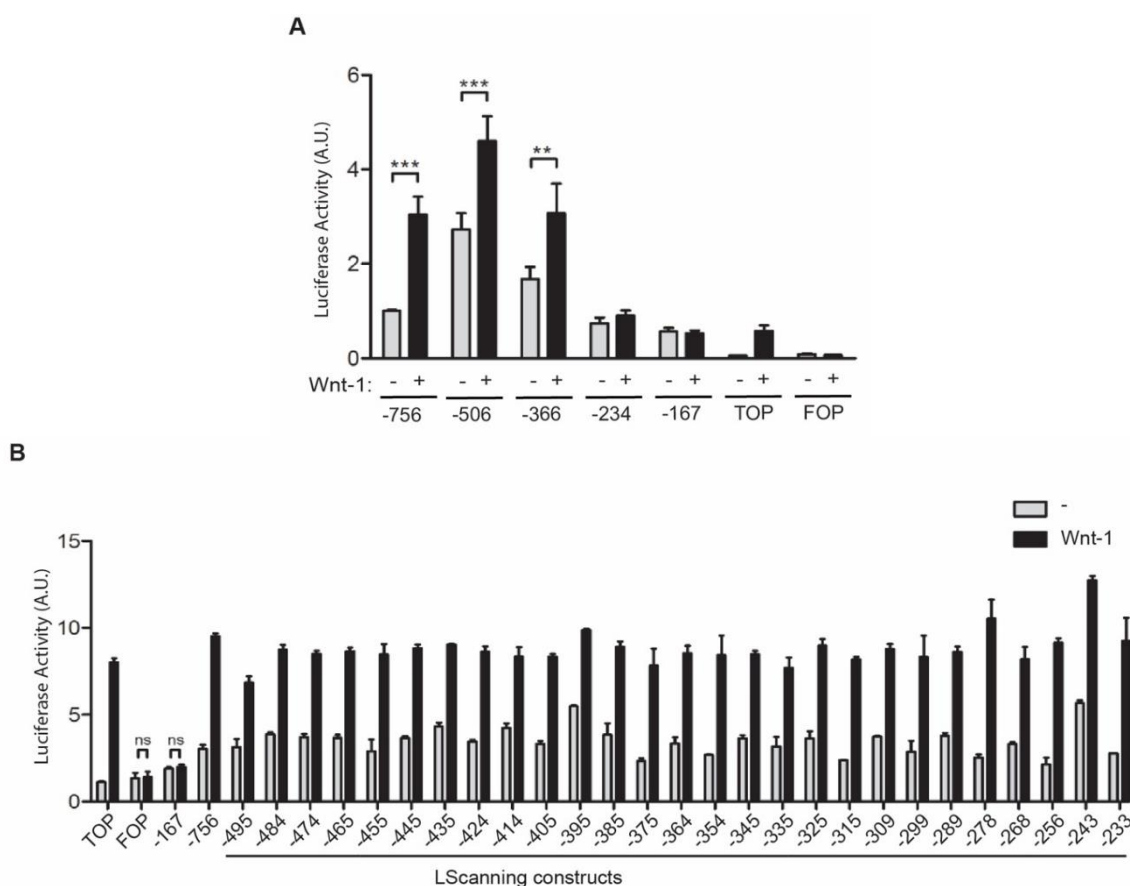# Supplementary Materials: SP1 and STAT3 Functionally Synergize to Induce the RhoU Small GTPase and a Subclass of Non-Canonical WNT Responsive Genes Correlating with Poor Prognosis in Breast Cancer

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**Figure S1.** RhoU promoter analysis in MEF cells. (**A**) Cells were transiently transfected with the indicated RhoU promoter constructs or with the TOP-flash and FOP-flash positive and negative control plasmids (TOP, FOP). A SEAP-expressing vector was included as an internal control for transfection efficiency. Transfected cells were co-cultured for 24 h with wild type or WNT1-expressing HEK-293 cells. Luciferase activity was normalized to SEAP activity. Data are shown as mean ± SEM of independent experiments. ** $p < 0.01$, *** $p < 0.001$. (**B**) The indicated linker scanning (Lscanning) constructs based on the −756 RhoU-promoter fragment, along with the wild type −756 construct and the TOP and FOPflash controls were transiently transfected as above and tested for WNT1-responsiveness. Induction was always significant ($p < 0.001$), with the exception of the −167 and FOP negative controls (ns, non significant). A.U., arbitrary units. Data are shown as mean ± SEM of 4 independent experiments.

**A**
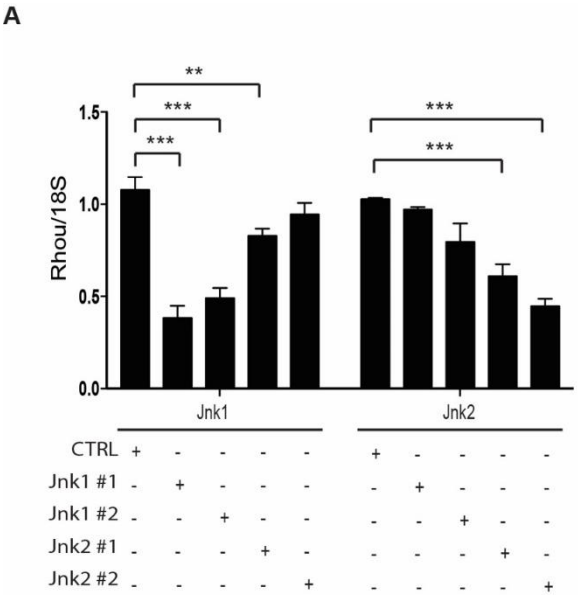


**Figure S2.** Downregulation of Jnk1 or Jnk2 mRNAs by the indicated shRNAs as measured by qRT-PCR in MEF cells. Data are shown as mean ± SEM of 3 independent experiments. ** $p < 0.01$, *** $p < 0.001$.
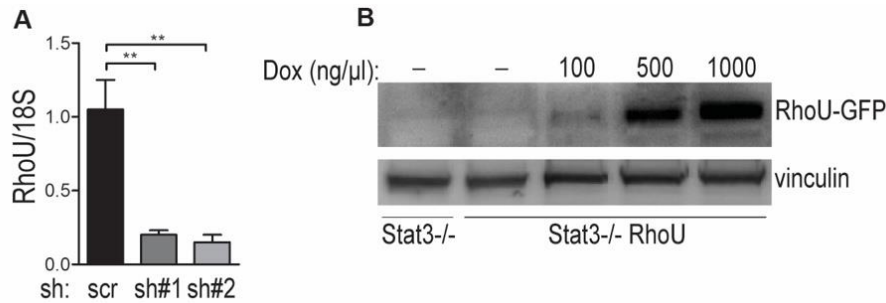


**Figure S3.** Manipulation of RhoU levels in MEF and MDA-MB-231 cells. (**A**) Lentiviral-mediated shRNA silencing of RhoU mRNA in MDA-MB-231 cells, measured by qRT-PCR upon infection with two distinct shRNA sequences (sh1, sh2) or with a control scrambled vector (scr). ** $p < 0.01$; $n = 4$. (**B**) RhoU-GFP expression levels in Stat3^{−/−} MEF cells either untransfected or stably transfected with a RhoU-GFP TetON construct (Stat3^{−/−} RhoU) and stimulated with the indicated doses of doxycycline (Dox), measured by Western blot with an anti-GFP monoclonal antibody.
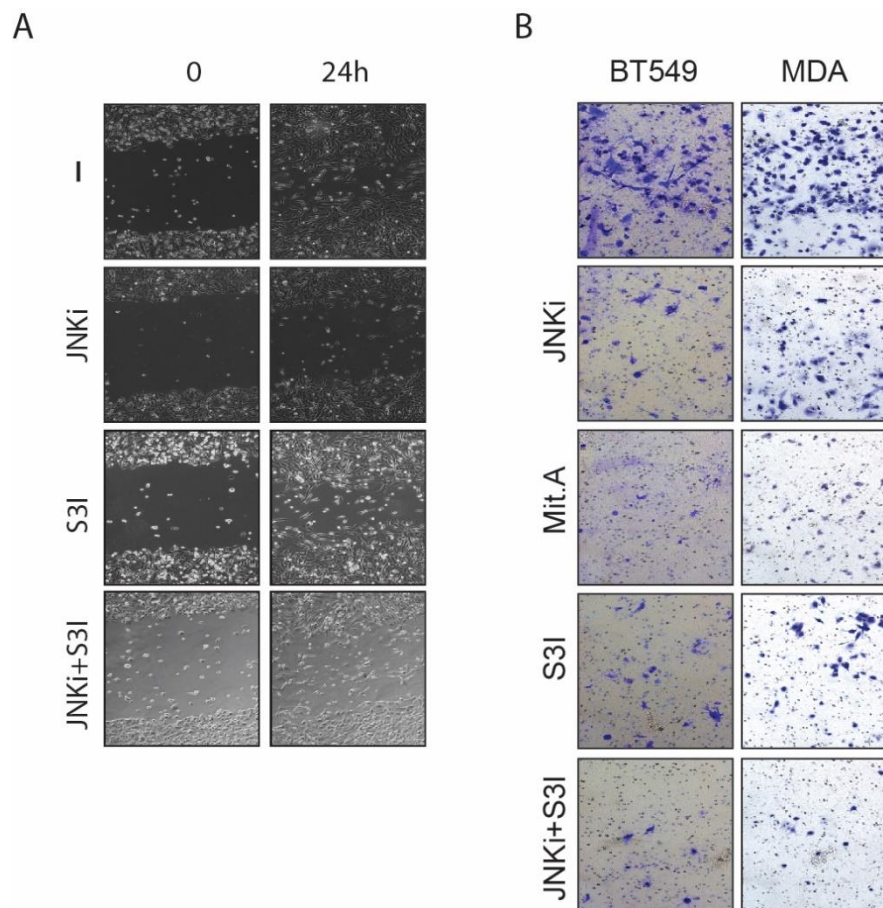
**Figure S4.** Migration and invasion assays upon treatment with various inhibitors. Representative pictures showing wound healing or transwell invasion assays with MDA-MB-231 (**A**,**B**) and BT-549 (**B**) cells treated either with the indicated inhibitors. Fields of View: 960 × 713 μm.
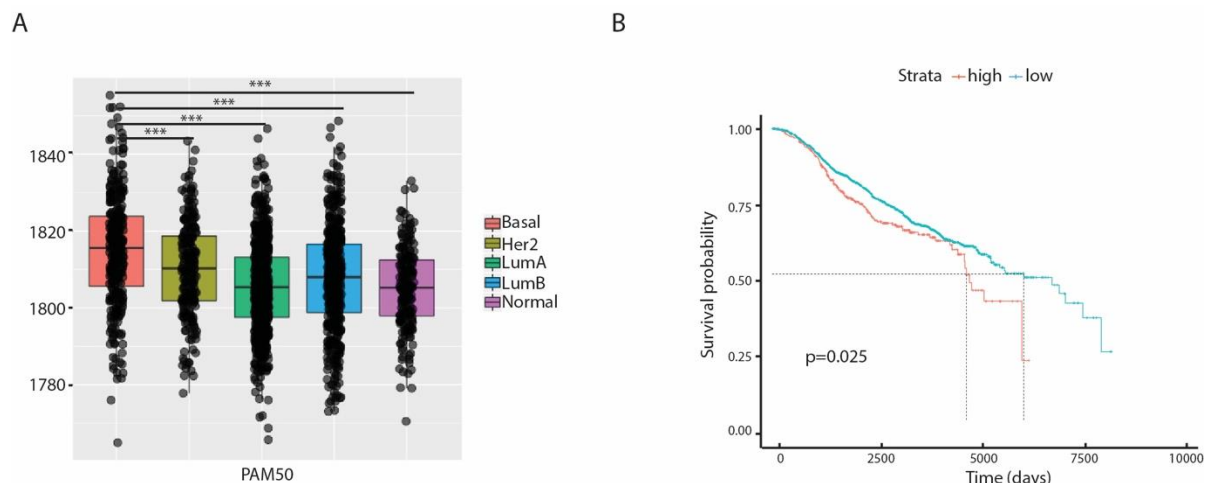


**Figure S5.** SP1 and STAT3 can functionally cooperate to enhance tumor aggressiveness independently of the tumor subtype. (**A**) Boxplot showing the distribution of the SP1-S3 score in the 5 breast cancer subtypes, significantly higher in basal-like tumors. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ between the indicated groups. (**B**) Kaplan-Meier plot of overall survival for breast tumor patients, as a function of time in days. Patient samples from the METABRIC database, excluding basal-like tumors, were subdivided according to low or high SP1-S3 score (median value). Even A high SP1-S3 score is predictive of poor prognosis.

**Table S1.** Total Binding Affinity of JASPAR Transcription Factors (TF) with the mouse RhoU promoter.

| TF | rank |
|---|---|
| MZF1_5-13 | 0.995208285029755 |
| ZNF354C | 0.99408764201252031 |
| ETS1 | 0.98334492619213232 |
| MZF1_1-4 | 0.95969549424221345 |
| Mycn | 0.95749285107040727 |
| Myc | 0.95617899373985626 |
| PPARG::RXRA | 0.95204420743488682 |
| Nr2e3 | 0.94292449184635596 |
| Myf | 0.94010356287193753 |
| znf143 | 0.93426849060978434 |
| INSM1 | 0.92453048921864134 |
| EWSR1-FLI1 | 0.91869541695648815 |
| ELK1 | 0.90644562949223273 |
| SP1 | 0.89539377077053872 |
| Arnt::Ahr | 0.87727026818146692 |
| Spz1 | 0.8758791251255893 |
| RREB1 | 0.87421748203106886 |
| ELF5 | 0.87309683901383417 |
| PLAG1 | 0.86865290980755849 |
| SPI1 | 0.85632583661797668 |
| Esrrb | 0.85168869309838469 |
| BRCA1 | 0.83766133395161912 |
| Sox17 | 0.83731354818764969 |
| PBX1 | 0.83553597650513955 |
| TLX1::NFIC | 0.82943040420434344 |
| Zfx | 0.82417497488213931 |
| REST | 0.81621454517350644 |
| ESR1 | 0.80983847283406751 |
| RUNX1 | 0.80879511554215933 |
| Egr1 | 0.80411932915990414 |
| ESR2 | 0.80033232861890413 |
| Klf4 | 0.79449725635675095 |
| USF1 | 0.79306747043821002 |
| GATA3 | 0.77548496792642396 |
| Pax5 | 0.76779503825643403 |
| NR3C1 | 0.76601746657392378 |
| Pax4 | 0.76439446634206665 |
| CTCF | 0.76358296622613808 |
| Gfi | 0.7626941803848829 |
| Sox2 | 0.75589303655614803 |
| GABPA | 0.75496560785222966 |
| SPIB | 0.74611639230234172 |
| MYC::MAX | 0.74244532034933153 |
| Foxd3 | 0.73232089033155578 |
| TFAP2A | 0.71002395857485123 |
| SOX9 | 0.70299095757013674 |
| Stat3 | 0.69924259989179993 |
| NHLH1 | 0.69456681350954474 |
| Myb | 0.68958188422598343 |
| SRF | 0.68757245536749367 |
| Tal1::Gata1 | 0.68223974031996293 |
| HNF4A | 0.67640466805780974 |

| | |
|---|---|
| HIF1A::ARNT | 0.65801066543009501 |
| E2F1 | 0.63888244841177833 |
| Hand1::Tcfe2a | 0.6313857330551047 |
| IRF2 | 0.62976273282324757 |
| RXR::RAR_DR5 | 0.62856480408068627 |
| GATA2 | 0.62728958961279857 |
| Zfp423 | 0.62543473220496171 |
| NFATC2 | 0.61187108741015539 |
| MAX | 0.61183244454749208 |
| FEV | 0.5974959424994204 |
| SRY | 0.59479094211299177 |
| NFKB1 | 0.58733286961898135 |
| NFIC | 0.58273436896205266 |
| Arnt | 0.58227065461009353 |
| TAL1::TCF3 | 0.58049308292758328 |
| TEAD1 | 0.57527629646804235 |
| Nkx3-2 | 0.5626014375144911 |
| REL | 0.53759950537135792 |
| ARID3A | 0.5313393616199088 |
| SOX10 | 0.5263930751990108 |
| Lhx3 | 0.52055800293685761 |
| NF-kappaB | 0.51823943117706162 |
| Hltf | 0.51584357369193912 |
| Nkx2-5 | 0.4831130690161527 |
| Gata1 | 0.48017621145374451 |
| Ddit3::Cebpa | 0.47078599582657082 |
| MIZF | 0.44914599273514183 |
| NR1H2::RXRA | 0.44377463482494783 |
| ELK4 | 0.44137877733982533 |
| EBF1 | 0.44033542004791715 |
| Pou5f1 | 0.43175670453667209 |
| NFE2L1::MafG | 0.42881984697426384 |
| STAT1 | 0.42093670299095759 |
| IRF1 | 0.41888863126980447 |
| En1 | 0.41884998840714122 |
| RELA | 0.41684055954865135 |
| Sox5 | 0.41363320194760028 |
| TP53 | 0.41119870159981453 |
| Mafb | 0.40710255815750829 |
| YY1 | 0.39280469897209985 |
| RXRA::VDR | 0.39210912744416104 |
| Pax6 | 0.38774248396321198 |
| Tcfcp2l1 | 0.38720148388592629 |
| NR2F1 | 0.36532962361851767 |
| NKX3-1 | 0.36053790864827268 |
| PPARG | 0.33855011979287425 |
| NR4A2 | 0.32552747507535357 |
| ZEB1 | 0.31265940180848595 |
| HNF1B | 0.28560939794419971 |
| RORA_1 | 0.28460468351495477 |
| Prrx2 | 0.27471211067315865 |
| Pax2 | 0.26791096684442384 |
| MEF2A | 0.26586289512327071 |
| T | 0.25512017930288278 |
| FOXC1 | 0.21535667362238195 |
| HNF1A | 0.213540045907720845 |

| | |
|---|---|
| Evi1 | 0.18467424066774868 |
| Foxq1 | 0.18254888322126903 |
| FOXI1 | 0.17779581111368731 |
| HOXA5 | 0.17327459618208516 |
| FOXO3 | 0.15248473606924801 |
| Nobox | 0.15202102171728882 |
| Pdx1 | 0.14823402117628873 |
| CREB1 | 0.13181080454440064 |
| NFYA | 0.12400494628642089 |
| FOXD1 | 0.12280701754385964 |
| CEBPA | 0.1195610170801453 |
| FOXL1 | 0.096877656696808104 |
| FOXF2 | 0.09571837081691012 |
| Ar | 0.091660870237267184 |
| NFE2L2 | 0.091081227297318185 |
| FOXA1 | 0.082966226138032312 |
| HLF | 0.079372439910348563 |
| RORA_2 | 0.064842723548960507 |
| TBP | 0.052322436046062293 |
| Foxa2 | 0.049385578483654068 |
| NFIL3 | 0.046989720998531569 |
| AP1 | 0.038449648349949767 |

The table shows a list of the 130 transcription factors described in the JASPAR Core Vertebrata database (downloaded November 2009), ranked according to their total binding affinity with the WNT-responsive region of the Rhou promoter (−756 to −235). A rank of 0.99 means that the affinity value with the Rhou promoter is higher compared to that shown by 99% of all gene promoters.

**Table S2.** See separated file.

**Table S3.** See separated file.

**Table 4.** Primers used to generate the RhoU promoter fusions.

| Primer | Sequence |
|---|---|
| -1756 forward | 5′-ACTAGGTACCGAATTCAGGTGGCCCAAGGGC-3′ |
| -1316 forward | 5′-GGCCGGTACCGCATCTCTGTACTTCAGGTTGTCC-3′ |
| -756 forward | 5′-GGCCGGTACCCGCTAACAAAGGGTGGTATCCTGC-3′ |
| -506 forward | 5′-GGCCGGTACCCCATGATCACCCTGCTTCTTCC-3′ |
| -366 forward | 5′-ACTAGGTACCCTGCCCTGCCCCCTCC-3′ |
| -234 forward | 5′-ACTAGGTACCGCTGGCTCCACCACG-3′ |
| -167 forward | 5′-GGCCGGTACCACAGCGCCCCCGTCTGC-3′ |
| reverse | 5′-TATCTCGAGGCCGCGAGACCAGCTGCC-3′ |

**Table S5.** Primers used to generate the Linker Scanning constructs.

| Linker | Forward primer 5′-3′ | Reverse primer 5′-3′ |
|---|---|---|
| LSc -605 | CCGAATTCAACACTCTCCCCCTCC | TTGAATTCGGTGCCTGCTTTTGCAG |
| LSc-595 | CCGAATTCAACTCCAAACCAAGACAC | TTGAATTCGGCTGTGGGGCCTG |
| LSc -585 | CCGAATTCAAAGACACTTGTGTGGAG | TTGAATTCGGGGGGGAGAGTGCTG |
| LSc-575 | CCGAATTCAAGTGGAGGAGCAAGG | TTGAATTCGGTGGTTTGGAGGGGG |
| LSc -565 | CCGAATTCAAAAGGGCAGCTGGAGG | TTGAATTCGGACAAGTGTCTTGGTTTGG |
| LSc-555 | CCGAATTCAAGGAGGAACAGGAAGAG | TTGAATTCGGGCTCCTCCACACAAG |
| LSc -545 | CCGAATTCAAGAAGAGTGGAAGAAGC | TTGAATTCGGAGCTGCCCTTGC |
| LSc-535 | CCGAATTCAAAGAAGCAGGGTGATCATGG | TTGAATTCGGCTGTTCCTCCAGCTGC |
| LSc -525 | CCGAATTCAATGATCATGGTGAGAGG | TTGAATTCGGTCCACTCTTCCTGTTC |

| | | |
|---|---|---|
| LSc-515 | CCGAATTCAAGAGAGGGAGACCG | TTGAATTCGGCCCTGCTTCTTCCAC |
| LSc-505 | CCGAATTCAACCGCGGAAAGGAG | TTGAATTCGGACCATGATCACCCTG |
| LSc -495 | CCGAATTCAAGAGGTTGTGGAGTAG | TTGAATTCGGTCTCCCTCTCACC |
| LSc-484 | CCGAATTCAAGTAGAAAGAGAATAGGGGG | TTGAATTCGGCCTTTCCGCGGTCTC |
| LSc -474 | CCGAATTCAAAATAGGGGGCGACC | TTGAATTCGGTCCACAACCTCCTTTCC |
| LSc-465 | CCGAATTCAACGACCTAAGGAGGG | TTGAATTCGGTCTTTCTACTCCACAACC |
| LSc -455 | CCGAATTCAAAGGGAGAAGGAGGAG | TTGAATTCGGCCCCCTATTCTCTTTC |
| LSc-445 | CCGAATTCAAAGGAGAGGGAAGAG | TTGAATTCGGCCTTAGGTCGCCC |
| LSc -435 | CCGAATTCAAAGAGAAAGAGCCGGG | TTGAATTCGGCCTTCTCCCTCCTTAG |
| LSc-424 | CCGAATTCAACGGGAGGGAAGG | TTGAATTCGGTTCCCTCTCCTCC |
| LSc-414 | CCGAATTCAAGGGGGAACAGAAGG | TTGAATTCGGGCTCTTTCTCTTCCCTC |
| LSc-405 | CCGAATTCAAGAAGGATGGGGAGG | TTGAATTCGGTCCCTCCCGGC |
| LSc -395 | CCGAATTCAAGAGGAGGGGGC | TTGAATTCGGTGTTCCCCCTTCCC |
| LSc-385 | CCGAATTCAACAGGGCAGGAGG | TTGAATTCGGCCCATCCTTCTGTTC |
| LSc -375 | CCGAATTCAAGGAACTGGAGAGC | TTGAATTCGGCCCCCTCCTCC |
| LSc-364 | CCGAATTCAAGCAAGAGGAGGAAAG | TTGAATTCGGCTCCTGCCCTGC |
| LSc -354 | CCGAATTCAAGAAAGGAAGCTTGGG | TTGAATTCGGTCTCCAGTTCCTCC |
| LSc-345 | CCGAATTCAACTTGGGAAGGAGCC | TTGAATTCGGTCCTCTTGCTCTCC |
| LSc -335 | CCGAATTCAAAGCCGGGAAAGG | TTGAATTCGGCTTCCTTTCCTCCTC |
| LSc-325 | CCGAATTCAAGGGGGGTGGG | TTGAATTCGGCCTTCCCAAGCTTCC |
| LSc -315 | CCGAATTCAAGGGATGAGAAGCC | TTGAATTCGGTTTCCCGGCTCC |
| LSc-309 | CCGAATTCAAAGAAGCCCACAGCAGG | TTGAATTCGGCCCCCCTTTCCCG |
| LSc -299 | CCGAATTCAAAGCAGGGGCCAG | TTGAATTCGGCATCCCCCCACC |
| LSc-289 | CCGAATTCAAAGGGGTAGCTCGG | TTGAATTCGGGTGGGCTTCTCATCC |
| LSc -278 | CCGAATTCAAGGGGTCCGCG | TTGAATTCGGTGGCCCCTGCTG |
| LSc-268 | CCGAATTCAAGGGTTGGCGTGG | TTGAATTCGGGAGCTACCCCTGG |
| LSc-256 | CCGAATTCAAGAGCCAGCTAGTCC | TTGAATTCGGCACGCGGACCCCG |
| LSc-242 | CCGAATTCAAGTCCAGCCGCAC | TTGAATTCGGCACCACGCCAACC |
| LSc-233 | CCGAATTCAAACCGTTTCGAGGCG | TTGAATTCGGTAGCTGGCTCCACC |

**Table S6.** Primers used for qRT-PCR.

| Gene | Forward primer 5'-3' | Reverse primer 5'-3' | Probe |
|---|---|---|---|
| *Mus musculus* RhoU | ACGGCCTTCGACAACTTCT | ACTCATCCTGTCCTGCAGTGT | 78 |
| *Homo sapiens* RhoU | GACTCCAACTCTGTGACACTGC | ATGAGGGGCTCACGACACT | 4 |
| *Homo sapiens* WNT5a | AGGGCTCCTACGAGAGTGCT | GACACCCCATGGCACTTG | / |
| *Homo sapiens* WNT5b | AGAAGAACTTTGCCAAAGGA | CTACGTCTGCCATCTTATACAC | / |
| *Mus Musculus* MAPK8 (JNK1) | AGAAACTGTTCCCCGATGTG | GGATAACAAATCTCTTGCCTGAC | 33 |
| *Mus Musculus* MAPK9 (JNK2) | GCTTGTTAAATGTGTTCACACCAC | TTCTCTCATGGTCCAGTTCCA | / |
| Homo sapiens Ror2 | GTGACCTTTGTAGACTTTCC | ATTCTGTACATCTTGGTCCC | / |
| Homo sapiens Ror1 | GATTAGAAACCTCGACACCAC | GCAAAGACTCCATAGACGG | / |