SUPPLEMENTAL INFORMATION

Methods

Reagents and antibodies. The pan-caspase inhibitor as well as inhibitors of caspases 2 and 3/7 were purchased from Calbiochem or R&D Systems; purified caspase 2 and caspase 3 were purchased from Calbiochem, and purified netrin-1 was provided by Dr. Arakawa (National Cancer Center Research Institute, Tokyo, Japan). The antibodies used in this study included anti-caspase 3 (Calbiochem), anti-E2F1 (C-20 & KH-95, Santa Cruz), anti-PARP (Santa Cruz), anti-NGF (Sigma-Aldrich), anti-Flag (M2, Sigma-Aldrich) and anti-actin (Sigma-Aldrich) antibodies.

Plasmid Construction. Human UNC5D cDNA was cloned as described previously (17). Human UNC5D, UnICD, and DD cDNAs were cloned and introduced into the pCMV vector, and deletion mutants of UnICD were in-frame inserted into the pM vector. Point mutations were generated using the Quick-change Kit (Stratagene). Nucleotide sequencing was performed to confirm the sequences of all constructs.

Semi-quantitative and quantitative RT-PCR. Total RNA was prepared using an RNeasy mini kit (Qiagen). Primers used in real-time RT-PCR for *UNC5D* include

5’-TGAACTGCAGATGCCATAGG-3’ (forward) and 5’-GGTTTCAGGGACACTGT GGT-3’ (reverse); probe: 5’-FAM-ACATGAAGGTCATCGAGCCCTACAGGAGAG TAMRA-3’. Quantitative RT-PCR analysis was performed using the ABI7700 Prism sequence detector (Applied Biosystems), according to the manufacturer’s instructions using the TaqMan Universal PCR Master Mix. Quantification of UNC5D mRNA was carried out by comparing with a standard curve generated using the UNC5D expression plasmid. As a control, the primers and probes for *GAPDH* were purchased from Applied Biosystems. GAPDH mRNA quantification was also performed for normalization of the initial RNA content in each sample.

For semi-quantitative RT-PCR, the sequences of primers are available upon request.

IP and IB. Whole cell lysates were prepared as described previously (24). For nuclear extracts, cells were lysed with lysis buffer A (10 mM Tris (pH=7.5), 1 mM EDTA, 0.1% (v/v) NP-40, 1 mM PMSF) supplemented with protease inhibitor cocktail (Sigma-Aldrich) for 10 minutes on ice and cleared by centrifugation. Resultant pellets were washed twice using lysis buffer A and then incubated with ice-cold RACK1 lysis buffer supplemented with protease inhibitor cocktail. After sonication, nuclear extracts were pre-cleaned with protein A Sepharose beads, and incubated with the indicated

antibody overnight at 4 °C, followed by incubation with pre-cleaned BSA-coated protein A beads for an additional 1 h. The immune complexes were extensively washed with the RACK1 lysis buffer, dissolved in 2x SDS sample buffer, and resolved by SDS-PAGE. Proteins were transferred onto Immobilon-P membranes (Millipore) and probed with the indicated antibodies. The membranes were developed with an ECL detection system.

In vitro *Caspase Cleavage Assay.* Human UNC5D and the D416N mutant were expressed *in vitro* using a T7 Quick Coupled Transcription/Translation System (Promega) in the presence of [^{35}S]methionine according to the manufacturer's recommendations. Prepared proteins were then incubated with caspase 2 or 3 for 1 h at 37 °C (58). Protein products were resolved by SDS-PAGE and detected by autoradiography.

Supplemental Figure 1

UNC5D is a novel favorable prognostic indicator for NB. (**A**) Expression levels of the UNC5 family and their ligand netrin-1 were detected by semi-quantitative RT-PCR in primary NBs. GAPDH expression is shown as an internal control. (**B**) Kaplan-Meier survival curves based on higher or lower expression levels of *UNC5A* mRNA in 102 patients with neuroblastoma.

Supplemental Figure 2

Enlarged images of immunohistochemical analysis shown in Figure 1B. F, favorable; UF, unfavorable. Original magnification, ×400.

Supplemental Figure 3 Unc5d knockdown attenuates NGF deprivation-induced cell death in PC12 cells. (**A**) FACS analysis of PC12 cells treated with NGF and subsequent withdrawal as indicated. Data represent mean ± SD. (**B**) Semi-quantitative RT-PCR using the cells in (**A**). (**C**) PC12 cells were transfected with siRNAs against Unc5d or a negative control and then subjected to NGF treatment (50 ng/mL) for 6 days with or without subsequent withdrawal for 2 additional days. Semi-quantitative RT-PCR was conducted to measure the expression of the genes indicated. (**D**) Representative light

microscopic images of the cells in (**C**). Original magnification, ×40. (**E-F**) Apoptotic cell death was detected by TUNEL assay in the cells in (**C**). Nuclei were stained with DAPI. Original magnification, ×100. TUNEL positive cells were counted and represented as the percentage of the total cells. Results are shown as mean ± SD (n=2). *, $P < 0.01$.

Supplemental Figure 4

UNC5D is cleaved by caspases 2 and 3. (**A**) *In vitro* caspase 2 cleavage assay. Full-length UNC5D, UnICD and the D416N mutant were translated *in vitro* in the presence of [^{35}S] methionine using a T7 Quick Coupled Transcription/Translation System (Promega). Proteins were incubated with or without an increasing amount of purified caspase 2 in the presence or absence of the caspase-2 specific inhibitor z-VDVAD-fmk (z-VDVAD) for 1 h at 37 °C. Protein products were resolved by SDS-PAGE and detected by autoradiography. All the lanes were run on the same gel but were noncontiguous. (**B**) The *in vitro* caspase 3 cleavage assay was performed as described in (**A**) using purified caspase 3 instead. (**C**) Double immunostaining was performed using the monoclonal anti-FLAG antibody and polyclonal anti-Unc5d antibody recognizing the C-terminus in U2OS cells transfected with N-terminal

FLAG-tagged mouse Unc5d. Nuclei were stained with DAPI. Original magnification, ×400. (**D**) Subcellular fractionations of UnICD-transfected HeLa cells. WCL, whole cell lysate; C, cytoplasmic fraction; N, nuclear fraction. (**E**) Colony formation assay of U2OS cells transfected with the indicated plasmids. Cells transfected with UNC5D were treated with or without a pan-caspase inhibitor (PI). Results are shown as mean ± SD of two independent experiments. *, $P < 0.01$.

Supplemental Figure 5 UNC5D is induced in response to various apoptotic stimuli. (**A**) FACS analysis of SH-SY5Y, U2OS and HeLa cells treated with ADR, CDDP or TNFα, respectively. (**B**) Semi-quantitative RT-PCR analysis of UNC5D expression in the cells treated with ADR, CDDP or TNFα, as indicated in (**A**). (**C**) UNC5D is the target of the p53 family. SH-SY5Y cells were transiently transfected with a mock, a p53 or a p73 expression vector. SK-N-AS cells were transiently transfected with a mock or a p63 expression vector. Forty-eight hours later, total RNAs were isolated and semi-quantitative RT-PCR was performed to determine the expression of the indicated genes. (**D**) Immunofluorescent analysis of U2OS and SH-SY5Y cells treated with ADR, CDDP or TNFα. Original magnification, ×400.

Supplemental Figure 6

Unc5d$^{-/-}$ MEFs have a resistance to CDDP treatment. (**A**) Semi-quantitative RT-PCR analysis of *Unc5d*$^{+/+}$ and *Unc5d*$^{-/-}$ MEF cells treated with CDDP (15 μM, 24 h) and TNFα/CHX (TNFα 30 ng/mL, CHX 10 μg/mL, 10 h), respectively. (**B**) Immunoblot analysis of MEFs treated in (**A**). PC, positive control. A crude protein extract isolated from wild-type mouse brain was used as a positive control. PI, pan-caspase inhibitor. All the lanes were run on the same gel but were noncontiguous. (**C**) Immunofluorescent staining was performed using an antibody recognizing the C-terminal region of mouse Unc5d protein in *Unc5d*$^{+/+}$ and *Unc5d*$^{-/-}$ MEF cells treated with CDDP (15 μM, 24 h). Nuclei were stained with DAPI. Original magnification, ×400. (**D**) MTT assay of *Unc5d*$^{+/+}$ and *Unc5d*$^{-/-}$ MEFs treated with CDDP at the indicated doses for 24 h. Results are shown as mean ± SD (n=3). **P* < 0.05, ** *P* < 0.01.

Supplemental Figure 7

UnICD interacts with E2F1 in the nucleus. (**A**) Expression of transfected UnICD in U2OS and HeLa cells. (**B**) Efficiency of E2F1 knockdown by siRNAs against E2F1. #1 and #4 siRNAs were used in the experiment of Figure 8E. (**C**) HeLa cells were co-transfected with full-length UNC5D or UnICD and E2F1 expression vectors and

processed for immunofluorescent staining with the indicated antibodies. Original magnification, ×400. (**D**) Confocal images of UNC5D and E2F1 immunostaining in HeLa cells treated with CDDP (20 μM, 24 h). Nuclei were stained with DAPI. Original magnification, ×400. (**E**) Nuclear extracts were prepared from HeLa cells co-transfected with E2F1 and UnICD, and subjected to immunoprecipitation (IP), followed by immunoblotting with the indicated antibodies. (**F**) U2OS cells were treated with or without CDDP (80 μM, 24 h), followed by the ChIP assay using the indicated antibodies or normal rabbit IgG. (**G**) Semi-quantitative RT-PCR using the cDNAs generated from U2OS or HeLa cells transfected with the indicated expression vectors. (**H**) Expression of E2F1 promoting proliferation and survival targets in SH-SY5Y, U2OS and HeLa cells transfected with the indicated expression vectors. (**I**) Expression of E2F1 targets in primary NB cells in Figure 3B.

Supplemental Table 1

Expression of UNC5D and TrkA in 11 primary NBs

Case	Age (mo)	Origin	*MYCN* (copy)	Histology (INPC)	Immunostaining	
					UNC5D	TrkA
1	6	Med	1	F	-	+++
2	7	Adr	1	F	+	+++
3	7	Adr	1	F	+++	+++
4	9	Adr	1	F	+++	+++
5	25	Adr	1	F	+	+++
6	29	Med	1	F	+++	+++
7	13	Adr	Amp	UF	-	-
8	13	Adr	Amp	UF	-	+
9	96	Adr	1	UF	-	++
10	18	Med	1	UF	-	++
11	20	Adr	1	UF	-	++

mo, months; INPC, International Neuroblastoma Pathology Classification; Med, mediastinum; Adr, adrenal; Amp, amplified; F, favorable histology; UF, unfavorable histology; +, positive; -, negative.

Supplemental Table 2

Prognostic significance of expression of UNC5D in primary NBs

	N	5-year survival (%)	*P* value
UNC5A (median value)			
Low expression	49	74.6	0.794
High expression	53	71.0	
UNC5D (median value)			
Low expression	54	57.0	0.003
High expression	54	89.2	
Age			
> 18 months	56	62.7	0.056
≤ 18 months	52	82.9	
Stage (INSS)			
1, 2, 4s	51	91.1	< 0.001
3, 4	57	54.7	
MYCN			
Amplified	18	28.6	< 0.001
Single copy	90	80.6	
TrkA			
Low expression	50	55.9	< 0.001
High expression	56	86.1	
DNA ploidy			
Aneuploidy	47	97.9	< 0.001
Diploidy	45	52.5	

Supplemental Table 3

Cox hazard model: UNC5D expression in neuroblastoma

	N	*P* value	H.R.	95% C.I.
UNC5D expression	108	0.006	3.630	(1.456, 9.048)
Age (1.0yr)	108	0.005	4.543	(1.563, 13.204)
UNC5D expression	108	0.018	3.021	(1.205, 7.574)
Stages (INSS)	108	0.003	5.199	(1.774, 15.243)
UNC5D expression	108	0.239	1.889	(0.655, 5.448)
MYCN amplification	108	0.001	0.202	(0.082, 0.502)
UNC5D expression	108	0.036	2.695	(1.064, 6.825)
TrkA expression	108	0.006	0.274	(0.108, 0.695)
UNC5D expression	92	0.060	2.889	(0.956, 8.729)
DNA ploidy	92	0.001	11.756	(2.635, 52.529)

H.R., hazard ratio; 95% C.I., 95% confidence interval.

Figure S1 Zhu *et al*.

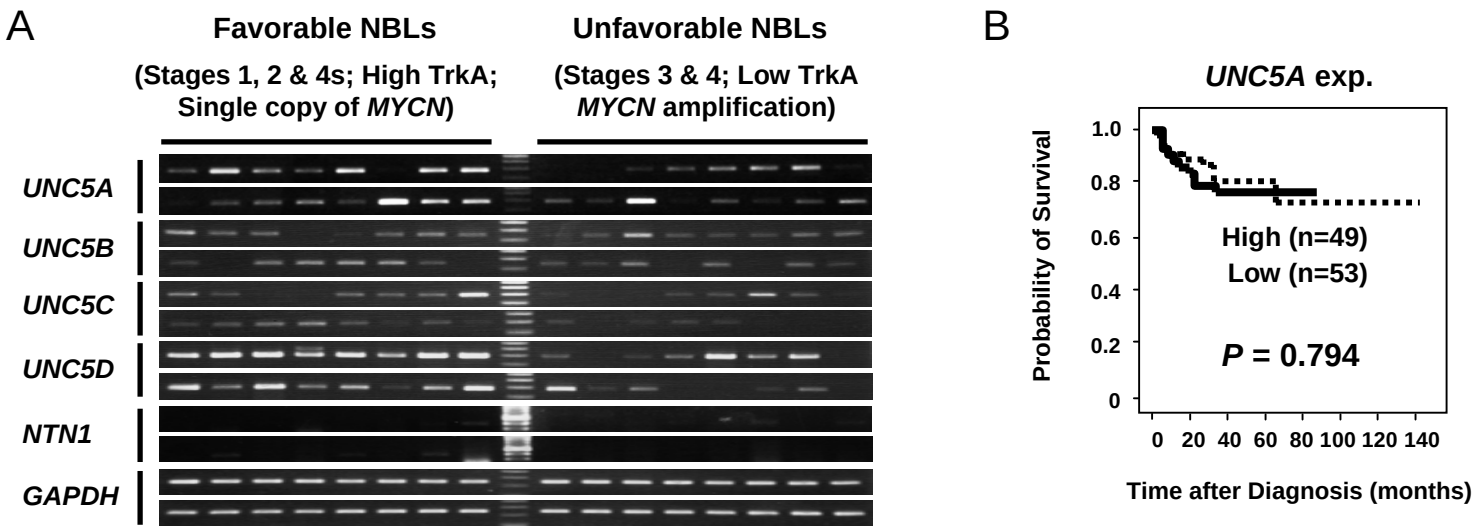


Figure S2 Zhu *et al*.

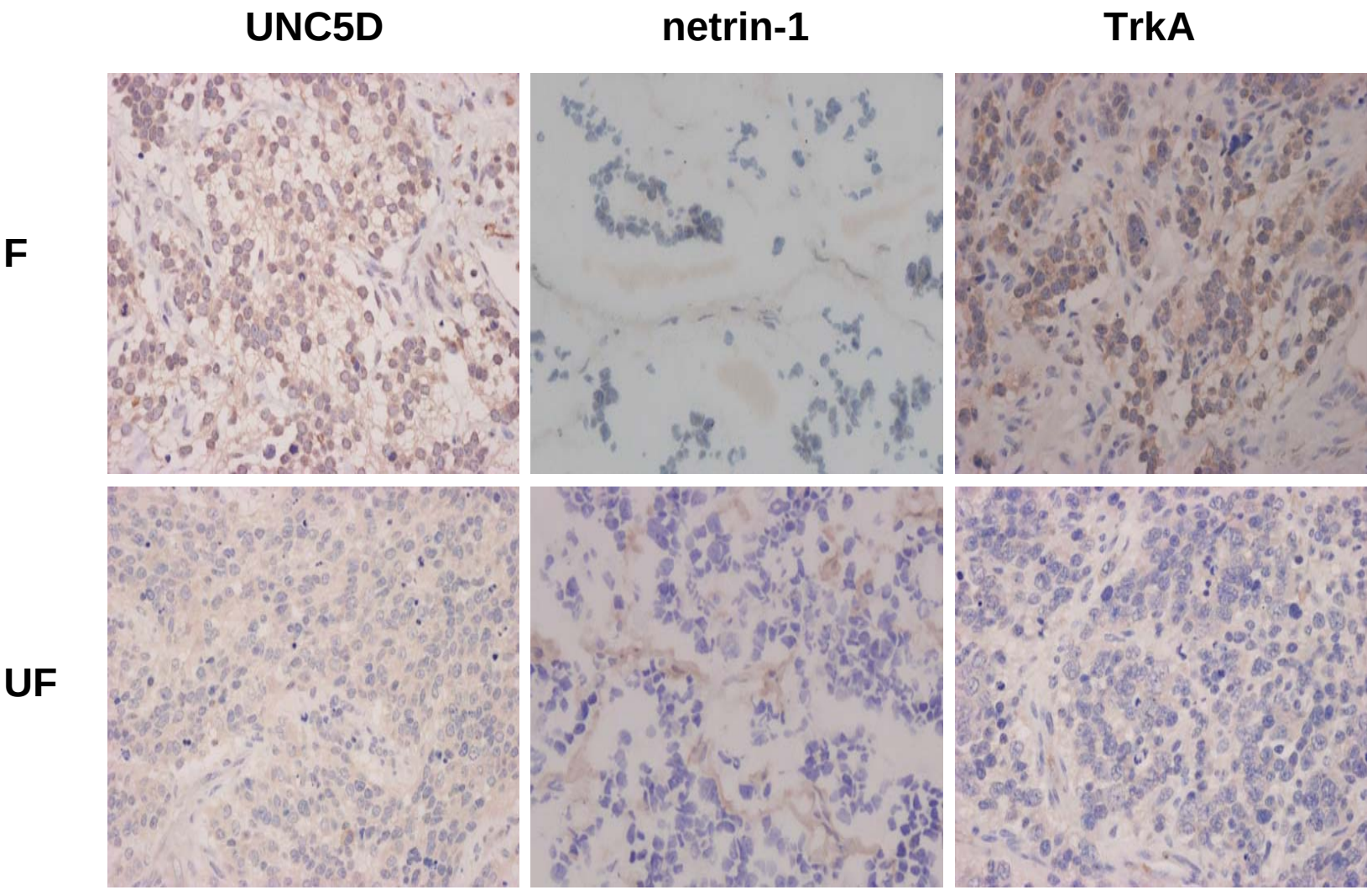
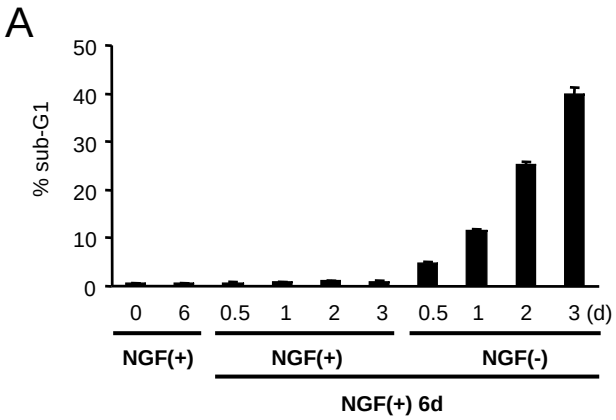
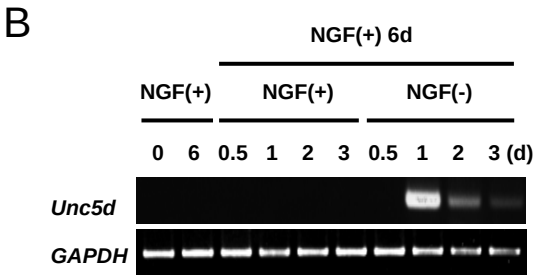
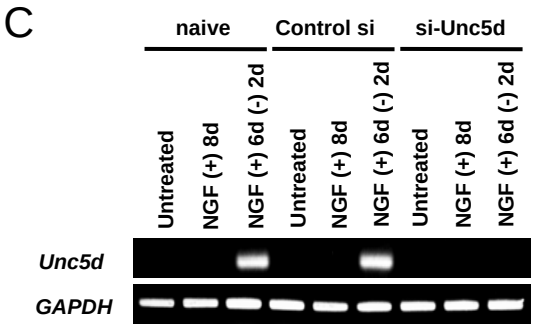
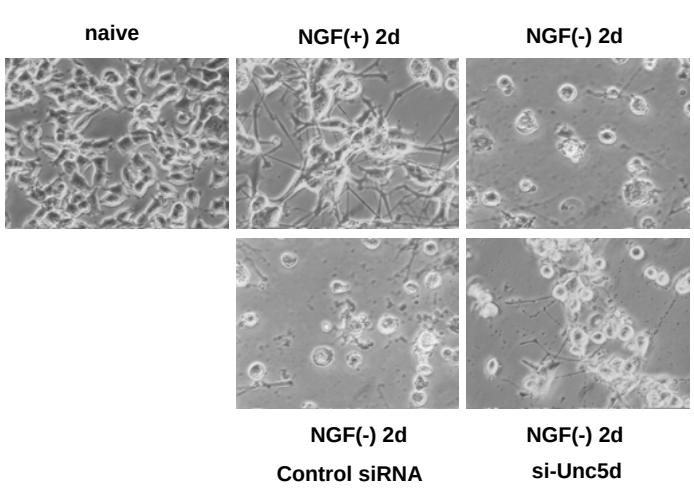


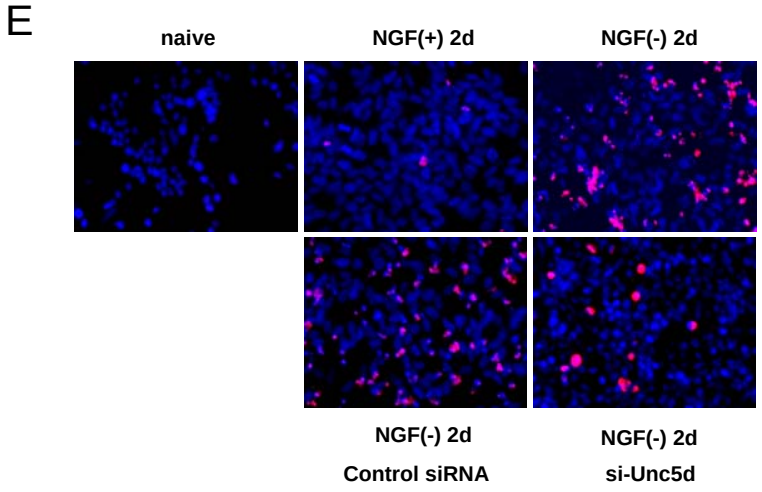
Figure S3 Zhu *et al*.











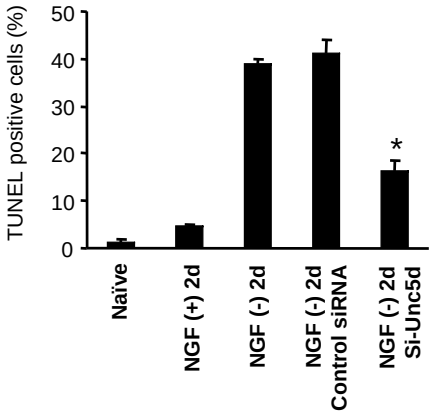


Figure S4 Zhu *et al*.

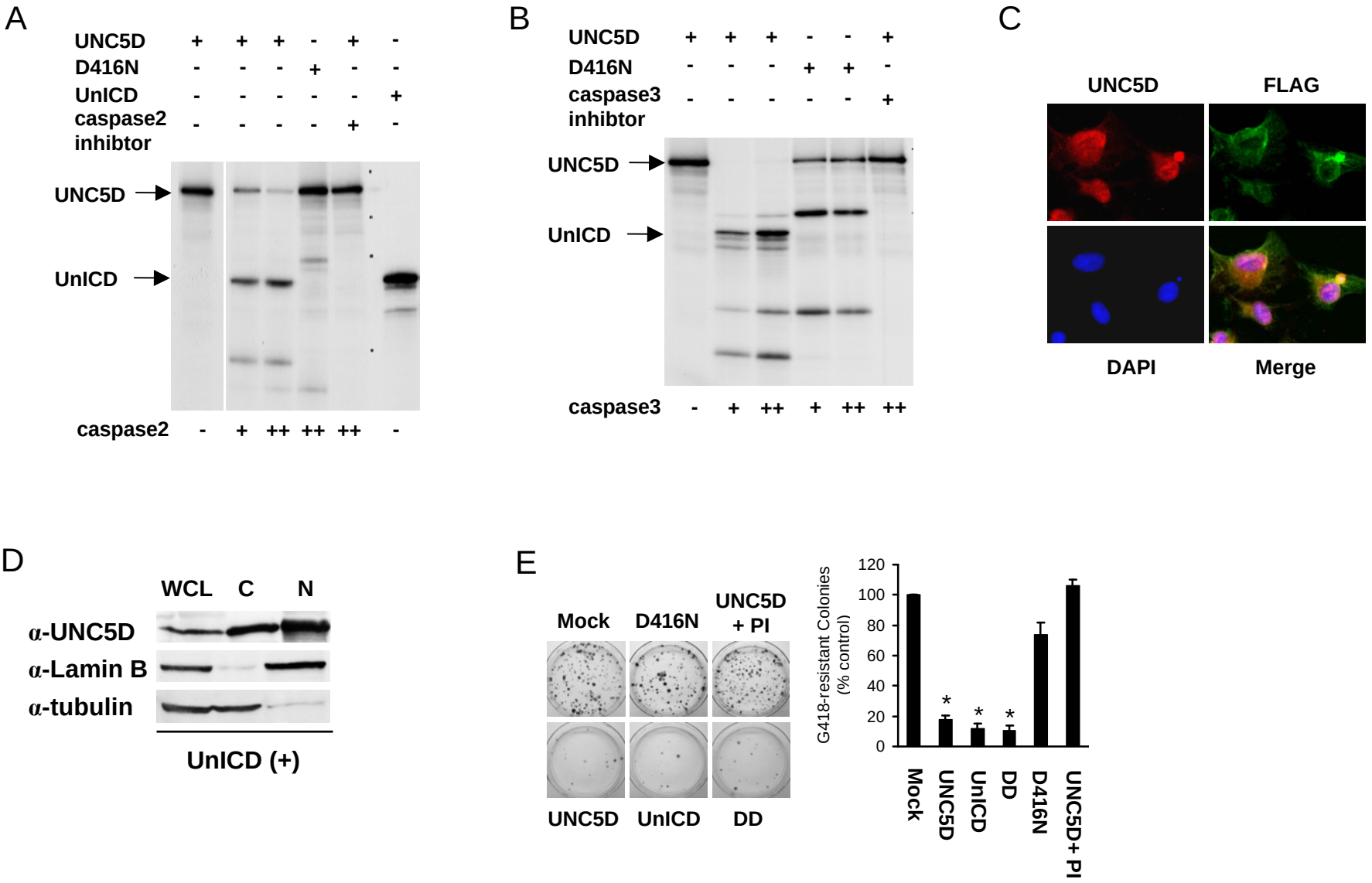


Figure S5 Zhu *et al*.

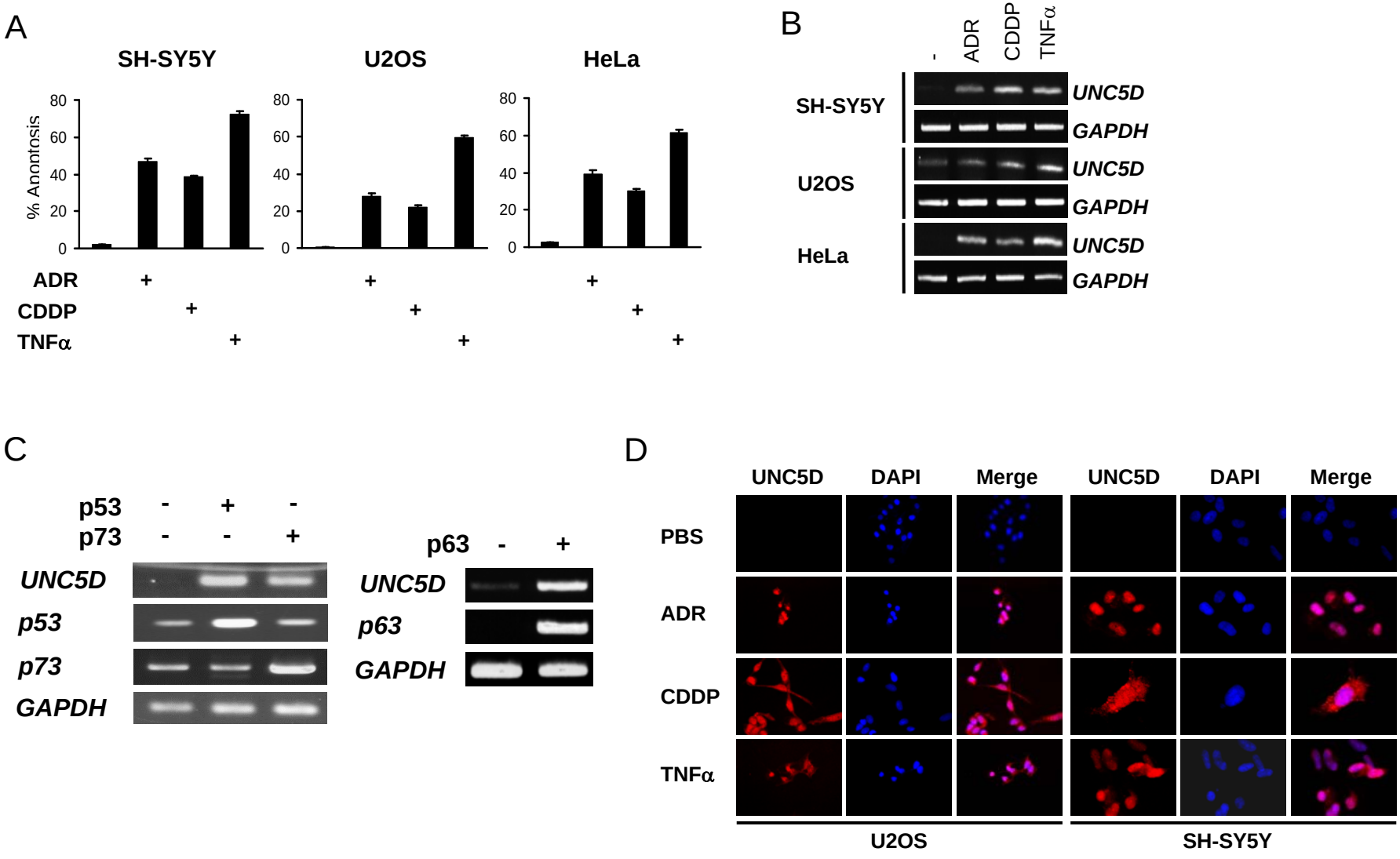


Figure S6 Zhu *et al*.

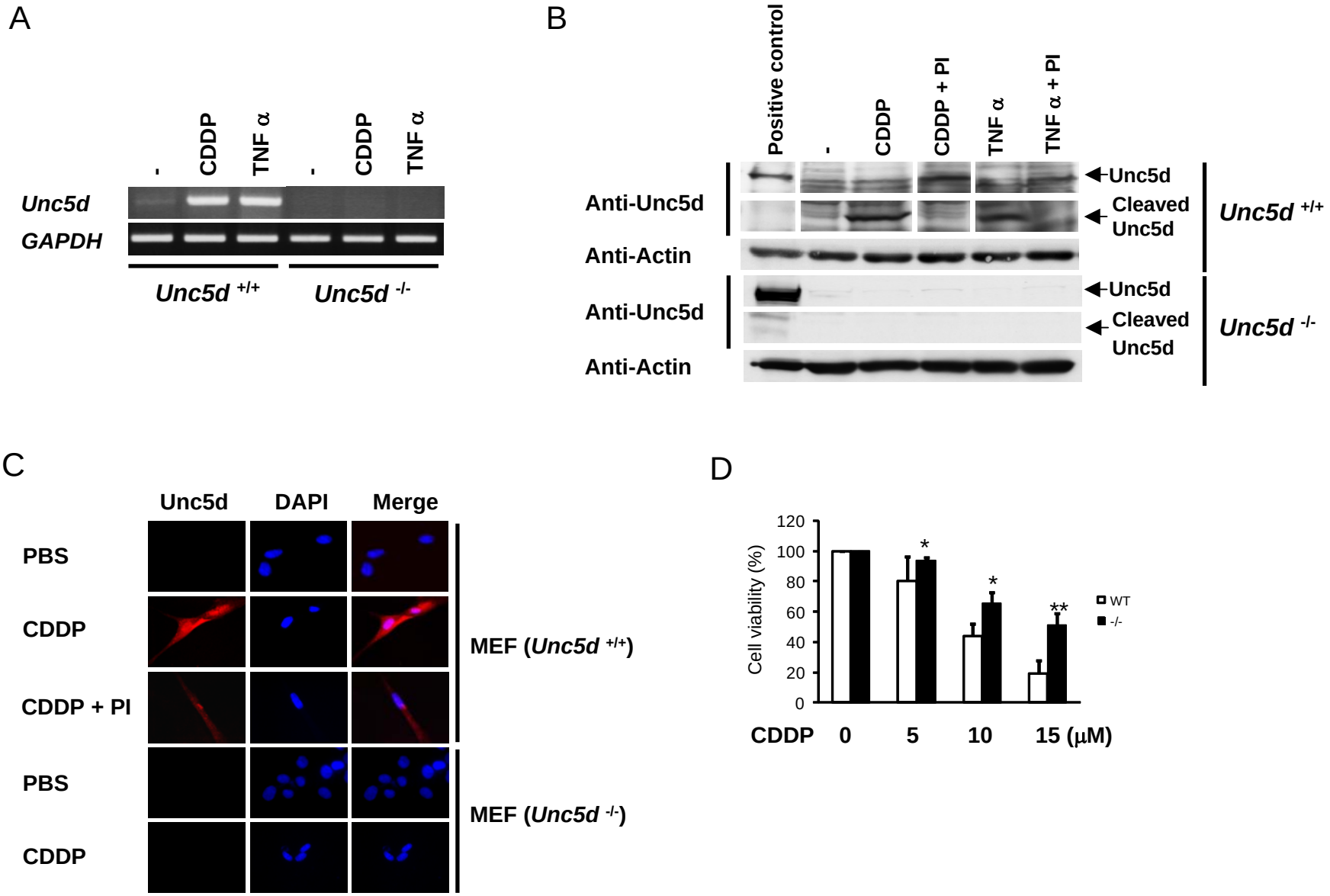


Figure S7 Zhu *et al*.