNA transfected with EWS/FLI and various NR0B1 mutant constructs, using the indicated antibodies. *WT*, NR0B1 wild-type allele; *N.5* and *C.5*, the amino- and carboxyl-terminal domains of NR0B1, respectively; *N.5 LXXLL*, the amino-terminal NR0B1 with all three LXXLL motifs mutated. All NR0B1 constructs are 3× FLAG-tagged. Input samples were used to ensure the appropriate expression of all constructs tested. *D*, coimmunoprecipitation of 293EBNA transfected with NR0B1 and EWS/FLI parental proteins EWSR1 or FLI1 using the indicated antibodies.

include regions from –5.5 to +2.5 kb relative to the transcriptional start site. We found that 250 genes were directly occupied by NR0B1 (Supplementary Table S3).

Analysis revealed a small but insignificant overlap between the NR0B1 ChIP-chip and NR0B1 microarray data sets. We believe this result may be due to a combination of factors. First, gene expression profiles are comprised of both direct and indirect transcriptional targets. Therefore, it is possible that only a small portion of NR0B1-regulated genes are direct targets. Second, a portion of directly occupied NR0B1 sites may be transcriptionally inert and these are not reflected in the NR0B1 gene signature. Third, the ChIP microarray only evaluates promoter region occupancy. If NR0B1 has many functional binding sites outside of the interrogated region, then the sensitivity of our data overlap analysis is reduced. Lastly, although

every effort was taken to identify genes that are statistically significantly different from background levels, the amount of “noise” present in each gene list is unknown. Such noise will reduce the ability to observe statistically significant similarities between data sets. Similar findings were also observed between the EWS/FLI gene expression profile and ChIP-chip data sets (17).⁶

Some genes are bound by both NR0B1 and EWS/FLI. We recently reported that EWS/FLI bound to ~900 genes, including *NR0B1* (11). In addition to promoter binding, we also noted strong binding to the *NR0B1* intron.⁶ Interestingly, our ChIP-chip analysis showed that NR0B1 bound this same region. Indeed, the pattern of

⁶ K. Gangwal, S. Sankar, and S.L. Lessnick, unpublished observations.

NR0B1 binding mirrored the EWS/FLI-binding pattern (Fig. 2A), and directed ChIP assays confirmed these results (Fig. 2B).

To determine if additional loci showed a similar binding pattern, we identified 20 genes bound by both proteins in the two ChIP-chip data sets ($P = 0.0003$; Fig. 2C). Evaluation of these dual-bound

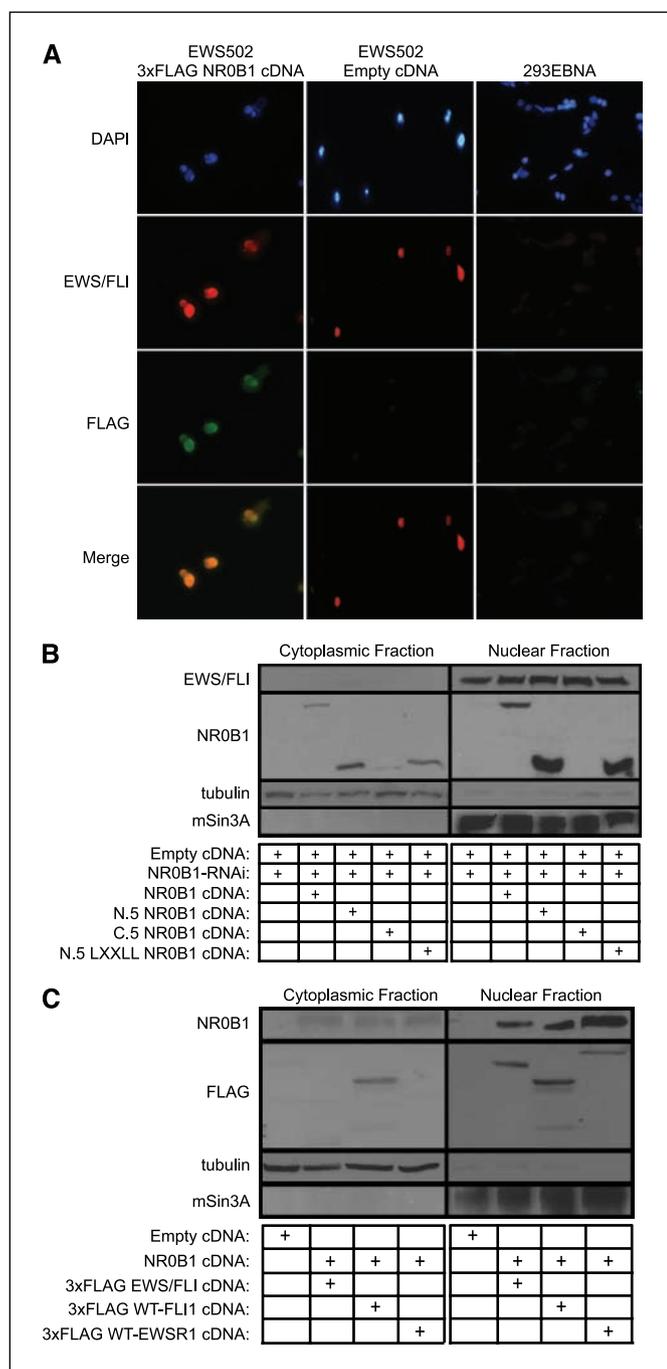


Figure 4. NR0B1 and EWS/FLI colocalize to the nucleus. **A**, immunofluorescence of EWS502 Ewing's sarcoma cells infected with the indicated cDNA constructs and detected with the indicated antibody. Nuclei are shown by 4',6-diamidino-2-phenylindole (DAPI) staining and 293EBNA cells are shown as a negative control. **B** and **C**, A673 cells infected or 293EBNA transfected, respectively, with the indicated cDNA constructs and subfractionated. Western blots were performed using the designated antibodies. Tubulin is used as a control for the cytoplasmic fraction, whereas mSin3A is used as a control for the nuclear fraction.

genes revealed that 90% displayed the same overlapping probe enrichment pattern for both NR0B1 and EWS/FLI (e.g., Fig. 2A). Importantly, our negative control ChIP-chip experiments did not show similar findings, demonstrating that the enrichment pattern is specific to NR0B1 and EWS/FLI (data not shown). Furthermore, a few genes had distinct probe-binding patterns for EWS/FLI and NR0B1, suggesting that such similarities were not simply technical artifacts (e.g., Fig. 2A). These data therefore show that NR0B1 and EWS/FLI occupy the same regions of genomic DNA at a subset of loci.

NR0B1 and EWS/FLI interact directly. NR0B1 is not believed to bind directly to DNA, rather, it is thought to predominantly function as a transcriptional coregulator by interacting with transcription factors to modulate gene expression (12). The identity of these transcription factors in Ewing's sarcoma is unknown. We have shown that both EWS/FLI and NR0B1 are bound to the same genomic loci (Fig. 2C). In addition, recent observations from various proteomic assays suggest that wild-type EWSR1 and NR0B1 may exist in a large protein complex (18, 19). We therefore speculated that NR0B1 and EWS/FLI interact directly. To test this hypothesis, we first performed directed yeast two-hybrid assays (Y2H). We fused NR0B1 to the GAL4 DNA-binding domain as bait, and EWS/FLI to the GAL4 activation domain as prey. Interactions were assessed through expression of a *HIS3* reporter gene placed downstream of GAL4 DNA-binding sites. Thus, bait-prey interactions enabled the growth of yeast on histidine-deficient plates. We found that when the two proteins were coexpressed as bait and prey, the *HIS3* reporter was activated, implying a direct protein-protein interaction between NR0B1 and EWS/FLI. Importantly, the *HIS3* reporter was also activated when EWS/FLI was used as bait and NR0B1 as prey (Supplementary Table S4).

To begin to identify the interacting regions of NR0B1 and EWS/FLI, mutant NR0B1 constructs were generated and tested using the directed Y2H approach (Fig. 3A). We found that both the amino- and carboxyl-termini of NR0B1 independently interacted with EWS/FLI, suggesting that both the amino and carboxyl domains have EWS/FLI-binding sites (Supplementary Table S4).

NR0B1 is known to interact with some of its protein-binding partners (e.g., SF1, ER, AR) through the three LXXLL motifs found in its amino-terminal half (20, 21). We therefore tested whether these contributed to the NR0B1-EWS/FLI interaction. We mutated each LXXLL motif to LXXAA, both singly and in combination, and analyzed these constructs using directed Y2H assays. The results using single and double LXXLL motif mutations were variable and not reproducible. However, when all three LXXLL motifs were mutated, NR0B1 was unable to interact with EWS/FLI (Supplementary Table S4).

To validate the Y2H data using a different experimental approach, we did coimmunoprecipitation experiments following transfection of EWS/FLI and 3x FLAG-tagged NR0B1. We found that EWS/FLI coimmunoprecipitated (Fig. 3B) and colocalized (Fig. 4A) with NR0B1. Furthermore, consistent with the Y2H data, both the amino- and carboxyl-termini of NR0B1 also immunoprecipitated EWS/FLI (Fig. 3C). Mutation of the NR0B1 LXXLL motifs again abolished this interaction without affecting its ability to localize to the nucleus (Figs. 3C and 4B). Introduction of mutations in the COOH-terminus of NR0B1 rendered the protein relatively unstable, and thus the EWS/FLI-interacting domain in this region could not be mapped. In addition, colocalization studies indicate that the carboxyl-terminal half of NR0B1 resides predominantly in the cytoplasm (Fig. 4B). Therefore, mapping studies using this mutant may not accurately reflect the native interaction between NR0B1 and EWS/FLI.

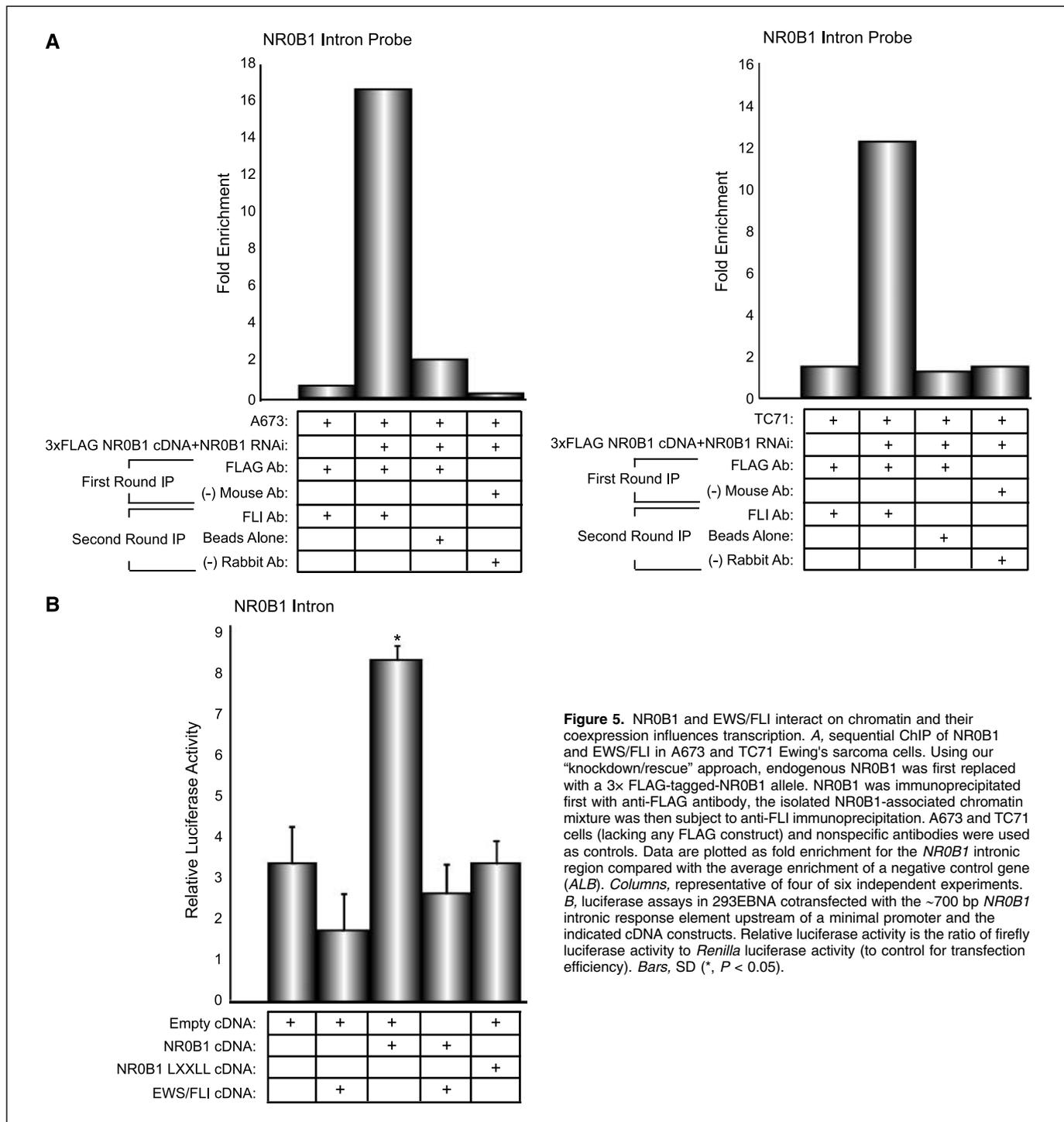


Figure 5. NR0B1 and EWS/FLI interact on chromatin and their coexpression influences transcription. **A**, sequential ChIP of NR0B1 and EWS/FLI in A673 and TC71 Ewing's sarcoma cells. Using our "knockdown/rescue" approach, endogenous NR0B1 was first replaced with a 3x FLAG-tagged-NR0B1 allele. NR0B1 was immunoprecipitated first with anti-FLAG antibody, the isolated NR0B1-associated chromatin mixture was then subject to anti-FLI immunoprecipitation. A673 and TC71 cells (lacking any FLAG construct) and nonspecific antibodies were used as controls. Data are plotted as fold enrichment for the *NR0B1* intronic region compared with the average enrichment of a negative control gene (*ALB*). Columns, representative of four of six independent experiments. **B**, luciferase assays in 293EBNA cotransfected with the ~700 bp *NR0B1* intronic response element upstream of a minimal promoter and the indicated cDNA constructs. Relative luciferase activity is the ratio of firefly luciferase activity to *Renilla* luciferase activity (to control for transfection efficiency). Bars, SD (*, $P < 0.05$).

We then repeated our Y2H assays using only the EWS or FLI domain of EWS/FLI to begin to identify the NR0B1 interacting site(s) on EWS/FLI. Neither construct activated the *HIS3* reporter. These data imply that the isolated domains are unable to interact with NR0B1 and suggest that both domains are required for the NR0B1-EWS/FLI interaction (Supplementary Table S4).

To determine if the protein interaction is indeed unique to the EWS/FLI fusion product, we did coimmunoprecipitation experiments with the full-length EWS/FLI parental proteins EWSR1 and FLI1. Although, FLI1 was predominantly nuclear, it did not in-

teract with NR0B1. In contrast, EWSR1 colocalized and coimmunoprecipitated with NR0B1 (Figs. 3D and 4C). The EWSR1 result is unexpected because the Y2H data suggest that both the EWS and FLI domain are required for the NR0B1-EWS/FLI interaction. This discordance may be due to improper folding of the isolated portions of the EWS or FLI domain mutant constructs or may reflect a lack of necessary cofactors in the yeast system. The EWSR1 data therefore suggest that a NR0B1-binding site is most likely present in the EWS domain of EWS/FLI. Indeed, the EWS amino-terminal domain is believed to lack a native structure, making the

region a highly amenable binding site for a multitude of protein interaction partners (22). Taken together, the coimmunoprecipitation and Y2H experiments show that EWS/FLI and NR0B1 participate in a protein-protein interaction, most likely mediated through the NR0B1 LXXLL motifs and the EWS domain of EWS/FLI.

NR0B1 and EWS/FLI interact on chromatin. Because NR0B1 and EWS/FLI interact directly, and are coordinately present at a subset of promoters, we next sought to determine whether NR0B1 and EWS/FLI interact on chromatin. We did a sequential ChIP assay with the same NR0B1 “knockdown/rescue” A673 Ewing’s cells we used for our ChIP-chip and directed ChIP studies. We first precipitated and eluted 3× FLAG-NR0B1-bound chromatin to isolate genomic loci directly affiliated with NR0B1. Following chromatin resuspension, we did a second immunoprecipitation with anti-FLI antibody to isolate genomic regions also bound by EWS/FLI. We then assessed occupancy at the *NR0B1* intronic region. We chose this region because our ChIP-chip experiments showed that NR0B1 and EWS/FLI enriched several probes within the *NR0B1* in-

tron and independent directed ChIP assays confirmed binding by both NR0B1 and EWS/FLI (Fig. 2A and B). Our sequential ChIP experiments showed enrichment of the *NR0B1* intronic region relative to a negative control region (Fig. 5A). Importantly, the intronic region was not enriched when the same technique was applied to A673 cells not expressing the 3× FLAG-NR0B1 construct, nor was it enriched with control antibodies (Fig. 5A). To further validate these results, sequential ChIP experiments were repeated using a different Ewing’s sarcoma cell line (TC71) and a similar trend was observed (Fig. 5A). These results, taken together with the Y2H and immunoprecipitation data, support the hypothesis that NR0B1 and EWS/FLI physically interact and concurrently occupy the same genomic region.

Transcriptional consequences of EWS/FLI and NR0B1 co-expression. Because both EWS/FLI and NR0B1 have transcriptional function, we next sought to determine if there was a transcriptional effect due to the NR0B1-EWS/FLI interaction. We cloned ~700 bp of the *NR0B1* intronic region upstream of a luciferase reporter construct containing a minimal promoter derived from SV40. The

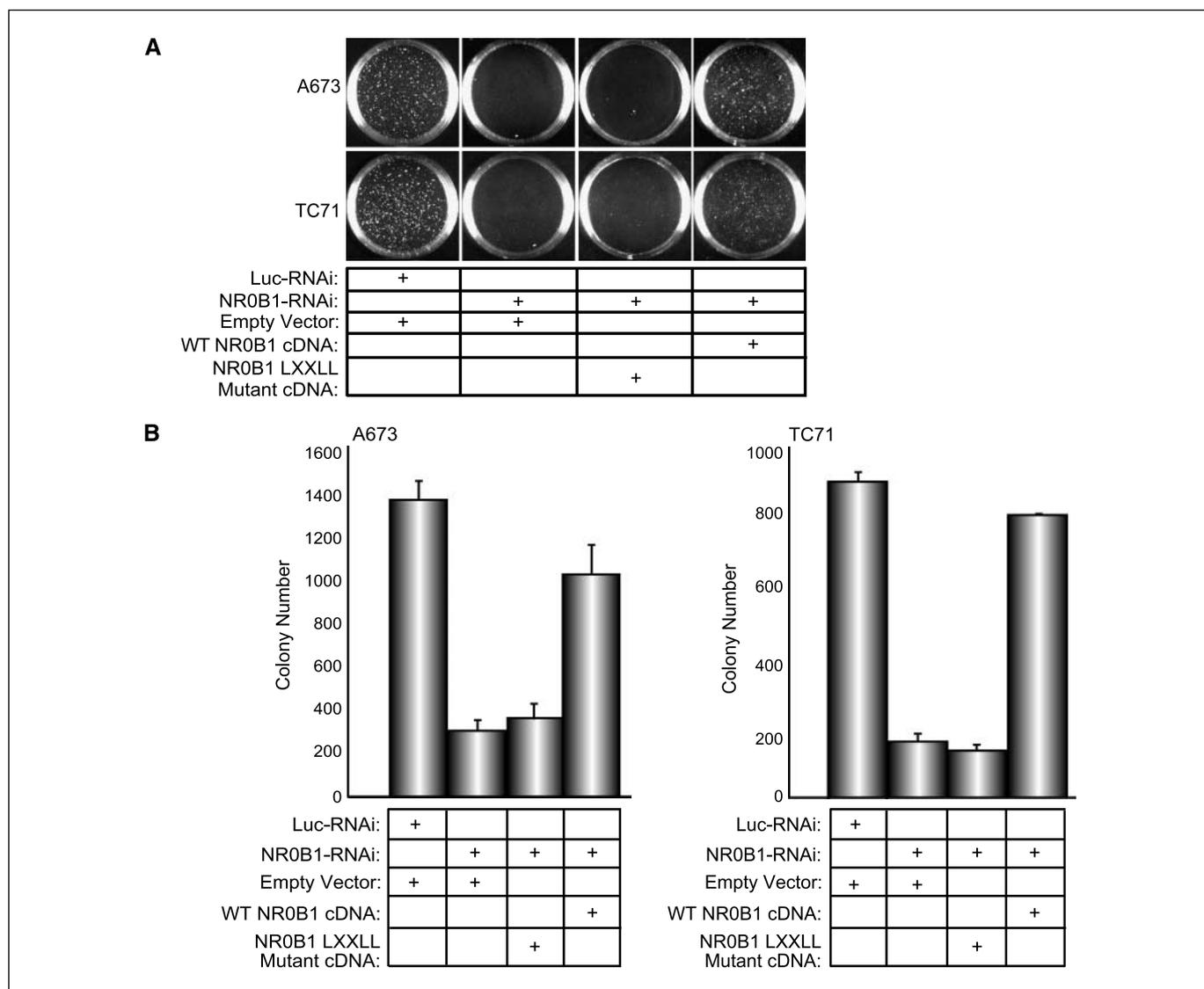


Figure 6. The NR0B1-EWS/FLI-interacting domain is critical for transformation. *A*, soft agar colony formation in A673 and TC71 Ewing’s sarcoma cells infected with the indicated constructs. *NR0B1* LXXLL Mutant cDNA, the full-length triple LXXLL NR0B1 mutant allele. *B*, quantification of colonies formed in soft agar. Bars, SD of duplicate assays.

NROB1 intronic region was chosen for these assays because it was identified as a mutual binding site for *NROB1* and *EWS/FLI* by our multiple ChIP studies. This construct was cotransfected into 293EBNA cells with *NROB1* and/or *EWS/FLI*, and luciferase activity determined. We found that the intron was not responsive to *EWS/FLI* by itself (Fig. 5B). In contrast, luciferase activity was increased ~3-fold with *NROB1* (Fig. 5B). When both proteins were coexpressed, luciferase activity was reduced to basal levels (Fig. 5B). In this setting, therefore, *EWS/FLI* inhibits *NROB1*-mediated transcriptional activity. Because a full-length triple LXXLL mutant form of *NROB1* did not stimulate activity from this reporter, the contribution of *NROB1*'s LXXLL motifs to the inhibitory effect of *EWS/FLI* could not be assessed. These results suggest that at the *NROB1* intron, *NROB1* binds an unidentified transcription factor through its LXXLL motifs to enable transcriptional activation, and that *EWS/FLI* abrogates this effect through direct interaction with *NROB1*.

The *EWS/FLI*-interacting region of *NROB1* is required for oncogenic transformation. We previously showed that *NROB1* expression is critical to the Ewing's sarcoma transformed phenotype (8). To assess the biological significance of the *NROB1*-*EWS/FLI* protein interaction to oncogenesis, we did "knockdown/rescue" soft agar colony formation experiments using mutant forms of *NROB1* in two different Ewing's sarcoma cell lines (A673 and TC71). "Knockdown" of endogenous *NROB1* abrogated colony growth, whereas re-expression of *NROB1* fully rescued transformation, as previously reported (8). In contrast, neither amino-terminal nor carboxyl-terminal *NROB1* mutants were capable of rescuing transformation (data not shown). These data suggest that both domains are necessary for the function of *NROB1* in Ewing's sarcoma. One limitation of this interpretation, however, is that it is dependent on data derived from large structural protein alterations. Indeed, relatively little is known about how the entire amino or carboxyl domains of *NROB1* function, rather most work has focused on subdomains within the protein (e.g., the LXXLL motifs). Therefore, we generated a full-length triple LXXLL *NROB1* mutant allele to test a construct with domain mutations incapable of interacting with *EWS/FLI* and to minimize the effect of large structural deletions on *NROB1* function. We found that this mutant was also unable to rescue transformation (Fig. 6A and B). The LXXLL mutant protein had similar expression levels, subcellular localization, and tissue culture growth patterns as wild-type *NROB1* (data not shown). These data show that intact LXXLL motifs are required for the participation of *NROB1* in the oncogenic phenotype of Ewing's sarcoma, and suggest that our experimental system may be a useful approach towards enhancing our understanding of the function of the structural domains of *NROB1* in tumorigenesis.

Conclusions. *NROB1* is an enigmatic protein, and this is particularly true in Ewing's sarcoma, in which a role for this protein has only recently been discovered (8, 23). The data in this report provides a new understanding of the mechanisms by which *NROB1* functions in cancer. We showed that *NROB1* influences both transcriptional repression and activation during Ewing's sarcoma oncogenesis. We have also shown that *NROB1* and *EWS/FLI* are

coordinately present at a subset of promoters and display a direct protein-protein interaction. In addition, we showed that the regions of *NROB1* required for the *EWS/FLI* interaction are also required for its transcriptional and tumorigenic functions. Taken together, our data suggest that *NROB1* and *EWS/FLI* physically interact to influence gene expression and mediate the transformed phenotype of Ewing's sarcoma.

EWS/FLI is the principal oncoprotein in Ewing's sarcoma. Most prior data suggest that the fusion functions as a transcriptional activator (5–7). However, recent studies (using RNAi-based approaches) have shown that *EWS/FLI* downregulates more genes than it upregulates (4, 8, 9). Some of this downregulated signature seems to be mediated by *EWS/FLI* target genes, such as *NKX2.2* (10), and as shown in this report, *NROB1*.

In contrast to its function as a transcriptional corepressor, emerging evidence suggests that *NROB1* may also have transcriptional activating functions in some settings (24, 25). Indeed, our own data supports this dual activity of *NROB1*. We showed that the *NROB1* transcriptional profile consists of both downregulated and upregulated genes, and we found that *NROB1* serves as an activator at the *NROB1* intron. In addition, the inhibition of the activating function of *NROB1* by interaction with *EWS/FLI* may be a method for modulating the transcriptional influence of both proteins and may dynamically affect gene target expression at specific loci. For example, the interaction at the *NROB1* intronic region could be a means to fine tune *NROB1* expression levels in Ewing's sarcoma cells.

Our data provide important insights into how the critical orphan nuclear hormone receptor, *NROB1*, contributes to Ewing's sarcoma tumorigenesis, and sets the stage for future work focused on understanding the biochemical mechanisms underlying these functions. Such an understanding may allow for the development of antagonists and/or synthetic ligands that modulate *NROB1* activity. Indeed, elucidating the role of other nuclear hormone family members (e.g., estrogen receptor, androgen receptor, and the retinoic acid receptor) in a variety of cancers has led to the development of more directed and effective therapies (26–28). Such an approach focusing on *NROB1* in Ewing's sarcoma may result in novel therapeutic options for patients affected by this devastating pediatric cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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EWS/FLI and Its Downstream Target NR0B1 Interact Directly to Modulate Transcription and Oncogenesis in Ewing's Sarcoma

Michelle Kinsey, Richard Smith, Anita K. Iyer, et al.

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