N-Cadherin Dynamically Regulates Schwannoma Migration and Represents a Novel Therapeutic Target in NF2-Related Schwannomatosis

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SUPPLEMENTARY METHODS AND MATERIALS

Cell culture

Mouse $NF2^{-/-}$ schwannoma cells (MD-MSC) and immortalized human schwannoma cells derived from a patient with NF2 (HEI-193) were cultured in DMEM supplemented with 10% fetal bovine serum (FBS, Gibco), 1% penicillin/streptomycin, and 10 μM forskolin under standard conditions (37^0C, 5% CO_2, humidified incubator). Details on primary VS culture have been described previously [1, 2]. Briefly, tumors were rinsed in PBS, sharply dissected into chunks less than 2 mm in diameter, treated with collagenase and maintained in SC medium (Sciencell) consisting of 500 ml of basal medium, 5% FBS, 1% SC growth supplement, and 1% penicillin-streptomycin, at 37 ºC, 5% CO_2. Collection of tumor conditioned media was performed after 72 hours.

Lentiviral shRNA and siRNA N-cadherin modulation

To generate stable N-cadherin knockdown, cells were exposed to lentivirus carrying a CDH2 hairpin, (Sigma-Aldrich MISSION TRC TRCN0000053978; pLKO.1-puro-CMV-tGFP). A non-targeting shRNA lentivirus served as control. Transfections were performed at an MOI 20, followed by puromycin selection (0.5 μM) for 48 h to obtain stable populations. For transient siRNA knockdown, MD-MSC cells were transfected with mouse *Cdh2* (N-cadherin) siRNA (Invitrogen/Thermo Fisher; Silencer™ pre-designed, Assay ID 160128, AM16708) or a non-targeting siRNA control using Lipofectamine RNAiMAX (Invitrogen/Thermo Fisher; 13778030) per the manufacturer. siRNA was used at 20 nM final concentration; cells were transfected for 4 h and harvested/analyzed 48–72 h post-transfection. The efficiency of gene downregulation was validated using Western Blot and qRT-PCR.

Animal models

All mouse studies were conducted under The Ohio State University Institutional Animal Care and Use Committee (Protocol#: 2022A00000043). Athymic female nude mice (6-8 week old, Jackson Labs, Bar Harbor, ME, strain code: 007850, homozygous) and immunodeficient NSG mice (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ; 6-8 week old, Jackson Labs, Bar Harbor, ME, strain code:005557) were obtained under the protocol approved by A merlin-deficient schwannoma allograft model was established by injecting MD-MSC cells subcutaneously in the flanks of adult athymic nude mice (5,000 cells per mouse). Mice were randomly assigned to vehicle control, Dasatinib (MedchemExpress, HY-10181) combine with brigatinib (MedchemExpress, HY-12757) (D+B), or D+B plus the N-cadherin antagonist ADH-1 (MedchemExpress, HY-13541). Treatments were initiated on day 10 post-implantation and continued through day 17, when tumors were harvested. Dasatinib (15 mg/kg diluted in 80nM citric acid at a concentration of 5 mg/mL) and brigatinib (25 mg/kg dissolved in 90% PEG400 + 10% 1-methyl-2-pyrrolidinone) were administered by daily oral gavage. ADH-1 (50 mg/kg) was prepared in PBS and was daily administered by intraperitoneal injection. A second ectopic xenograft model was established by injecting HEI-193 cells expressing either shRNA targeting *N-cad* or a control shRNA into the subcutaneous flanks of adult athymic nude mice (10×10^6 cells per mouse). Combination therapy with dasatinib + brigatinib (D+B) was orally administered beginning on day 12 post-implantation and continued through day 17. A third mouse xenograft schwannoma model was established by injecting HEI-193 cells expressing *shNcad* or *shCtrl* subcutaneously into the flanks of NSG mice (10×10^6 cells per mouse). Mice received dasatinib + brigatinib (D+B) treatment beginning on day 12 through day 17.

Immunoblotting

Cells were lysed in protein extraction buffer consisting of 50 mM HEPES, 1% Triton-X-100, 0.5%

sodium deoxycholate, 0.1% SDS, 0.5 mM EDTA, 50 mM NaF, and protease cocktail inhibitor (Cell signaling, #5871). Proteins were separated by either 4–12% acrylamide Bis-Tris and were transferred onto a PVDF membrane (Immobilon-P, Millipore), blocked with Intercept (TBS) blocking buffer (Li-COR), incubated with primary antibodies (all from Cell Signaling, Danvers MA)**:** N-cadherin (1:1000, Abcam ab76011); pSTAT3 (1:1000 Abcam ab76315); STAT3 (1:1000, Novus NB222471); NF-kB p65 (1:1000, Novus NB100-56712); IkBα (1:1000, Novus NB100-56507). pERK1/2 Thr^{202}/Tyr^{204} (1:2000, #4696); pAKT Ser^{473} (1:2000 #4060); pFAK Tyr^{397} (1:1000 # 8556); pSRC Tyr^{530} (1:1000 #2105); FAK (1:1000 #3285); AKT (1:2000 #2920); ERK (1:1000 #9102); SRC (1:1000 #2109); p-p70S6K (1:1000 #9234); GAPDH (1:1000, #97166S) overnight at 4 °C, and probed with fluorescently labeled secondary antibodies (1:5000, Li-COR, IRDye®800CWand 1:5000, Li-COR IRDye®680RD). Signal was visualized using the Li-COR Odyssey FC system.

Immunofluorescence

Cells were fixed in 4% paraformaldehyde, permeabilized, and incubated with primary antibodies against N-cadherin (1:200, Proteintech 22018-1-AP), NF-κB p65 (1:200, Novus NB100-56712), MMP9 (1:100, ThermoFisher MA532705), and S100 (1:1, Dako Omnis GA50461). For mouse tissues/cells, mouse-specific MMP9 (1:100, R&D Systems AF909) and SOX10 (1:250, Abcam Ab155279) were used. For endocytosis assays, Rab4 (1:500, BD 610888), Rab5 (1:500, BD 610724), and Rab11 (1:500, BD 610656) were used.

FFPE sections were deparaffinized in xylene, rehydrated through graded ethanol, subjected to antigen retrieval in Tris–EDTA (pH 9), quenched with hydrogen peroxide, and blocked with 5% goat or donkey serum. Fluorescent secondary antibodies were applied, and images were acquired on an Olympus FV3000 spectral confocal microscope. For spheroid migration, schwannoma cells

were labeled with CellTracker Green (10 μM, ThermoFisher C2925) and astrocytes with CellTracker Red (10 μM, ThermoFisher C34552) for 30 min at 37°C. At least 10–15 representative ROIs per slide were imaged, and all analyses were performed blinded to condition. Confocal images (10–15 high-power fields/sample) were acquired on an Olympus FV3000 and analyzed in FIJI (NIH).

Quantitative RT-PCR

Total RNA was isolated, reverse-transcribed to cDNA, and analyzed by SYBR Green qPCR. Relative expression was calculated using the $2^{-}\Delta\Delta CT$ method with housekeeping gene (GAPDH) normalization. Primer sequences are provided in Supplementary Table S1.

ELISA and in vitro N-cadherin digest assay

IL-6 levels in conditioned media from primary VS cultures was measured by ELISA per manufacturer's instructions (Abcam, ab46027). In vitro N-cadherin digestion was performed as previously described, using 2.5μg of recombinant N-cad and 0.5μg of recombinant metalloproteases including: MMP-2(BML-SE237); MMP-9 (BML-SE360) and MMP-14 (BML-SE259) (all from Enzo Biochem Inc). Reactions were terminated by addition of denaturing loading buffer at 95 °C for 5 minutes followed by freezing. Digestion products were resolved by electrophoresis on a 4–15% Tris-glycine polyacrylamide gradient gel and then transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was subsequently visualized with Coomassie Blue.

Cell aggregation, adhesion, and migration

MD-MSC cells were first seeded onto a 96-well plate and subjected to siRNA transfection targeting N-cadherin or non-targeting control. Cells were then harvested, counted, and re-plated under the

indicated assay conditions with N-cadherin blocking antibody GC-4 with (10-50 µg/mL) or ADH-1 (0.2 1 mg/mL). For cell aggregation, 5000 cells/well in 50 uL were seeded into non-adherent/low-attachment 96-well plates in the presence or absence of GC-4 or ADH-1 and allowed to aggregate for the indicated period before imaging. For cell adhesion, 2 x 10^5 MD-MSC cells were seeded onto a black 96-well plates were coated with Matrigel (Corning Basement Membrane Matrix) with GC-4 or ADH-1 during the attachment period. Cell adhesion was quantified using the Vybrant Cell Adhesion Assay (Thermo Invitrogen/Fisher Scientific; V13181). After 30 min binding to the coated surface, non-adherent cells were removed and fluorescence from adherent cells were measured using a microplate reader (Biotek Cytation 1) using 494 nm excitation and 517 emissions. For Transwell migration assay, a total of 1 x 10^5 MD-MSC cells were seeded into the upper chamber of a transwell membrane (5-µm pore size, Corning) with or without ADH-1 at 1mg/mL.The lower compartment was filled with DMEM with 10% FBS and 1% penicillin/streptomycin. After migration, membranes were fixed and stained with DAPI, migrated cells were quantified by counting DAPI-positive nuclei on the underside of the membrane from 5-10 random ROIs at 10x magnification using FIJI/ImageJ.

Spheroid migration and invasion assay

Tumor spheroids were generated from mouse and human schwannoma cell lines by seeding 5,000-10,000 cells per well in ultra-low attachment round-bottom plates and culturing until compact spheroids formed (~150 µm). For 3D astrocyte co-culture, 10,000 immortalized murine astrocytes were plated on Matrigel-coated coverslips and allowed to form a 3D astrocyte scaffold, after which spheroids were transferred onto the scaffold and maintained in migration medium for 24–72 h before fixation and staining to delineate tumor cells. For 3D ECM invasion, spheroids were embedded in growth factor–reduced Matrigel by overlaying polymerized Matrigel with an

additional Matrigel layer and then adding culture medium on top after gelation at 37 °C. Spheroid outgrowth was imaged at 0 h and every 24 h, and invasion/migration was quantified in FIJI as the area of cell outgrowth (or effective radius calculated from outgrowth area) with ≥3 spheroids per condition, measured by a blinded evaluator

Drug synergy analysis

Schwannoma cells were plated in 96-well format and evaluated for response to kinase inhibitors (brigatinib, dasatinib and pictilisib) as single agents or in combination. To determine dose ranges from individual IC_{50}, cells were exposed to a matrix of drug concentrations for 72 hours. For each compound, single agent dose response curves were generated using serial dilutions, normalized viability data were fitted using a four-parameter logistic model, and $IC_{50\ values}$ were derived from the fitted curves. For each drug pair (brigatinib + dasatinib, dasatinib + pictilisib, or brigatinib + pictilisib), cells were exposed to a 5x5 concentration matrix spanning 0 to 1000 nM for each compound. Cell viability was measured using a commercial bioluminescent ATP assay (CellTiter-Glo, Promega) normalized with vehicle controls. Synergy scores were generated with Combenefit software (Cancer research UK) to visualize areas of greater than additive inhibition. A synergistic interaction was defined by an excess over Loew or HSA expectation (synergy score > 10 or combination < 1). Normalized cell viability values were organized as two-way tables, with drug A concentrations on one axis and drug B concentrations on the other, and plotted as heat maps (GraphPad Prims v10.3).

Statistical analysis

Data are presented as mean ± SD unless stated otherwise. Unless otherwise noted, non-parametric one-way ANOVA with Dunnett's multiple comparisons test and non-parametric two-way ANOVA

with Tukey's multiple comparisons test were employed. Summary data in figures are presented as mean ± standard deviation. The Benjamini– Hochberg correction for multiple comparisons was performed. Two-tailed independent Student's t-test or Mann-Whitney U test (two-tailed) was used for binary comparisons.

SUPPLEMENTARY FIGURE LEGENDS

Supplemental Figure 1. N-cad regulates context-dependent migration and cell–cell cohesion in schwannoma cells.

(A) Representative fluorescence images of transwell migration of HEI-193 cells (DAPI) under control conditions or following pharmacologic N-cadherin inhibition with ADH-1 (1 mg/mL) or siRNA-mediated N-cadherin knockdown (si*Ncad*). (B) Quantification of migrated cells from (A) (n = 12 fields per condition; Bars represent mean ± SD; ****$P < 0.0001$). (C) Immunoblot confirming N-cad knockdown in HEI-193 cells expressing sh*Ncad* relative to sh*Ctrl*; GAPDH serves as a loading control. (D) Representative immunofluorescence images showing reduced N-cad expression following sh*Ncad* knockdown. Scale bar, 10 μm. (E) Relative mRNA expression of adhesion- and ECM-associated genes in sh*Ctrl* versus sh*Ncad* cells(Bars represent mean ± SD; ****$P < 0.0001$). (F) Representative images of MD-MSC spheroid migration in 2D laminin (top) or 2D astrocyte co-culture (bottom) comparing si*Ctrl* and si*Ncad* conditions. Scale bar, 200 μm. (G–H) Quantification of cumulative migration distance on laminin (G) and astrocyte co-culture (H) (n = 8–10 spheroids per condition; Bars represent mean ± SD; *$P < 0.05$, **$P < 0.01$). (I) Representative images of HEI-193 spheroid outgrowth in 3D Matrigel (top) or 3D astrocyte co-culture (bottom) under sh*Ctrl* or sh*Ncad* conditions; dashed outlines indicate spheroid boundaries. Scale bar, 200 μm. (J–K) Quantification of cumulative migration distance in 3D Matrigel (J) and astrocyte co-culture (K) (n = 6–8 spheroids; Bars represent mean ± SD; **$P < 0.01$). (L) Representative brightfield images from cell aggregation assays of HEI-193 cells treated with vehicle, ADH-1 (1 mg/mL), or si*Ncad*. Scale bar, 100 μm. (M) Quantification of relative cell aggregation from (L) (n = 8; Bars represent mean ± SD; ***$P < 0.001$). (N) Quantification of MD-

MSC cell adhesion following treatment with the N-cadherin–blocking antibody GC-4 at indicated concentrations (n = 10; Bars represent mean ± SD ; ****$P < 0.0001$).

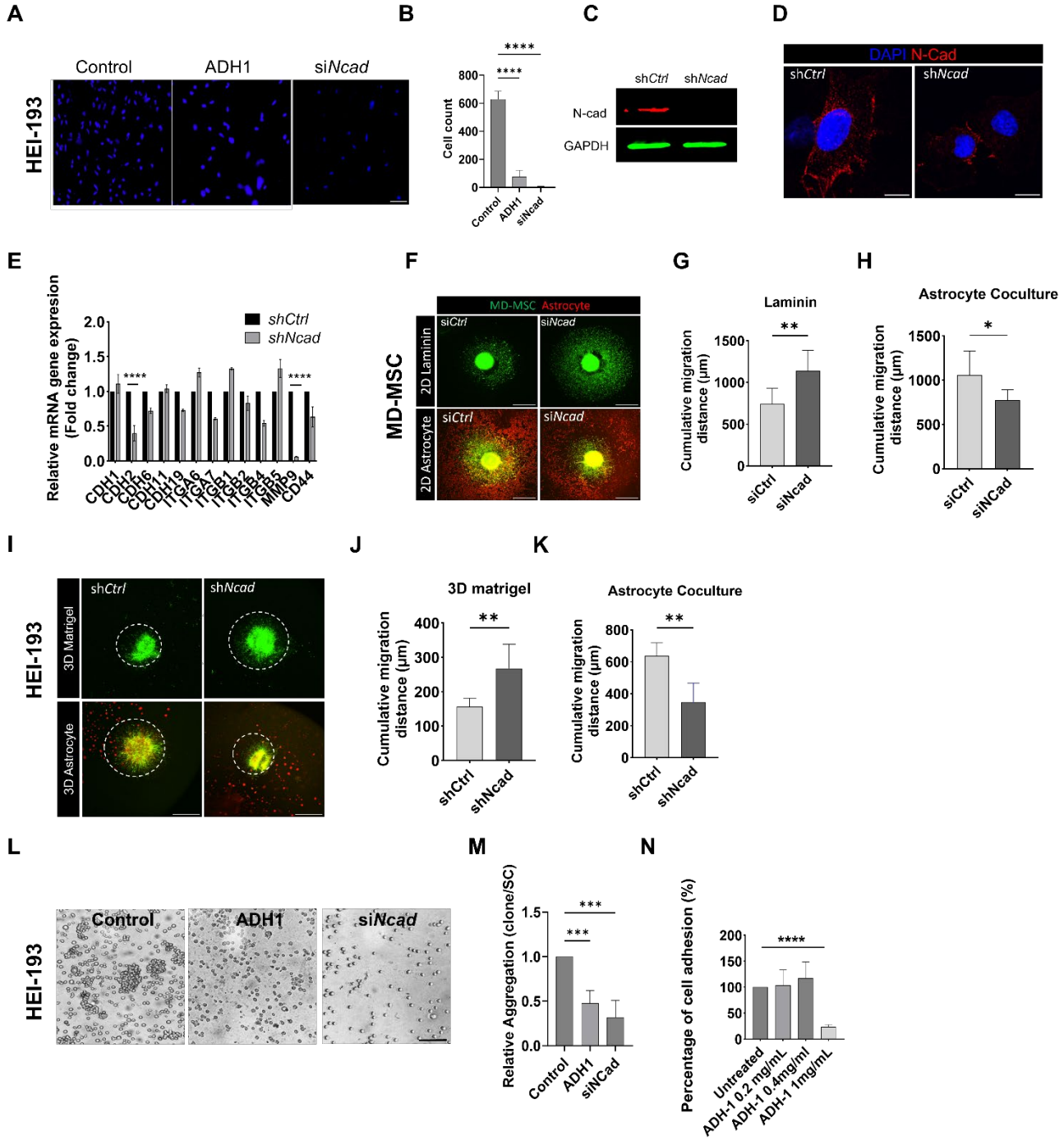
Supplemental Figure 2. ERK signaling is maintained following N-cad depletion and kinase inhibitor treatment.

(A) Representative immunoblots showing phosphorylated ERK1/2 (pERK), total ERK1/2, and GAPDH in sh*Ctrl* and sh*Ncad* schwannoma cells treated with vehicle, dasatinib, brigatinib, or a combination of dasatinib and brigatinib. (B) Quantification of pERK normalized to total ERK and GAPDH (Bars represent mean ± SD). ERK phosphorylation was not significantly altered by single-agent treatment in either sh*Ctrl* or sh*Ncad* cells, while combination treatment with dasatinib and brigatinib increased pERK selectively in sh*Ctrl* cells (****$P < 0.0001$; ns, not significant). (C-D) Synergy landscapes for dasatinib + pictilisib and brigatinib + pictilisib combinations calculated using highest single agent (HSA) and Loewe additivity models for both sh*Ctrl* and sh*Ncad* cells.

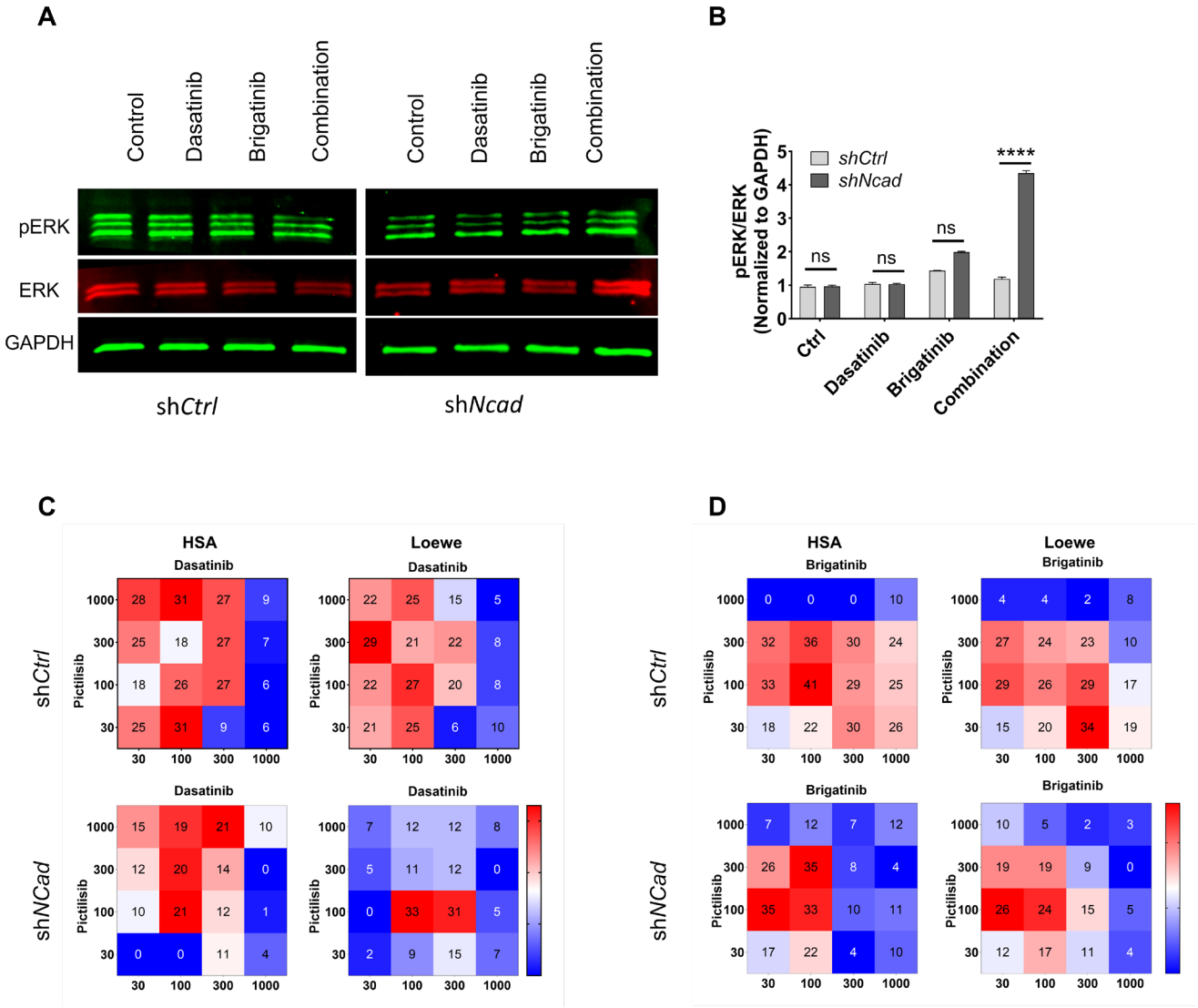
SUPPLEMENTARY TABLE LEGENDS

Supplementary Table 1. Sequence of primers used in qRT-PCR.

Supplementary Figure 1



Supplementary Figure 2



Supplementary Table 1

Genes	Primer	Sequence (5'- 3')
CDH1	Forward	GCCTCCTGAAAAGAGAGTGGAAG
	Reverse	TGGCAGTGTCTCTCCAAATCCG
CDH2	Forward	CCTCCAGAGTTTACTGCCATGAC
	Reverse	GTAGGATCTCCGCCACTGATTC
CDH6	Forward	AGATGCTGCCAGGAATCCTGTC
	Reverse	CCATAGCAGTGTTTCTCGGTCAA
CDH11	Forward	GATCGTCACACTGACCTCGACA
	Reverse	CTTTGGCTTCCTGATGCCGATTG
CDH19	Forward	ATTGGTCAGCCAGGAGCGTTGT
	Reverse	GCAGATTCAGAGACAGTCAAGCG
ITGA6	Forward	CGAAACCAAGGTTCTGAGCCCA
	Reverse	CTTGGATCTCCACTGAGGCAGT
ITGA7	Forward	CCTGTCCAATGAGAATGCCTCC
	Reverse	TCTACCTCCAGTTCCGTGGTCT
ITGB1	Forward	GGATTCTCCAGAAGGTGGTTTCG
	Reverse	TGCCACCAAGTTTCCCATCTCC
ITGB2	Forward	AGTCACCTACGACTCCTTCTGC
	Reverse	CAAACGACTGCTCCTGGATGCA
ITGB4	Forward	AGGATGACGACGAGAAGCAGCT
	Reverse	ACCGAGAACTCAGGCTGCTCAA
ITGB5	Forward	GCCTTTCTGTGAGTGCGACAAC
	Reverse	CCGATGTAACCTGCATGGCACT
MMP9	Forward	GCCACTACTGTGCCTTTGAGTC
	Reverse	CCCTCAGAGAATCGCCAGTACT
CD44	Forward	CCAGAAGGAACAGTGGTTTGGC
	Reverse	ACTGTCCTCTGGGCTTGGTGTT

References:

1 Wu L, Vasilijic S, Sun Y, Chen J, Landegger LD, Zhang Y *et al*. Losartan prevents tumor-induced hearing loss and augments radiation efficacy in NF2 schwannoma rodent models. *Science translational medicine* 2021; 13: eabd4816.

2 Landegger LD, Sagers JE, Dilwali S, Fujita T, Sahin MI, Stankovic KM. A unified methodological framework for vestibular schwannoma research. *Journal of visualized experiments: JoVE* 2017: 55827.