Supplement

Loss of Neuropilin-2 in Murine Mesenchymal-like Colon Cancer Organoids Causes Mesenchymal-to-Epithelial Transition and an Acquired Dependency on Insulin-Receptor Signaling and Autophagy

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Table S1. Nrp2 single guide RNA (sgRNA) sequences

sgRNA	Exon	Strand	Sequence 5′- 3′
mNrp2- 1	1	Forward	TGGATATGTTTCCTCTTACC
mNrp2- 2		Forward	GGATATGTTTCCTCTTACCT
mNrp2- 3	2	Reverse	GGAATTCAGCCGACCTCCGC
mNrp2- 4		Reverse	TTCAGCCGACCTCCGCAGGG
mNrp2- 5	3	Forward	CACCTCAGACTACGCCCGGC
mNrp2- 6		Forward	AGTTCACCTCAGACTACGCC
mNrp2- 7	4	Forward	GCATGACCCTCTACAAGTGG
mNrp2- 8		Forward	GAGATTCAATGGTCCCATTG
mNrp2- 9	6	Forward	CAATGGCTGGACACCCAATT
mNrp2- 10		Forward	CCATGGAGCCGGCTCTGTTG

Table S2. Nrp2 oligo sequences to clone into LentiCRISPRv2 sgRNA scaffold

sgRNA oligo	Sequence 5′- 3′
mNrp2- 1F	CACCGTGGATATGTTTCCTCTTACC
mNrp2- 1R	AAACGGTAAGAGGAAACATATCCAC
mNrp2- 2F	CACCGGGATATGTTTCCTCTTACCT
mNrp2- 2R	AAACAGGTAAGAGGAAACATATCCC
mNrp2- 3F	CACCGGGAATTCAGCCGACCTCCGC
mNrp2- 3R	AAACGCGGAGGTCGGCTGAATTCCC
mNrp2- 4F	CACCGTTCAGCCGACCTCCGCAGGG
mNrp2- 4R	AAACCCCTGCGGAGGTCGGCTGAAC
mNrp2- 5F	CACCGCACCTCAGACTACGCCCGGC
mNrp2- 5R	AAACGCCGGGCGTAGTCTGAGGTGC
mNrp2- 6F	CACCGAGTTCACCTCAGACTACGCC
mNrp2- 6R	AAACGGCGTAGTCTGAGGTGAACTC
mNrp2- 7F	CACCGGCATGACCCTCTACAAGTGG
mNrp2- 7R	AAACCCACTTGTAGAGGGTCATGCC
mNrp2- 8F	CACCGGAGATTCAATGGTCCCATTG
mNrp2- 8R	AAACCAATGGGACCATTGAATCTCC
mNrp2- 9F	CACCGCAATGGCTGGACACCCAATT
mNrp2- 9R	AAACAATTGGGTGTCCAGCCATTGC
mNrp2- 10F	CACCGCCATGGAGCCGGCTCTGTTG
mNrp2- 10R	AAACCAACAGAGCCGGCTCCATGGC

Table S3. Nrp2 genomic DNA primer[1] sequences for five Nrp2 CRISPR-Cas9 plasmids

Primer	Sequence 5′- 3′
NRP2_1.1_F	GGCTGGGAGACACCGAATAA
NRP2_1.1_R	TAGCAGCTGTGGAAATGGGG
NRP2_1.1_1F	GTCGGCACCACAAAACCAC
NRP2_1.1_1R	TTTTGTAGTCCAGCCCCTGC
NRP2_5.1_F	ATGGTGCATCTGGCATCTGT
NRP2_5.1_R	GGGGCACCTTGAGTTCTGTT
NRP2_5.1_1F	TGGTGCATCTGGCATCTGTAG
NRP2_5.1_1R	GGCAGGGCTCTCTCCAATAG
NRP2_3.1_F	ATTTATGAGGGCTTGAAACGTGC
NRP2_3.1_R	AGCCTTGGGTGATGTACCTTTT
NRP2_3.1_1F	TCTCTGTACCTCCACCCCAC
NRP2_3.1_1R	TGATGATAGCAGCTAACTTAACACA
NRP2_6.1_F	AGCATCTGCTTCCACAGTCC
NRP2_6.1_R	GAGCCCATCCCCAACAAACT
NRP2_6.1_1F	TCCAAGGCAGGCCATCATTT
NRP2_6.1_1R	CAGAGCCCATCCCCAACAAA
NRP2_2.2_F	AACGATGCTCCTGCTCTTACG
NRP2_2.2_R	TGGTAACATGGACTGCCAAGG
NRP2_2.2_1F	GATGCTCCTGCTCTTACGTG
NRP2_2.2_1R	GTAACATGGACTGCCAAGGG

[1] Primers were designed based on mouse Nrp2 NCBI reference sequence NC_000067.6 using NCBI primer design tool. Primer quality and annealing temperatures were checked using NCBI and NEB Tm calculator tools.

Table S4. Murine organoid culture medium composition

Component	Supplier (catalog number)	Concentration
Advanced DMEM/F12 basal medium	Gibco (12634-010)	1x (500ml total)
HEPES buffer	Lonza (BE17-737E)	10mM
Penicillin-Streptomycin	Gibco (15070-063)	50U/ml
GlutaMAX	Gibco (35050-038)	2mM
Noggin conditioned medium	293T-mNoggin-Fc cell line	100ng/ml
B27, serum free	Invitrogen (17504-044)	1x
Recombinant murine FGF-basic	PeproTech (450-33)	10ng/ml

Table S5. The list of antibodies

Assay	Antibody (clone)	Supplier (catalog number)	Dilution
Western blot	TJP2	Invitrogen (71-1400)	1:150
	LC3II (0510)	Nanotools (0231-100/LC3-5F10)	1:1000
	Nrp2 (D39A5)	Cell Signaling (3366)	1:1000
	IR-β (4B8)	Cell signaling (3025)	1:1000
	phospho IGFIRβ-IRβ	Cell signaling (3021)	1:1000
	PI3 kinase p85	Cell signaling (4292)	1:1000
	phospho PI3 kinase p85/p55	Cell signaling (4228S)	1:1000
	p44/42 MAPK (Erk1/2) (137F5)	Cell signaling (4695)	1:1000
	phospho P44/42 MAPK (Erk1/2)	Cell signaling (9101)	1:1000
	PARD3/ Par3	Novus Biologicals (NBP1-88861)	1:150
	Occludin (OCLN)	Thermofisher (40-4700)	1:250
	β-actin (AC-15)	Novus Biologicals (NB600-501)	1:10000
	α-tubulin (B-5-1-2)	Sigma (T5168)	1:10000
	Goat anti-rabbit IgG HRP	Dako (P0448)	1:1000
	Goat anti-mouse IgG HRP	Dako (P0447)	1:2000
IHC	ZEB1 (2A8A6)	Novus Biologicals (NBP2-23484SS)	1:250
	Slug	Novus Biologicals (NBP2-27182SS)	1:100
	TJP2	Novus Biologicals (NBP1-86850)	1:100
	LC3II (0510)	Nanotools (0231-100/LC3-5F10)	1:100
	IR-β (E9L5V)	Cell signaling (23413S)	1:50
	PARD3/ Par3	Novus Biologicals (NBP1-88861)	1:500
	Occludin (OCLN)	Thermofisher (40-4700)	1:100
IF	LC3II (0510)	Nanotools (0231-100/LC3-5F10)	1:50
	p62/SQSTM1	Novus Biologicals (NBP1-48320)	1:200
	Cleaved caspase-3	Cell Signaling (9661S)	1:400
	PARD3/ Par3	Novus Biologicals (NBP1-88861)	1:50
	TJP2	Invitrogen (71-1400)	1:300
	IR-β (E9L5V)	Cell signaling (23413S)	1:50
	Nrp2 (D39A5)	Cell Signaling (3366)	1:1000
	DAPI	Sigma (D9542)	1:1000
	Phalloidin AlexaFluor488	ThermoFisher (A12379)	1:500
	Alexa Fluor 568	ThermoFisher (A11036)	1:500

Figure S1. Generation and characterization of Nrp2$^{-/-}$ CRC organoids. (**a**) Schematic view of lentiviral plasmids used for Nrp2$^{-/-}$ (left) or control (right) CRC organoid generation. (**b**) Sanger sequencing data used to analyze % of DNA with insertions and/or deletions in CRC organoid genomic DNA targeted location. Inference of CRISPR Edits (ICE) tool from Synthego was used. (**c**) Principal component analysis (PCA) for RNAseq data. Samples were sequenced in three replicates. (**d**) Cell cycle analysis by quantification of DNA content (propidium iodide) in CRC organoids. (**e**) Immunofluorescence analysis for Nrp2 expression in CRC organoids. Representative organoid images are depicted, where DAPI-stained nuclei are shown in blue, and membranous Nrp2 is shown in red. Scale bars: 15μm. (**f**) Western blot analysis for Nrp2 expression in CRC organoids.

Figure S2. Increased receptor tyrosin kinase (RTK) and tight junction protein (TJP) RNA expression in Nrp2$^{-/-}$ CRC organoids. (**a**) RTK and (**b**) TJP RNA expression in control and Nrp2$^{-/-}$ CRC organoids determined by RNA sequencing, represented in heatmaps. Samples were sequenced in three replicates.

Figure S3. Increased tight junction protein expression in Nrp2$^{-/-}$ CRC organoids. (**a**) TJP2 RNA and protein expression analysis in CRC organoids via RNAseq and western blotting, respectively. (**b**) PARD3 RNA and protein expression analysis in CRC organoids via RNAseq and western blotting, respectively.

Figure S4. Increased autophagic flux in Nrp2$^{-/-}$ CRC organoids. Immunofluorescence analysis of CRC organoids after treatment with an early autophagy inhibitor (Spautin-1) or late autophagy inhibitor (Chloroquine). Each inhibitor was used at 30μM concentration for 24h. Representative organoid images are depicted, where DAPI-stained nuclei are shown in blue, and p62 and LC3 puncta are shown in red. Mean fluorescence intensity was quantified relative to the control (n=5). Scale bars: 15μm. Error bars are presented as SD from the arithmetic mean. Statistical significance on graphs is indicated $p \leq 0.05$ by *, $p \leq 0.01$ by **, $p \leq 0.001$ by ***, $p \leq 0.0001$ by ****.

Figure S5. Decreased viability and regeneration in Nrp2$^{-/-}$ CRC organoids. (**a**) CellTiter-glo 3D viability analysis of CRC organoids after single or combination treatment with early autophagy inhibitor (Spautin-1) (30μM) and IR signaling inhibitor (Linsitinib) (5μM or 15μM) for 24h. Relative luminescence signal was quantified relative to the 100% untreated control. (**b**) Colony forming assay by CRC organoids. Number of colonies were counted two weeks after single cell seeding, and quantified relative to the control (n=3). Error bars are presented as SD from the arithmetic mean. Statistical significance on graphs is indicated $p \leq 0.05$ by *, $p \leq 0.0001$ by ****.

Figure S6. Immunoblots used to generate Figure 4b. Representative blots from protein analysis of insulin-stimulated CRC organoids. Bands used for figure generation are highlighted.

Figure S7. Immunoblots used to generate Figure 5b. Representative blots from protein analysis of the control and Nrp2$^{-/-}$ CRC organoids. Bands used for figure generation are highlighted.

Figure S8. Immunoblots used to generate Figure S1. Representative blots from protein analysis of the control and Nrp2$^{-/-}$ CRC organoids. Bands used for figure generation are highlighted.

Figure S9. Immunoblots used to generate Figure S3. Representative blots from protein analysis of control and Nrp2$^{-/-}$ CRC organoids. (**a**) TJP2 expression blot, (**b**) PARD3 expression blot. Bands used for figure generation are highlighted.