Supplementary Materials

Ninjurin1 drives lung tumor formation and progression by potentiating Wnt/β-Catenin signaling through Frizzled2-LRP6 assembly

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Methods

Preparation of control and Wnt3a conditioned media

Control and Wnt3a conditioned media were obtained by using L and L-Wnt3a cells, respectively, according to the protocol available in the Wnt homepage (http://web.stanford.edu/group/nusselab/cgi-bin/wnt/).

In silico analysis

We used publicly available datasets deposited in the GEO (National Center for Biotechnology Information): GSE31210 for evaluation of the level of the NINJ1 expression in NSCLC tumors derived from NSCLC patients by comparison with that in the corresponding control tissues; GSE31210, GSE9893, and GSE17537 for analysis of the association of the NINJ1 expression with prognosis of patients with lung, colon, and breast cancer; GSE77803, GSE9893, and GSE85043 for analysis of the correlation of the NINJ1 expression with CSC markers (POU5F1, NANOGR, or SOX2). Raw data comprising gene expression levels and clinical information for each patient sample (such as histology, survival status, and duration of survival) were manually downloaded and analyzed. A Kaplan-Meier survival curve was used to show differences in the survival of lung cancer patients. For survival analysis, the top and bottom 25% percentile were used to determine the Ninj1\textsuperscript{high} and Ninj1\textsuperscript{low} groups, respectively. The log-rank test was used to determine significance.

Immunohistochemistry

Sections of formalin-fixed and paraffin-embedded tissue specimens were deparaffinized, rehydrated, and then subjected to antigen retrieval using the citrate-based antigen unmasking solution (Vector Laboratories). After treatment with 0.3% hydrogen peroxide solution, slides were incubated with blocking buffer (5% normal serum in TBS containing 0.025% Triton X-100) for 1 h at room temperature. Slides were incubated with primary antibodies overnight at 4\textdegree C and then
with a biotinylated secondary antibody (Bethyl laboratories) for 1 h at room temperature. Solutions A and B (ABC-Elite, Vector Laboratories) were added simultaneously for 30 min, and signals were detected using a 3,3'-diaminobenzidine (DAB) substrate kit (Vector Laboratories). Slides were further counterstained with hematoxylin.

**Immunofluorescence**

Sections of formalin-fixed, paraffin-embedded (FFPE) tissue specimens were deparaffinized, rehydrated, and treated with citrate-based antigen unmasking solution (Vector Laboratories, Burlingame, CA, USA) for antigen retrieval. The slides were treated with 0.3% hydrogen peroxide solution and then incubated with blocking solution (5% normal serum in TBS containing 0.025% Triton X-100) for 1 h at room temperature. The slides were incubated with primary antibodies (1:100 dilution) overnight at 4°C. The slides were washed multiple times with wash buffer (TBS containing 0.025% Triton X-100), incubated with fluorochrome-labeled secondary antibodies (Thermo Fisher Scientific) for 1 h at room temperature, and then washed several times with wash buffer. The slides were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and observed under a fluorescence microscope (Zeiss Axio Observer Z1, Carl Zeiss AG, Oberkochen, Germany).

**MTT assay**

Cells were seeded into 96-well plates at a density of $2 \times 10^3$ to $1 \times 10^4$ cells/well and allowed to attach for 24 h. Cells were treated with vehicle or the indicated concentrations of test compounds diluted in complete media for 2 days, after which they were treated with MTT solution (final concentration of 500 μg/mL) and incubated for 2-4 h at 37°C. The formazan products were dissolved in DMSO, and the absorbance of each well was measured at 570 nm. The data are presented as a percentage of the control group.

**Sphere formation assay**
Cells were seeded on ultra-low attachment 96-well plates (Corning, Corning, NY, USA) in spheroid medium [DMEM-F12, supplemented with B27 supplements (Thermo Fisher Scientific, Waltham, MA, USA), EGF, bFGF, and antibiotics]. Cells were incubated at 37°C and 5% CO2 for 2 weeks or until spheres formed and reached above 150 µm². Spheres were imaged, and the diameter of spheres and the number of spheres above 30 or 100 µm in diameter were determined using ImageJ software (National Institutes of Health, Bethesda, MA, USA).

**Aldehyde dehydrogenase (ALDH) assay**

1 x 10⁶ NSCLC cells were suspended in AldeRed buffer and stained with AldeRed A588 at 37°C for 40 min. Each group contained a blank sample (AldeRed A588 alone) and a positive control sample (AldeRed A588 plus DEAB). The fluorescence intensity was obtained by flow cytometric analysis, and the sorting gates were established using a sample with DEAB treatment (negative control). The ALDH\textsuperscript{high} and ALDH\textsuperscript{low} populations were sorted using a FACS Aria III flow cytometer (BD Biosciences) for further in vitro experiments.

**Anchorage-dependent colony formation assay**

Cells were seeded into 6-well plates at a density of 300 cells/well and treated for two weeks with various concentrations of test materials diluted in complete medium. The drug-containing medium was changed once or twice a week. After incubation, colonies were fixed with 100% methanol, stained with 0.02% crystal violet solution, and washed with deionized water several times. Colonies were imaged and counted using ImageJ software.

**Soft agar colony formation assay**

Cells were mixed with sterile 1% agar solution (final concentration of 0.4%) and poured onto 1% base agar in 24-well plates. Test materials diluted in complete medium was added to the agar after solidification of the top agar. Cells embedded in the top agar were incubated for 2 weeks at 37°C
with 5% CO₂, and the medium was changed twice a week. After incubation, colonies were stained with the MTT solution (final 250-500 μg/mL). Colonies were imaged and counted using ImageJ software.

**Western blot analysis**

Total cell lysates were prepared with modified RIPA lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 0.25% sodium deoxycholate, 1% Triton X-100) containing various protease and phosphatase inhibitors (100 mM NaF, 5 mM Na₃VO₄, 1 mM PMSF, 1 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 μg/mL pepstatin). Equal amounts of protein (25-50 μg) were subjected to SDS-PAGE though 6-12% gels and electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes (ATTO Corp., Tokyo, Japan). Membranes were submerged into blocking buffer [5% nonfat dry milk in Tris-buffered saline (TBS) containing 0.01% Tween-20 (TBST)] for 1 h at room temperature before they were incubated overnight at 4°C with primary antibodies diluted in 3% BSA in TBST (1: 1,000). Membranes were then washed multiple times with TBST and incubated with secondary antibodies diluted in 5% nonfat dry milk in TBST (1: 5,000) for 1 h at room temperature. Finally, the membranes were washed multiple times with TBST, and protein bands were visualized using an enhanced chemiluminescence (ECL) detection kit (Thermo Fisher Scientific).

**Real-time PCR**

Total RNA was prepared using an easy-BLUE total RNA extraction kit (Intron Biotechnology, Sungnam-si, Kyunggi-do, Republic of Korea) according to the manufacturer’s recommended procedure. We used a SYBR Green-based qPCR master mix solution (Enzymomics, Daejeon, Republic of Korea) and gene-specific primers. All real-time PCR assays were performed on an Applied Biosystems 7300 Real-Time PCR System (Thermo Fisher Scientific). The following thermocycler conditions for real-time PCR were applied: pre-incubation at 95°C for 15 min; 40-70
cycles of 95°C for 10 sec, 60°C for 15 sec, and 72°C for 30 sec; and a final melt curve analysis to
determine reaction specificity. Relative quantification of mRNA expression was performed using
the comparative CT (cycle threshold) method as described in a previous report [1]. The primer
sequences used in the PCR assays are shown in Table S3.

**Plasmids, shRNAs, and Transfection**

The plasmid constructs for hNinj1 expression are described in our previous report [2]. The plasmid
construct for the FLAG-tagged hNinj1 protein was generated by using pCS+GFP hNinj1 vector as
a template and subcloning them into the pCMV-Tag2B vector using the following primers: FLAG
Ninj1 forward: 5'-TGTGAATTCATGGACTCGGGAACC-3', reverse: 5'-TATCTCGAGCTACTGCTGGGTCG-3'.

The plasmid constructs for the myc-tagged full-length hNinj1 protein and its domain
constructs were generated by cloning them into the pCS2+MT vector. NT (1-100 aa), ∆Cyto (1-
100 linked with 111-152 aa) and CT (111-152 aa) domain constructs were generated using the
template pCS2+MT-FL-hNinj1 and the following primers: NT forward: 5'-CAGGAATTCGATGGACTCGGGAAC-3', reverse: 5'-TCTCGAGTCAGAAGATGAGCAGCAC-3';
CT forward: 5'-CAGGAATTCGAAGCACGCCAAGCTG-3', reverse: 5'-TATCTCGAGCTACTGCTGGGTCG-3'. For ∆Cyto mutant hNinj1, PCR products of NT
fragments and fragments were ligated and subcloned into pCS2+MT vector.

For GST-tagged bacterial protein expression, pCMV-Tag2B-hNinj1 was subcloned into the
pGEX-4T-2. NT Ninj1 mutant was generated using the template pGEX-4T-2-FL-hNinj1 with the
following primers: NT GST Ninj1 forward: 5'-CAGGAATTCAATGGACTCGGGAAC-3', reverse: 5'-TCTCGAGTCAGAAGATGAGCAGCAC-3'.

His-tagged bacterial protein expression, LRP6 and FZD2 domain constructs were subcloned into the
pET32a. LRP6 constructs were generated by using LRP6-pCS2 vector as a
template and FZD2 construct was generated by using pCMV6-AC-GFP FZD2. PE1 LRP6 (1-328
aa), PE2 LRP6 (329-631 aa), PE3 LRP6 (632-932 aa), PE4 LRP6 (933-1247 aa) and Ext FZD2 (24-247 aa, no signal peptide) were generated using the following primers: PE1 LRP6 forward: 5' - TATGGATCCATGGGGGCCGTCCTG-3', reverse: 5' - TATGTCGACTCATGTGGCACCATC-3'; PE2 LRP6 forward: 5' - TATGGATCCGAATTATTGCTTTA-3', reverse: 5' - TATGTCGACTCTCTGGGACAAAT-3'; PE3 LRP6 forward: 5' - TATGGATCCGCTTTCCTTTTGTTT-3', reverse: 5' - TATGTCGACTCAAGGAGCACTACA-3'; PE4 LRP6 forward: 5' - TATGGATCCACGACTTTCCTGCTC-3', reverse: 5' - TATGTCGACTCATGGAGGTTCTCC-3'; Ext FZD2 forward: 5' - ATGAATTCCAGTTCCACGG-3', reverse: 5' - ATAAAGCTTTTACCAGAGGC-3'.

The pCS+GFP hNinj1 expression vector was kindly provided by Dr Kyu-Won Kim (Seoul National University, Seoul, Korea). The LRP6-pCS2 vector was purchased from Addgene (Plasmid #27242, Watertown, MA, USA). The pCMV6-AC-GFP FZD2 vector was purchased from OriGene Technologies (Cat #: RG220921, Rockville, MD, USA) (Origene-Technologies). The scrambled small interfering RNA (siRNA) for silencing hNinj1 and control siRNA were purchased from Integrated DNA Technologies (Coralville, IA, USA).

For transient transfection, cells were transfected with expression vectors or siRNAs using the JetPrime transfection reagent (Polyplus-Transfection SA). To generate stable cell lines knocked down Ninj1 expression, H460 and A549 cells were transduced with lentiviral particles containing control vector (shCon; pLKO.1) or NINJ1 shRNAs (Sigma-Aldrich). The stable transfectants were selected by culturing cells in a medium containing 1 – 2 µg/mL puromycin for 3 weeks. To establish stable cell lines overexpressing Ninj1, H1299 and H226Br cells were transfected with pCMV-Tag2B (EV) or pCMV-Tag2B-Ninj1 (Ninj1) vectors using JetPRIME for 48 h. Transfected cells were selected using G418.

Immunoprecipitation and pulldown assay
For immunoprecipitation analysis, cells were washed with ice-cold PBS twice and then harvested...
by IP lysis buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% NP-40, 1 mM MgCl₂, 10% glycerol, 100 mM NaF, 5 mM Na₃VO₄, 1 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 μg/mL pepstatin] for 10 min on ice. After centrifugation at 13,000 rpm for 10 min at 4°C, supernatants were harvested, and protein concentration was determined by the BCA assay. 1 mg of protein was immunoprecipitated with primary antibodies overnight at 4°C in lysis buffer. Protein G agarose beads were added and incubated for additional 2 h. The beads were collected by centrifugation (3,000 rpm for 2 min at 4°C) and washed six times (three times with lysis buffer and three times with PBS). Bound proteins were extracted by boiling with 5x SDS-PAGE sample buffer for 5 min at 95°C. Proteins were resolved by SDS-PAGE, transferred onto PVDF membranes, and then subjected to Western blot analysis as described above.

The expression and purification of hexahistidine (6xHis, His)-tagged recombinant LRP6 and FZD2 or glutathione-S-transferase (GST)-tagged Ninj1 proteins were performed as described previously [3]. For a pull-down assay, the recombinant Ninj1 protein bound to the Ni-NTA agarose or the recombinant vimentin protein bound to the glutathione-agarose were incubated with 1 mg of cell lysates or purified proteins in TNE binding buffer [50 mM Tris-HCl (pH 8.0), 120 mM NaCl, and 0.1 M EDTA) with rotation for 2 h or overnight at 4°C. The pull-down complexes were centrifuged and washed thrice with the lysis buffer before being resolved using SDS-PAGE and analyzed by Western blot analysis.

**Limiting dilution assay**

Cells were harvested by trypsinization. Live cells, as confirmed using a trypan blue exclusion assay, were diluted in PBS, mixed with Matrigel (ratio 1:1), and then inoculated into the right flanks of NOD/SCID mice. The incidence of tumor formation was determined. Tumor-initiation fraction of vehicle- or Evo-treated groups was determined by using Extreme Limiting Dilution Analysis (ELDA) online software (http://bioinf.wehi.edu.au/software/elda/)[4].
**Hanging drop assay**

Cells (suspended in a complete medium) were seeded in 50 μL drops on the inner surface of a multi-well plate lid and cultured for a day. The drops were observed under an inverted microscope, and the non-aggregated cells were manually counted. Cells in at least four fields per sample were counted.

**Cell adhesion assay**

A 96-well culture plate was coated with type I collagen (10 μg/mL) and fibronectin (10 μg/mL) overnight at 4°C. After washing with PBS, the plates were blocked with 3% BSA at room temperature for 1 h. Suspended cells in serum-free media were added to each coated well. After incubation for 30 min at 37°C, non-adherent cells were removed by streaming PBS over the plate three times. The remaining adherent cells were stained with 0.2% crystal violet and washed with PBS several times. The stained cells were dissolved in DMSO and the absorbance was measured at 570 nm.
| Histology¹ | Name       | Genetic alterations² |
|-----------|------------|----------------------|
| ADC       | Calu-1     | WT       Mut   Null  WT   WT   WT   WT   |
| ADC       | H1975      | Mut      WT       Mut  WT   WT   WT   Mut |
| ADC       | HCC827     | Mut      WT       Mut  WT   WT   WT   WT   |
| ADC       | H1944      | WT       Mut      WT   Mut  WT   WT   WT   WT   |
| ADC       | H292       | WT       WT³     WT   WT   WT   WT   WT   WT   |
| ADC       | H522       | WT       WT       Mut  WT   WT   WT   WT   WT   |
| ADC       | PC-9       | Mut      WT       Mut  WT   WT   WT   WT   WT   |
| ADSQC     | A549       | WT       Mut      WT   Mut  WT   WT   WT   WT   |
| SQCC      | H226B      | WT       WT       WT   Un   Un   WT   Un   |
| SQCC      | H226Br     | WT       Mut      Mut  Un   Un   Un   Un   |
| SQCC      | HCC15      | WT       WT       Mut   Del  WT   WT   WT   |
| LCC       | H1299      | WT       WT       Null  WT   WT   Null⁴  WT   |
| LCC       | H460       | WT       Mut      WT   Mut  WT   WT   WT   Mut   |

¹ADC: Adenocarcinoma; ADSQC: Adenosquamous carcinoma; SQCC: Squamous cell carcinoma; LCC: large cell carcinoma

²Genetic alterations were retrieved from the Catalogue of Somatic Mutations in Cancer (COSMIC) database, the Cancer Cell Line Encyclopedia (CCLE) database, and literatures. WT: wild type; Mut: mutated; Un: unknown; Del: deletion; ND: not determined.

³KRAS G12S mutation has been identified in a previous study, but this mutation might be an artifact caused by a long-term culture and should be further validated[5].

⁴Undetectable expression caused by promoter methylation[6]
## Table S2. Antibodies used in this study.

| Target                          | Vendor                    | Catalogue Number | Clone | Dilution ratio                      | Application   |
|---------------------------------|---------------------------|------------------|-------|--------------------------------------|---------------|
| Ninjurin1 (human)               | Provided by Dr. Shin YK lab (SNU) |                  | D12   | 1:100 (IF) 1:1000 (IP) 1 μg /10⁵ cells (FACS) | IF, IP, FACS  |
| Ninjurin1 (mouse)               | Provided by Dr. Kim KW lab (SNU) | MAB5105          |       | 1:100                               | IHC           |
| Ninjurin1 (human)               | R&D system                | MAB5105          | 758943 | 1:1000                              | WB            |
| Ninjurin1 (human)               | R&D system                | MAB51051         | 758926 | 1:100                               | IF            |
| Nanog                           | Cell Signaling            | 4903S            | D73G4 | 1:1000 (WB) 1:100 (IF)               | WB, IF        |
| Sox2                            | Abcam                     | ab97959          |       | 1:1000                              | WB            |
| Oct4                            | Abcam                     | ab19857          |       | 1:1000                              | WB            |
| Actin                           | Santa Cruz                | sc-47778         | C4    | 1:1000                              | WB            |
| GAPDH                           | Cell Signaling            | 5174             | D16H11 | 1:1000                              | WB            |
| OctA-probe                      | Santa Cruz                | sc-166355        | H-5   | 1:1000 (WB) 1:100 (IF) 1:1000 (IP)   | WB, IF, IP    |
| CC10                            | Santa Cruz                | sc-9772          | T-18  | 1:100                               | IF            |
| Prosurfactant protein C        | Merck                     | AB3786           |       | 1:100                               | IF            |
| β-catenin                       | Santa Cruz                | sc-7963          | E-5   | 1:1000 (WB) 1:100 (IF)               | WB, IF        |
| Dvl3                            | Cell Signaling            | 3218             |       | 1:1000                              | WB            |
| Actin                           | Santa Cruz                | sc-47778         | C4    | 1:1000                              | WB            |
| Cleaved PARP (Cl-PARP)          | BD                         | 552596           | F21-852 | 1:1000                              | WB            |
| PARP                            | Cell Signaling            | 9542             |       | 1:1000                              | WB            |
| Protein/Antibody | Supplier          | Catalog Number | Dilution | Format |
|------------------|-------------------|----------------|----------|--------|
| Cleaved caspase-3 (Cl-Cas3) | Cell Signaling | 9661S D175 | 1:1000 | WB     |
| β-tubulin        | Cell Signaling    | 2128 9F3     | 1:1000 | WB     |
| His              | Santa Cruz        | sc-8036 H-3  | 1:1000 | WB     |
| GST              | Santa Cruz        | sc-459 Z-5   | 1:1000 | WB     |
| LRP6             | Cell Signaling    | 2560 C5C7    | 1:1000 | WB     |
| LRP6             | Santa Cruz        | sc-25317 C-10| 1:1000| IP     |
| phospho-LRP6 (pLRP6, S1490) | Cell Signaling | 2568 | 1:1000 | WB     |
| FZD2             | LSBio             | LS-C166295   | 1:1000 | WB     |
| Axin1            | Cell Signaling    | 2087 C76H11  | 1:1000 | WB     |
| GSK3β            | Cell Signaling    | 9832 3D10    | 1:1000 | WB     |
| HRP-conjugated goat anti-mouse IgG | GeneTex | 213111-01 | 1:5000 | WB     |
| HRP-conjugated goat anti-rabbit IgG | GeneTex | 213110-01 | 1:5000 | WB     |
| Alexa Fluor 488-conjugated goat anti-rabbit IgG | Thermo Fisher Scientific | A-11034 | 1:1000 | IF     |
| Alexa Fluor 488-conjugated goat anti-mouse IgG | Thermo Fisher Scientific | A11001 | 1:1000 | IF     |
| Alexa Fluor 594-conjugated goat anti-rabbit IgG | Thermo Fisher Scientific | A11012 | 1:1000 | IF     |
| Alexa Fluor 594-conjugated donkey anti-mouse IgG | Thermo Fisher Scientific | A21203 | 1:1000 | IF     |
| Antibody                      | Manufacturer       | Catalog Number | Dilution | Technique |
|------------------------------|--------------------|----------------|----------|-----------|
| FITC-conjugated Donkey anti-Human IgG | Jackson ImmunoResearch | 709-095-098    | 1:1,000  | IF        |
| Goat anti-Rabbit IgG (H+L) Biotinylated | Bethyl            | A120-101B      | 1:1,000  | IHC       |

1) Application - WB: Western blot analysis; IF: Immunofluorescence staining; IHC: Immunohistochemistry; IP: Immunoprecipitation; FACS: Fluorescence-Activated Cell Sorting
| Gene   | Forward sequence (5´-3´)                      | Reverse sequence (5´-3´)                      | Application   |
|--------|-----------------------------------------------|-----------------------------------------------|---------------|
| NINJ1  | CTCGACCCACTCAAGGACAC                        | GTAGGGCCAAAGGCAGTTCT                          | Real-time PCR  |
| ALDH1A1| CGGAAAGAAGAAATCTGAAGAGGG                      | GATGCAGCTATAAACAACACTGGGC                     | Real-time PCR  |
| POU5F1 | CTGGGTGTCCCTCGGACCT                        | CCATCGGAGTTGCTCTCCA                         | Real-time PCR  |
| NANOG  | TTTGTGGGCTGAAGAAAACT                       | AGGGCTGTCTGAATAAGCAG                        | Real-time PCR  |
| SOX2   | GCCGAGTGGAATTTTTGTCG                        | GCCGAGTGTAATTTCTCT                           | Real-time PCR  |
| MYC    | CAGCTGCTTAGACGGTGGATT                      | GTAGAAATACGGTGCACCGA                        | Real-time PCR  |
| AXIN2  | AGTGTGAGGTCCAGGAAAC                      | CTTCACAAGCAGATCGATTT                        | Real-time PCR  |
| GLI1   | AGCCTTGCGCAATGCCAGTGAC                     | GTCAGGACATGCAGTCTGGTTG                     | Real-time PCR  |
| GLI2   | GTTCAGACCATCAAGACGAGAG                      | GCATCTCACGCACGACTGTATT                     | Real-time PCR  |
| HES1   | TCAACACGAGCACCGGATAA                       | GCGCGAGCTATCTTCTCA                         | Real-time PCR  |
| HEY1   | GTTCCGGCTCTAGGTTGTCATGT                   | CGTCGCGCTTTCAATTATTC                        | Real-time PCR  |
| HEY2   | AAGGCGTCCGGACTCGGATA                       | AGAGCGTGTGGGCTCAAGTAG                      | Real-time PCR  |
| CTGF   | CTTGCGAAGCTGACCTGGAAGA                     | CCGTCGGTACATACTCCAGACA                     | Real-time PCR  |
| AXL    | GTTGGAGCTGCTGATGGAGGCC                      | CGCTTCACTCAGGAAATCTCC                       | Real-time PCR  |
| CYR61  | GGAAGGAGCAGCTCAGTAAGGC                     | GGAAGATAGCTACCCACGTC                        | Real-time PCR  |
| LRP5   | AGACCGTACGGCCTCATAC                       | TAGTGCAGTGCACACGTC                        | Real-time PCR  |
| LRP6   | AAACAGACGGACTTGGCCATGAG                     | AAACACAAAGCTCCACGACAG                      | Real-time PCR  |
| DVL2   | CTGGAGGCTGAGAGACGAAAC                    | CATGCTACAGTCTCTCCTC                        | Real-time PCR  |
| DVL3   | GCAGCGGACCCAGCTATAAGT                     | TGAAGCAGGTGATGCTCTGGA                      | Real-time PCR  |
| WNT1   | CTCTTCGGCAAGATGCTGACACC                   | CGATGGAACCTTCTGAGACAGA                     | Real-time PCR  |
| WNT2   | AGGATGCCAGACCCCTGTGAGA                    | AGCCAGATGCTCAGTGAGACG                      | Real-time PCR  |
| WNT3   | CGCTGTATCGTGGCAAGAGTT                    | TGAGGTGCACTGTGGTCCAGAG                      | Real-time PCR  |
| WNT3A  | ATGAACCGCCACAACAACGAGG                    | GTCTTCGGGAAGAGTCCACGAG                      | Real-time PCR  |
| WNT4   | GCTGGGAAGAGTGGCAGCTGAGA                   | CCACAAAGCATGCTGAGAAAGGC                    | Real-time PCR  |
| WNT5A  | TAGAGAGATGCTGCGATCCTCA                    | TGCTTCAAGGCTACAGAGG                        | Real-time PCR  |
| WNT6   | TGGCGCTTACGGAGGAAACAGT                   | ATGTAGACTAAACCTCACCCACC                     | Real-time PCR  |
| Gene   | Primer Sequence                     | Method       |
|--------|-------------------------------------|--------------|
| WNT7A  | AGGAGAAGGCTCACAAATGGGC CGGCAATGATGGCTAGGTGAA | Real-time PCR |
| WMT8A  | AGGCATGTGACCATGTCCA               | Real-time PCR |
| FZD1   | GGGGCTTAACAACGGTGGAC CAGAAAGGACGTGCCGATAAAA | Real-time PCR |
| FZD2   | GTGCCATCCTATCTCAGCTACA CTGCATGTCTCACAAGTACGTG | Real-time PCR |
| FZD3   | GGCTCTCATAGTTGCGATTCCTGCAGGCTGCTC | Real-time PCR |
| FZD4   | CCTCGGCTCAACAGCTGACC TGCACATTGCGACATAACACA | Real-time PCR |
| FZD5   | CCGTTCGTGCAAGTGTC GAAACCTTCAATGCTGAGT | Real-time PCR |
| FZD6   | GGCAGCTGTATCTGAAAGTGAGCG | Real-time PCR |
| FZD7   | GTCTTCCAGCGTGTCTCAGCAG  ACGGCATAGCTCTTGACATCT | Real-time PCR |
| FZD8   | GCTCTACAACCGCGTCAAGACA  AAGGTTGACAGCAGCAGAGCA | Real-time PCR |
| FZD9   | GTTCCAGTACGTGGAGAAAGGC CAGCAAGAAGGTGAGCAGTCG | Real-time PCR |
| FZD10  | GAACACGGACAAGCGTGGAGAGAG GGCGTTCGACAAATGGAGCAAG | Real-time PCR |
| ACTB   | GCGAGAAGTGGACCGCAGATGC ACTCTAATGCTGGCATGACAT | Real-time PCR |

| Gene   | Primer Sequence                     | Method       |
|--------|-------------------------------------|--------------|
| LRP5   | GGACACAAACATGCTGAGTGAAGGACTCAGACTTCC | RT-PCR       |
| LRP6   | CAGGGGAGTGTTGCTGAAAGAGG GACCGCCATGCTCAGT | RT-PCR       |
| DVL2   | CAGGATTCGGGGTGGTGGAA  AGCAGCCTCTGCTCTGC | RT-PCR       |
| DVL3   | GTCTGTGATGCTGCTGCTC  TCCATTTGACGGGTTGCA | RT-PCR       |
| WNT1   | CAAGATCGTCAACCGAAGGCT  AAGGTTGACAGGAGGAAGCCGA | RT-PCR       |
| WNT2   | CTGGGGGCGGCCCTTCAATAAA  AGGCAGCCTCCTCATTTTCA | RT-PCR       |
| WNT3   | CTGCTCTACCCAAATTTTGGCTTCTC | RT-PCR       |
| WNT3A  | AAGCAGGCTCTTGGGCAGCTA  GACGGTGTTGCAACATTC | RT-PCR       |
| WNT4   | GAGCAACTGCTGCTGACCTGG  TGCCCAAGAGATGGCGGCTACA | RT-PCR       |
| WNT5A  | CTCCTTCCGCTGGGCGCAT  GGAACCTTTCGACGCGAGG | RT-PCR       |
| WNT6   | CGTAGGGGCAGCTCAGTGAGT  AACTGGAACCTGGCAGTTCG | RT-PCR       |
| WNT7A  | ACTTACGGGTTGAGGAGGCCA  TGAAGCTTAACAGGCTGGGC | RT-PCR       |
| WMT8A  | CTTTCCCTCAGCGCAGAG  TTGGACATGCTGACATGCT | RT-PCR       |
| FZD1   | CTCTACTTCTGAGCATGGCCA  TCCACGTTGTAAGCCCA | RT-PCR       |
| Gene   | Primer 1                        | Primer 2                        | Method    |
|--------|--------------------------------|--------------------------------|-----------|
| FZD2   | CCATCCTATCTCAGCTACAAGTCTCTG    | GCAGCCCTCCTTCTTTG              | RT-PCR    |
| FZD3   | TCCCCCTCTGCTGTATGTGGTGA        | GCTGCTCACCTTTGCTTGGGA          | RT-PCR    |
| FZD4   | CTGGGCTACAAGGTTGACCAAGAT      | AATATGATGGGCGCTCAAGGGTA        | RT-PCR    |
| FZD5   | GTGCCATTCTGAAAGGAGTGCAC       | TCCATGTCGAGGAGGGAAGGTG         | RT-PCR    |
| FZD6   | ACTCTTGCCACTGTGCTTTT          | GTCGAGCTTTTGCTTTTGCCT          | RT-PCR    |
| FZD7   | CAAGACCAGAAGGCTGGAGGA         | TGCCGACGATCAGTGCAT             | RT-PCR    |
| FZD8   | GGAATACAACCGCCACCGACCT       | ACCACAGGCGATCCAGGAAGAC         | RT-PCR    |
| FZD9   | TCAAGGTCAGGGAAGTGGCA          | AGCTCCAGAGAAGCGCAACA           | RT-PCR    |
| FZD10  | CAGGTGTGCAGCCGTTAAGTAA       | AAGCACCACATCTTTAGCTTCCGG       | RT-PCR    |
| ACTB   | ACTACCTCATGAGATC             | GATCCACATCTGGCTGGA             | RT-PCR    |
| Ninj1  | CAAGCTTGATCCAGTACTAGTGAAC   | CGCTCCTTTGTTGGCTAAATGGTT      | RT-PCR    |
| Actb   | CATTGCTGACAGGATGCAAAGGG      | TGCTGGAAGGTGACAGTGAGG          | RT-PCR    |
Fig. S1. Increased expression of Ninj1 in lung tumor in response to urethane treatment, schematic diagram for generating the conditional Ninj1 overexpression in mice using the Cre-LoxP system, and breeding strategies for the establishment of mouse models. (a) Immunohistochemical analysis of Ninj1 expression in normal lung tissues from vehicle-treated mice and lung tumor tissues from urethane-treated mice. Scale bars: 100 μm. (b) The construct for LoxP-Stop-LoxP (LSL)-Ninj1^{Tg/+} for the transgenic (Tg) mouse model harboring conditional Ninj1 overexpression using the Cre-LoxP system. A breeding strategy between LSL-Ninj1^{Tg/+} and Scgb1a1-CreER^{TM} mice to obtain Scgb1a1-CreER^{TM};LSL-Ninj1^{Tg/+} mice. Ninj1: Ninjurin1.
Fig. S2. Association of Ninj1 with Oct4 or Nanog protein expression in xenograft tumors from NSCLC cells and the regulation of Nanog expression by tamoxifen-mediated Ninj1 induction in mice. (a) Western blot analysis examining Oct4 and Nanog protein expression in xenograft tumors of H1299-Ninj1 (left) or H460-shNinj1 cells (right) and in those of their control tumors. Densitometric analysis was performed using ImageJ software. (b) Immunofluorescence analysis examining the lungs from Scgb1a1-CreERT2;LSL-Ninj1Tg/+ mice (left) and Sftpc-CreERT2;LSL-Ninj1Tg/+;KrasG12D/+ mice (right) for correlation of Ninj1 and Nanog expression levels. The Ninj1+ cells were determined using ImageJ software. Scale bars: 20 μm. The significance of the correlation was determined using the Pearson correlation test. Statistic significance of the difference between two groups was determined by a two-tailed Student’s t-test or Mann-Whitney test. Ninj1: Ninjurin1. TM: tamoxifen.
Fig. S3. Induction of Ninj1 expression in hazardous environments and increased survival capacity in Ninj1\textsuperscript{high} populations. (a) Western blot analysis examining Ninj1, cleaved caspase 1 (Cl-Cas1), and cleaved caspase 3 (Cl-Cas3) in primary cultured patient-derived xenograft (PDX)-derived tumor cells under culture conditions of hypoxia (1% O\textsubscript{2}), serum starvation (0% FBS), glucose deprivation (1 mM 2-deoxy-L-glucose [2-DG]), and chemotherapy treatment (10 nM...
paclitaxel and 10 μM cisplatin in combination; Pc/Cs). (b-f) The basal Ki67 positivity (b), anchorage-dependent (AD) (c) and -independent (AID) (d) colony formation, and anchorage-dependent (e) and -independent (f) colony formation under hypoxia (1% O2), serum starvation, glucose deprivation, and exposure to chemotherapy (Pc/Cs) in the Ninj1low and Ninj1high populations of two PDX-derived primary cultured cancer cells. Scale bars: 50 μm (b). (g) Representative images of IF analyses examining the levels of Ninj1 and Nanog expression in three PDX tumors treated with chemotherapy (paclitaxel and cisplatin in combination; Pc/Cs). The quantification analysis results for the IF images are included in Fig. 4l. Scale bars: 50 μm. Scale bars (inset): 10 μm. The bars represent the mean ± SD; *P < 0.05, **P < 0.01, and ***P < 0.001, as determined by a two-tailed Student’s t-test by comparison to the indicated group. Ninj1: Ninjurin1.
Fig. S4. Role of Ninj1 in the activation of the Wnt/β-catenin signaling pathway. (a) Real-time PCR analysis examining MYC and AXIN2 in the indicated stable NSCLC cells with knockdown of Ninj1 expression in the absence or presence of Wnt3a conditioned medium (Wnt3a). The bars represent the mean ± SD; *P < 0.05, **P < 0.01, and ***P < 0.001, as determined by one-way ANOVA with Tukey’s post-hoc test. (b, c) Representative immunofluorescence analysis images
revealing the levels of Ninj1 and β-catenin (β-cat) expression in FACS-sorted Ninj1\textsuperscript{high} and Ninj1\textsuperscript{low} populations of three patient-derived xenograft (PDX)-derived primary cultured cancer cells (b), CCSP\textsuperscript{+} club cells (c, top) and SPC\textsuperscript{+} type II alveolar epithelial cells (c, bottom). The quantification analysis results for the IF images are included in Fig. 5h and 5i. Scale bars: 50 μm. Ninj1: Ninjurin1.
Fig. S5. Analysis of prognostic impact of Ninj1 expression in patients with breast or colorectal cancers and the association of Ninj1 expression levels with CSC marker gene expression in patient-derived tumors. (a) The Kaplan–Meier estimates for the association of NINJ1 expression with overall survival (OS) of patients with breast and colorectal cancers. The P value was determined using the log-rank test. (b) Analysis of GEO datasets for the relationship between NINJ1 and SOX2 expression in breast and colorectal cancers. The significance of the correlation was determined using the Spearman rank correlation test. (c) Real-time PCR examining changes in the mRNA expression of NINJ1 and CSC markers (POU5F1, NANOG, and SOX2) in the Ninj1low and Ninj1high populations of patient-derived xenograft (PDX)-derived primary breast and colon tumor cells. The bars represent the mean ± SD; *P < 0.05, **P < 0.01, and ***P < 0.001, as determined by a two-tailed Student’s t-test by comparison to the indicated group.
Fig. S6. mRNA and protein expression levels of the Wnt/β-catenin signaling components and the role of Ninjurin1 in β-catenin protein stability, activation of the Wnt signaling pathway, and formation of the LRP6-FZD2 signalosome. (a) RT-PCR analysis examining mRNA expression of Wnt ligands (WNT1-WNT8A), Frizzled receptors (FZD1-FZD10), LRP (LRP5 and LRP6), DVL (DVL2 and DVL3), and β-catenin (CTNNB1) in H1299-EV and H1299-Ninj1 cells. (b) Western blot (WB) analysis examining the regulation of Ninj1 expression in primary patient-derived xenograft tumor cells transfected with Ninj1 siRNAs. (c, d) WB analysis examining active β-catenin (β-cateninact) protein in H460-shCon and H460-shNinj1 cells after treatment with cycloheximide (CHX, 100 μg/mL) for the indicated time-points (c) or after treatment with MG132 (10 μM) (d). The bars represent the mean ± SD; **P < 0.01, as determined by a two-tailed Student’s t-test. (e) WB analysis examining the expression of the indicated canonical Wnt/β-catenin signaling components and the phosphorylation of EGFR, IGF-1R, and ROR1 in H460-shCon and H460-shNinj1 cells. (f) WB analysis examining the expression of the indicated canonical Wnt/β-catenin signaling components in H460-shNinj1, H1299-Ninj1, and their control
cells in the absence or presence of Wnt3a conditioned medium (Wnt3a). (g) WB analysis of IgG and anti-Ninj1 immunoprecipitates examining the indicated Wnt/β-catenin signaling proteins to determine interactions among Ninj1, LRP6, FZD2, and other Wnt/β-catenin signaling components in H460-shCon and H460-shNinj1 cells.
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