

Supplementary Materials for

Nucleolar Localization of a Netrin-1 Isoform Enhances Tumor Cell Proliferation

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Published 7 August 2012, *Sci. Signal.* **5**, ra57 (2012)

DOI: 10.1126/scisignal.2002456

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Fig. S1. Depletion of Δ N-netrin-1 affects B23 localization within the nucleolus in IMR32 cells.

Fig. S2. Depletion of Δ N-netrin-1 affects B23 localization in the nucleolus in H358 cells.

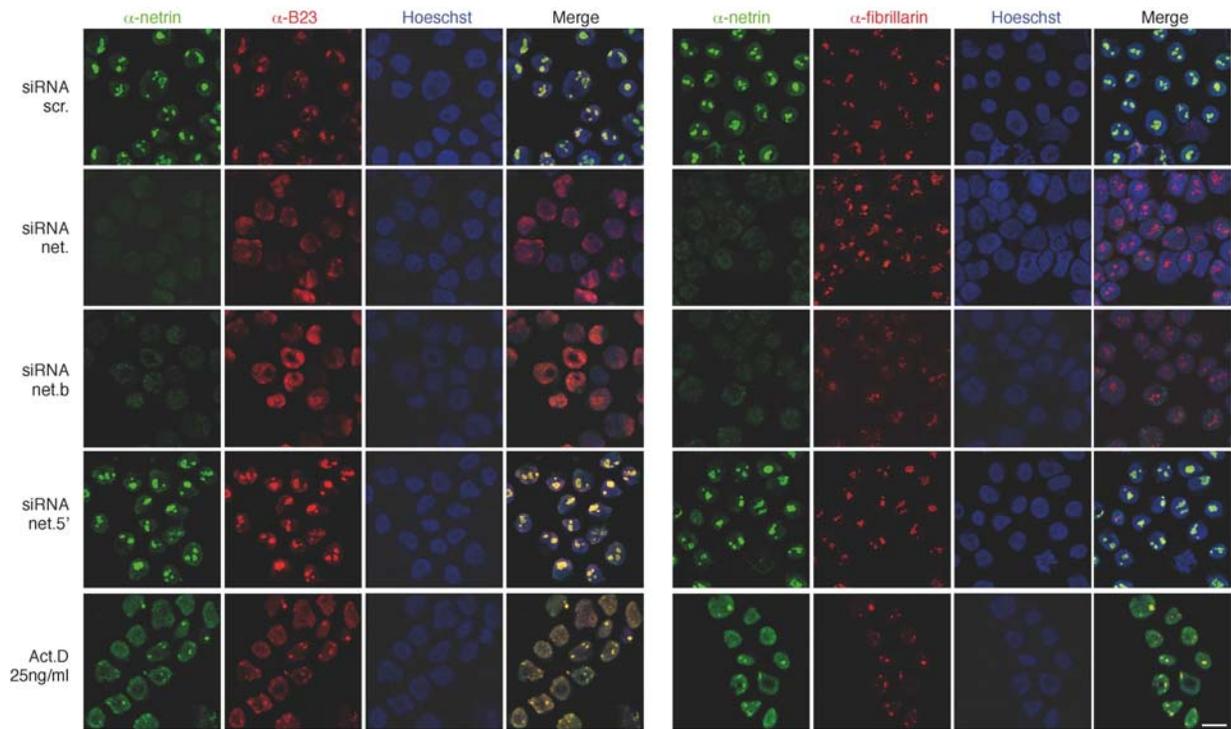
Fig. S3. Netrin-1 colocalizes with B23 and nucleolin in the nucleolus.

Fig. S4. Characterization of the netrin-1 alternative promoter and Δ N-netrin-1.

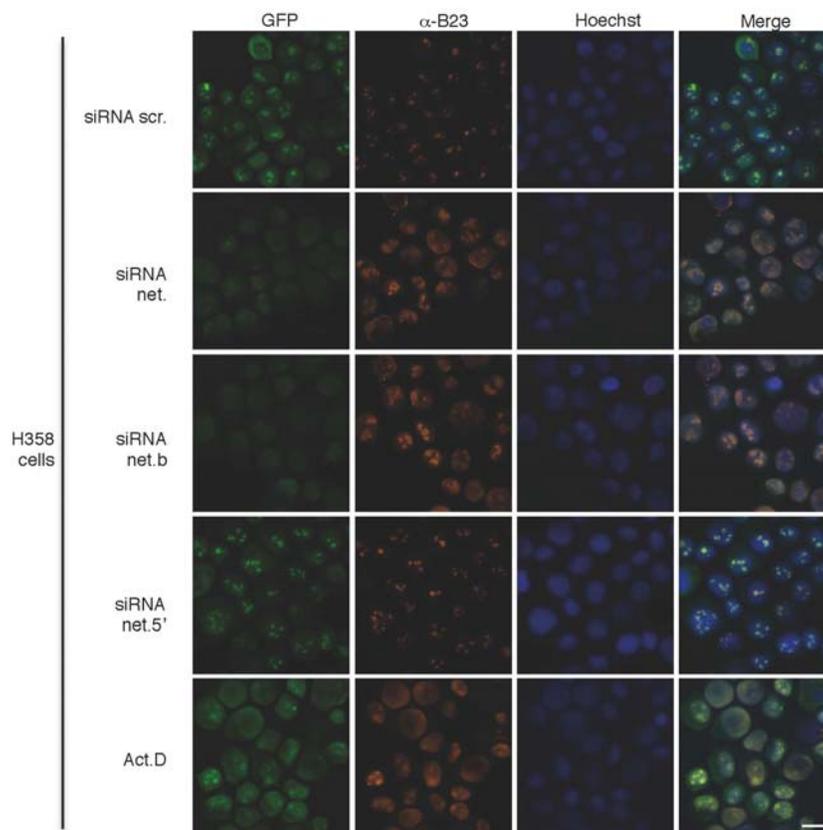
Fig. S5. Δ N-netrin-1 specifically associates with ribosomal DNA.

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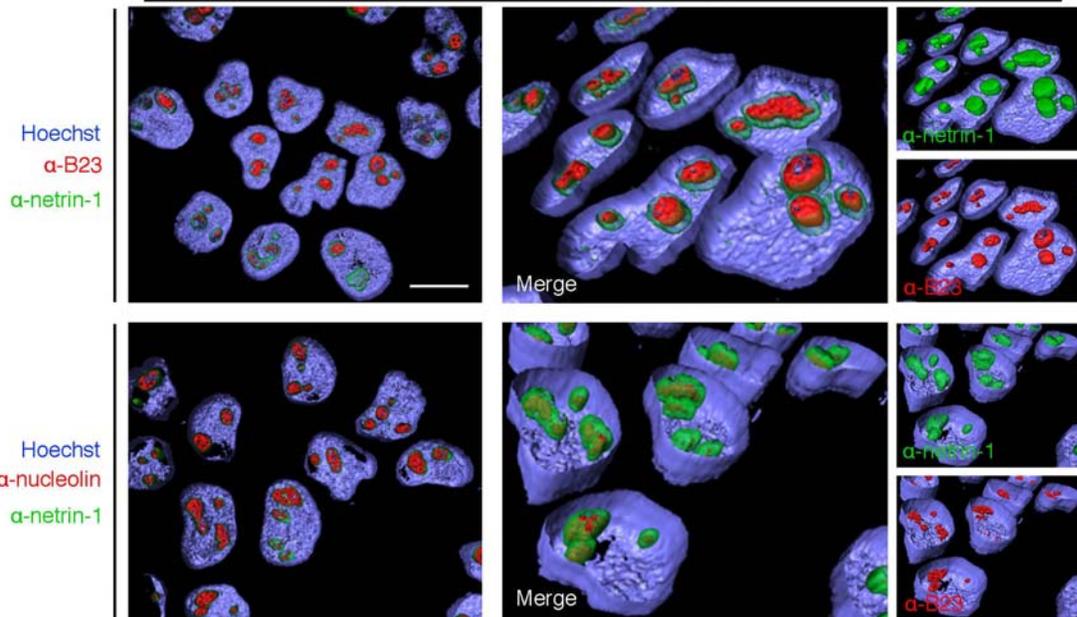


Supplementary Figure 1: Depletion of Δ N-netrin-1 affects B23 localization within the nucleolus in IMR32 cells. IMR32 cells were transfected with scrambled siRNA, siRNA specific for full-length netrin-1 (siRNA net.5') or siRNAs targeting both full-length and nucleolar netrin-1 transcripts (siRNA net.a and siRNA net.b). Cells were immunostained with antibodies specific to netrin-1 (green) and B23 (red; left) or fibrillarilin (red; right) and nuclei were visualized with Hoescht staining (in blue). Immunostaining was analyzed by confocal microscopy. IMR32 cells treated for 1 hour with 25 ng/ml actinomycin D were used as controls. Images are representative of 4 sets of cells imaged per condition. Scale bar, 10 μ m.



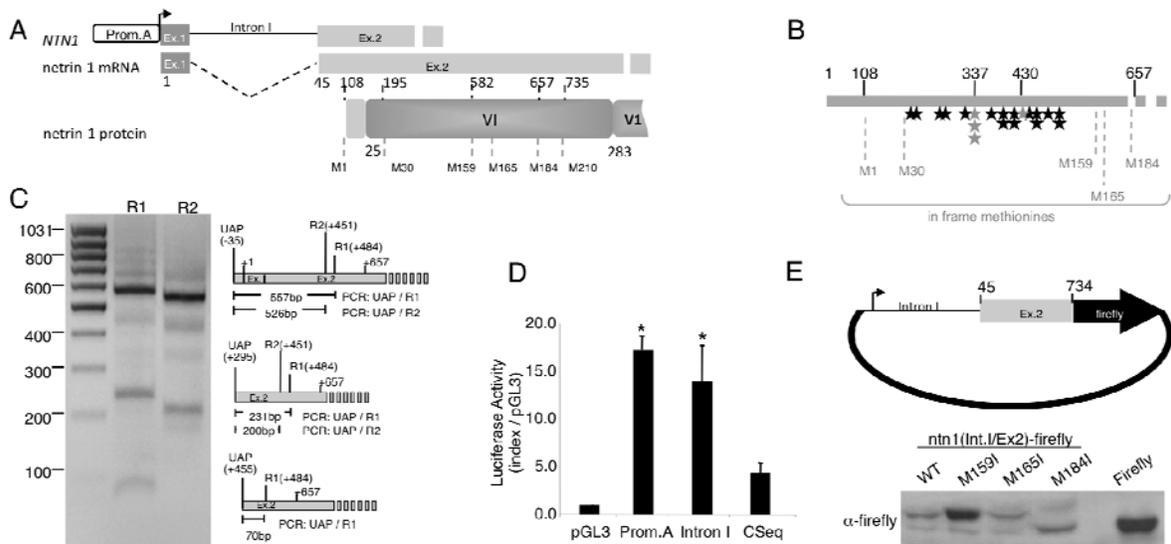
Supplementary Figure 2: Depletion of Δ N-netrin-1 affects B23 localization in the nucleolus in H358 cells. H358 cells were transfected with scrambled siRNA, siRNA specific for full-length netrin-1 (siRNA net.5') or siRNAs targeting both full-length and nucleolar netrin-1 transcripts (siRNA net. and siRNA net.b). Cells were immunostained with antibodies specific to netrin-1 (green) or anti-B23 (orange) and nuclei were visualized with Hoescht staining (blue). Immunostaining was analyzed by fluorescence microscopy. H358 cells treated for 1 hour with 25 ng/ml actinomycin D were used as controls. Images are representative of 3 sets of cells imaged per condition. Scale bar, 10 μ m.

3D reconstruction - IMR32 cells



Supplementary Figure 3: Netrin-1 colocalizes with B23 and nucleolin in the nucleolus.

IMR32 cells were immunostained with antibodies specific to netrin-1 (green), B23 (red, upper panels) or nucleolin (red, lower panels) and nuclei were visualized with Hoescht staining (in blue). Immunostaining was analyzed by confocal microscopy and 3D reconstruction was performed on a stack of images collected by depth profiling. Images are representative of 4 cells imaged per condition. Scale bar, 10 μ m.



Supplementary Figure 4: Characterization of the netrin-1 alternative promoter and ΔN -netrin-1.

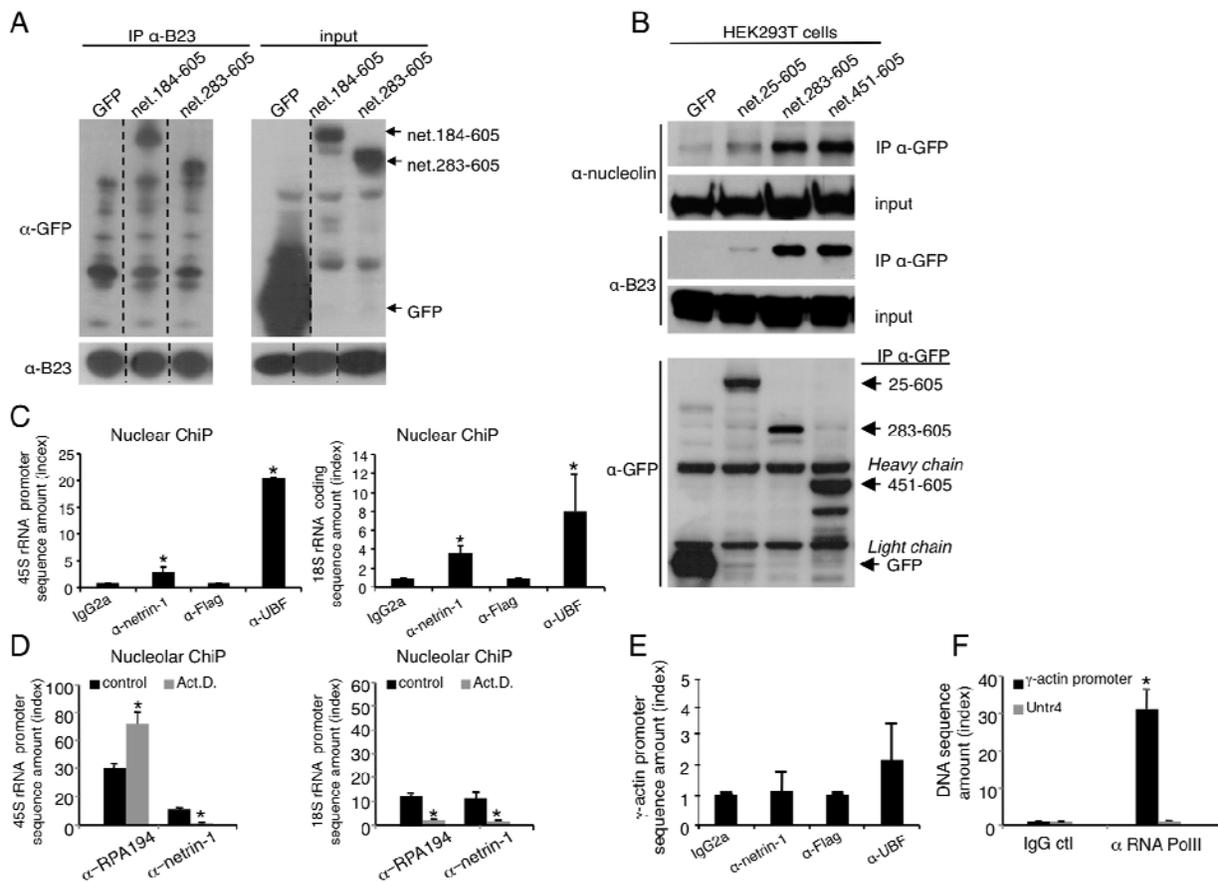
A. Schematic representation of the netrin-1 gene (*NTN1*), mRNA and protein sequence. In-frame ATG codons are annotated. **B.** Putative transcription start sites in the 5' region of human netrin-1 cDNA found by 5'-RACE. Grey asterisks indicate those identified in IMR32 cells, and black asterisks indicate those identified in other cell lines. **C.** Netrin-1 transcripts obtained from 5'-RACE were amplified using netrin-1 specific reverse primers R1 and R2 (located in exon 2) and adapter primer UAP. 5'-end positions of major bands were determined by sequencing. N=3 experiments. **D.** Relative luciferase activity from IMR32 cells transfected with netrin-1 promoter A or intron I sequences cloned upstream the luciferase reporter gene. Netrin-1 sequence corresponding to nt 921-1919 was used as a control sequence (CSeq). *, p<0.05 by Mann-Whitney test compared to luciferase activity in pGL3-transfected condition (N=3 independent experiments). **E.** Firefly luciferase immunoblot from HEK293T cells transfected with a construct bearing the netrin-1 intron I and the first 689 bp of exon 2 (Ex.2 45-734) cloned in frame with the firefly luciferase coding sequence (upper panel) or this construct with Met-to-Ile mutations at indicated ATG codons. A CMV-firefly luciferase was used as a control. N=4 experiments.

As described in the main text, the netrin-1 gene (*NTN1*), which is located on chromosome 17, contains 7 exons encoding a 5954 bp transcript. The translation initiation site ATG¹⁰⁸ and most of domain VI are encoded by exon 2 (Fig. S4A). A netrin-

1 promoter spanning a 1.7 kb genomic fragment upstream of the first non-coding exon of netrin-1 has been described (42) (Prom.A, Fig. S4A). In a search for a potential second transcript, 5'-RACE (rapid amplification of cDNA ends) was performed with cDNAs obtained by the RNA ligase-mediated RACE (RLM-RACE) method from different cell lines (IMR32, SKBR7, LAN5, HBL100, HCT116, H322, and H358 cells). This demonstrated the presence of several transcription initiation sites in a region corresponding to netrin-1 cDNA nt 295-455 (Fig. S4B). IMR32 cells produced a transcript including the expected +1 transcription site but also two main shorter transcripts, starting at +295 and +455 in reference to the numbering of the canonical *NTN1* transcript (Fig. S4C). These data support the existence of short netrin-1 transcripts that do not contain the usual ATG¹⁰⁸ codon found in full-length netrin-1.

To assay whether these shorter transcripts may be the products of an alternative promoter located in intron I, the "classic" *NTN1* promoter A (Prom.A), the whole *NTN1* intron I or a similar size unrelated DNA sequence (CSeq) were cloned upstream of the firefly luciferase coding sequence. When transfected in IMR32 cells, the Prom.A sequence drove the expression of firefly luciferase compared to the empty vector or to the Cseq DNA sequence (Fig. S4D). Similarly, the intron I sequence triggered expression of the firefly luciferase compared to the canonical promoter (Fig. S4D). Thus, these data indicate the existence of an alternative promoter which includes the first intron of the *NTN1* gene.

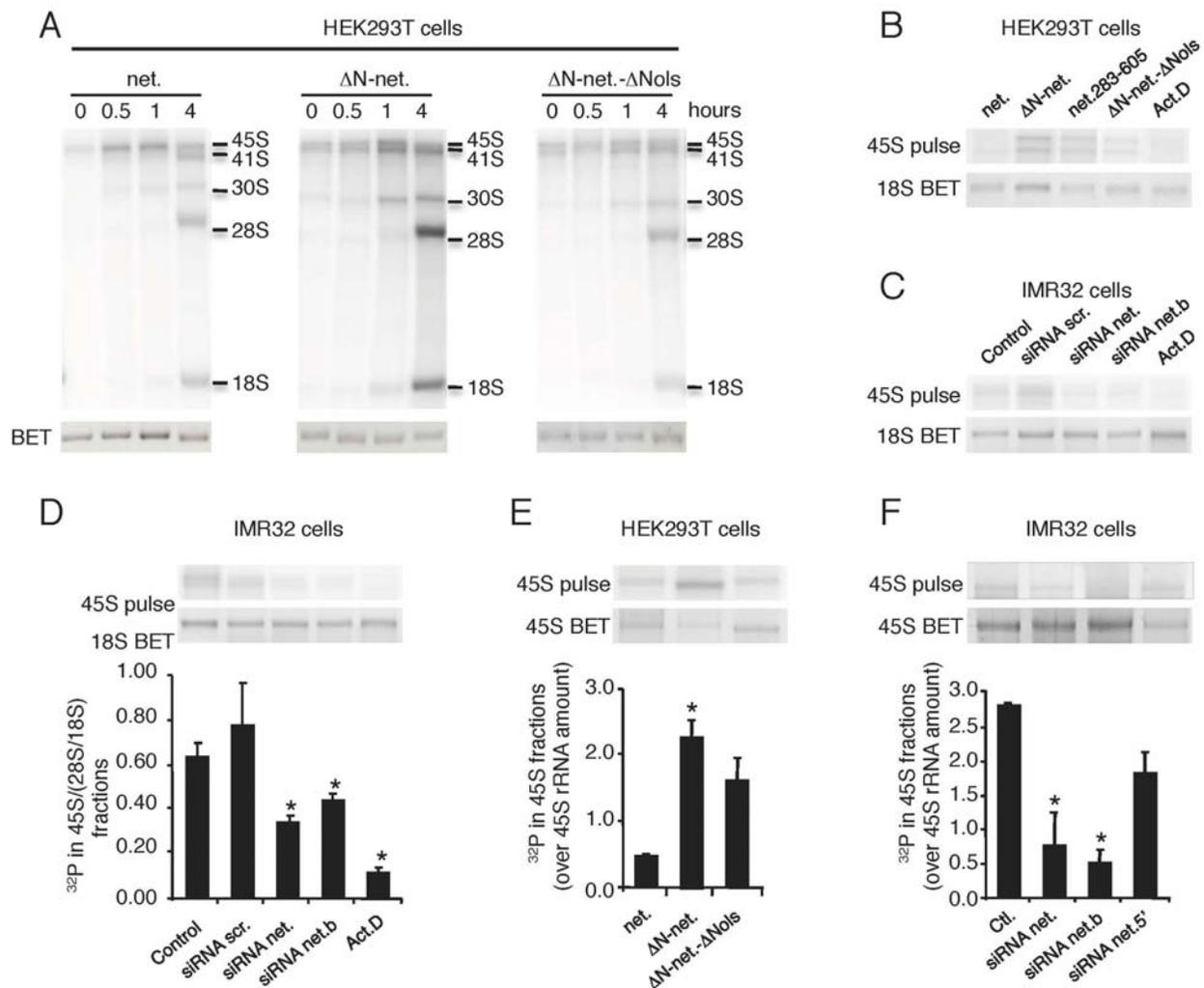
To determine the nature of the netrin-1 protein encoded by this alternative promoter, we inserted the firefly luciferase cDNA in frame in a construct that included the intron1-exon2 *NTN1* sequence (Fig. S4E). Transfection of this construct led to the production of a luciferase protein with a higher molecular weight than wild-type luciferase (Fig. S4E). This observation supports the presence of an in-frame ATG codon within nt 531-734 of netrin-1 cDNA sequence which was used instead of the usual firefly ATG codon. Methionines 159, 165 or 184 within the netrin-1 protein sequence appeared to be in frame with the firefly coding sequence and were independently mutated in the intron1-exon2-firefly construct. Although mutation of methionine 159 (M159I) or 165 (M165I) into isoleucine did not affect protein size, mutation of methionine 184 (M184I) was associated with the production of a firefly-only protein (Fig. S4E). Analysis of the netrin-1 sequence by 2 different programs [NetStart 1.0 prediction server (58) and ATGpr (59)] predicts that ATG⁶⁵⁷ (which encodes Met184) is more reliable for translation initiation than ATG⁵⁸² or ATG⁶⁰⁰ (which respectively encode Met159 and Met165).



Supplementary Figure 5: ΔN-netrin-1 specifically associates with ribosomal DNA.

A. HEK293T cells were transfected with constructs encoding different GFP-tagged netrin fragments or GFP alone and cell lysates were subjected to immunoprecipitation with a B23 specific antibody. GFP-fusion proteins were detected by Western blot in immunoprecipitated and input fractions. N=3 experiments. **B.** HEK293T cells were transfected with constructs encoding different GFP-tagged netrin fragments and cell lysates were subjected to immunoprecipitation with a GFP specific antibody. Nucleolin and B23 were detected by Western blot in immunoprecipitated and input fractions. N=3 experiments. **C.** Chromatin immunoprecipitation of total nuclear chromatin from IMR32 cell lysate was performed with an anti-netrin-1 specific antibody. 45S rRNA promoter (left panel) and 18S rRNA coding sequence specific primers (right panel) were used to quantify immunoprecipitated rDNA sequences. Anti-UBF antibody was used as a positive control. Rat IgG2A and anti-Flag antibody were used as negative controls for netrin-1 and UBF antibodies respectively. **D.** Chromatin immunoprecipitation was performed in IMR32 cells lysate after enrichment in nucleolar chromatin with anti-netrin-1 and anti-RPA194 specific antibodies in control condition or after treatment for 1 hour with actinomycin D (50 ng/ml). 45S rRNA promoter (left panel) and 18S rRNA coding

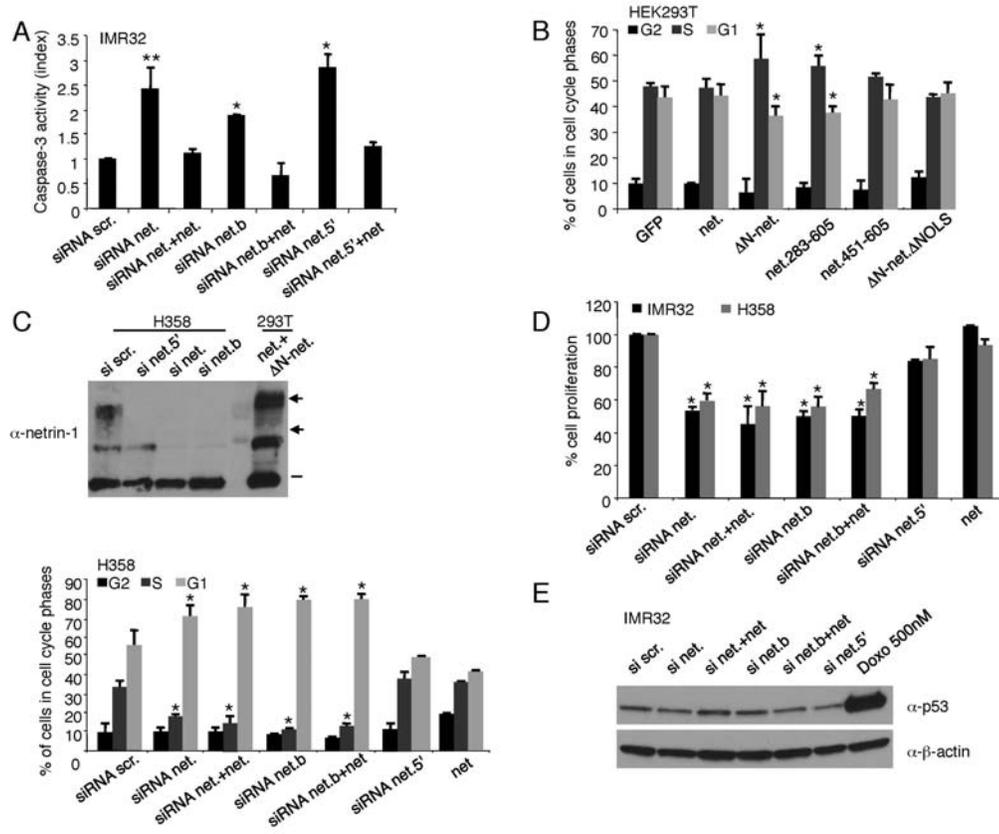
sequence specific primers (right panel) were used to quantify immunoprecipitated rDNA sequences. **E.** Chromatin immunoprecipitation of total nuclear chromatin from IMR32 cell lysate was performed with an anti-netrin-1 specific antibody. γ -actin promoter specific primers were used to quantify immunoprecipitated DNA sequences. Anti-Flag and anti-UBF antibodies were used as negative controls. **F.** Chromatin immunoprecipitation of total nuclear chromatin from IMR32 cell lysate was performed with an anti-RNA polymerase II specific antibody or an isotypic unrelated antibody. γ -actin promoter (or untranscribed 4 as a negative control) specific primers were used to quantify immunoprecipitated DNA sequences. For **C-F**, *, $p < 0.05$ by Mann-Whitney test compared to each negative control antibody condition (N=4 independent experiments).



Supplementary Figure 6: ΔN-netrin-1 promotes ribosome biogenesis.

A. Analysis of rRNA intermediates produced in HEK293T cells transfected either with full-length netrin-1 (net.), ΔN-netrin-1 (ΔN-net.) or ΔN-netrin-1 lacking its nucleolar localization sequence (ΔN-net.-ΔNols) at different times after ³²P labeling. Cells were labeled for 1 hour with ³²P (pulse) and then harvested at different times after the medium was replaced with ³²P-free medium. Detection of total 18S rRNA on an ethidium bromide agarose gel is also shown. (N=3 experiments). **B, C.** Representative autoradiograms showing ³²P-labeled 45S rRNA and ethidium bromide agarose gels showing total 18S rRNA are presented for HEK293T cells (B) and IMR32 cells (C) after respectively 30 min and 4 hours chase periods. **D.** Quantification of 45S rRNA produced in IMR32 cells in ³²P-pulse-chase experiment. Quantification of 45S rRNA 1 hour after the chase is shown. *, p<0.05 by Mann-Whitney test compared to the control-transfected condition (N=3 independent experiments). Representative autoradiogram

showing ^{32}P -labeled 45S rRNA and ethidium bromide agarose gel showing total 18S rRNA 1 hour after the chase are also presented. **E,F.** Quantification of 45S rRNA produced in HEK293T cells transfected with different GFP-netrin-1 fusion constructs (**E**) and IMR32 cells transfected with siRNAs targeting ΔN -netrin-1 or full-length netrin-1 (**F**) in a short ^{32}P -pulse-chase experiment. Quantification of 45S rRNA 15 min after the chase is shown. ^{32}P -labeled 45S rRNA amounts were quantified upon normalization to the amount of total 45S rRNA measured on an ethidium bromide agarose gel. Representative autoradiogram showing ^{32}P -labeled 45S rRNA and ethidium bromide agarose gel showing total 45S rRNA are shown. *, $p < 0.05$ by Mann-Whitney test compared to the netrin-1- (**E**) or to the control- (**F**) transfected condition. N=3 independent experiments.



Supplementary Figure 7: Nucleolar Δ N-netrin-1 promotes cell proliferation.

A. Caspase-3 activity was measured in IMR32 cells 24 hours after transfection with scrambled siRNA, siRNA specific for full-length netrin-1 (siRNA net.5') or siRNAs targeting both full-length and nucleolar netrin-1 transcripts (siRNA net. and siRNA net.b) with or without addition of recombinant full-length netrin-1 in the culture medium (net.). **, $p < 0.01$ by Mann-Whitney test compared to caspase-3 activity in scramble-transfected condition (N=5 experiments). **B.** Cell cycle analysis of HEK293T cells 24 hours after transfection with GFP-netrin-1 fragment expressing constructs. Percentage of cells in G2, S, and G1 phases is presented for each condition. *, $p < 0.05$ by Mann-Whitney test compared to percentage of cells in the corresponding phase in GFP-transfected condition (N=3 experiments). **C.** Cell cycle analysis in synchronized H358 cells transfected with scramble siRNA, or netrin-1 siRNAs (siRNA net.5' or siRNA net. and siRNA net.b) with or without the addition of recombinant netrin-1 (net.). Percentage of cells in G2, S and G1 phases is presented. Representative netrin-1 immunoblot is shown. Arrows point at full-length netrin-1 and Δ N-netrin-1. *, $p < 0.05$ by Mann-Whitney test compared to percentage of cells in the corresponding phase in scrambled-siRNA-transfected condition (N=4 experiments). **D.** Cell proliferation measured by MTS assay in IMR32 and H358 cells transfected with scrambled siRNA, siRNA specific for full-length netrin-1 (siRNA net.5') or siRNAs targeting both full-length and nucleolar netrin-1 transcripts (siRNA net.a and siRNA net.b) with or without addition of recombinant full-length netrin-1 in the culture medium (net.). *, $p < 0.05$ by Mann-Whitney test compared

to the proliferation rate of scrambled siRNA-transfected cells (N=4 experiments). **E.** Western blot analysis of p53 abundance in IMR32 cells 24 hours after transfection with scrambled siRNA, siRNA specific for full-length netrin-1 (siRNA net.5') or siRNAs targeting both full-length and nucleolar netrin-1 transcripts (siRNA net. and siRNA net.b) with or without addition of recombinant full-length netrin-1 in the culture medium (net.). IMR32 cells treated for 2 hours with 500 nM doxorubicin were used as a positive control. N=2 independent experiments.