Supplementary Information for:

Cancer cell specific inhibition of Wnt/β-catenin signaling by forced intracellular acidification

Svitlana Melnik[1,2,12], Dmytro Dvornikov[3,4,12], Karin Müller-Decker[5], Sofia Depner[3], Peter Stannek[6], Michael Meister[4,7], Arne Warth[4 8], Michael Thomas[4,7], Thomas Muley[4,7], Angela Risch[1, 4,9, 10], Christoph Plass[1], Ursula Klingmüller[3,4], Christof Niehrs[6,11], Andrey Glinka[6*].

*To whom correspondence should be addressed:
E-mail: glinka@dkfz.de

This pdf file includes:

Supplementary Tables 1-2
Supplementary Figures 1-10

Supplementary Table 1. Summary of clinical and mRNA expression data for the lung cancer patients cohort used in this study

Category	Non-diabetic	Metformin-taking diabetic
Number of patients	66	42
Age, median (range)	63 (43 – 80)	69 (53 – 79)
Male/Female	52/14	33/9
NS/FS/S/U	5/35/26/0	2/23/16/1
ECOG 0/1/unknown	55/9/2	38/4/0
p-stage I/II/III	20/24/22	9/13/18*
Histology		
Squamous cell	26	19
Adenocarcinoma	30	16
Large cell	4	2
Other (lung metastases and secondary malignant lung tumors)	6	5
Postoperative chemotherapy	36	16
Postoperative radiotherapy	18	5
Recurrence (local, distant, 2nd tumor)	29	20
Dead	29	15
mRNA expression, Mean value ± SEM		
AXIN2	1.89±0.45	2.22±0.22
SOX4	3.77±0.45	3.01±0.28
DDIT3	4.03±0.58	5.09±2.23
VIM	1.59±0.16	2.01±0.31
ZEB1	1.30±0.10	2.36±0.36

* – no stage was assigned
NS – non-smoker
FS – former smoker
S – smoker
U – unknown

Supplementary Table 2. List of primers used in this study

Gene Name	Sequence	UPL	Assay
DDIT3	CCCATCGATGCCACCATGGCAGCTGAGTCATTGCC CCCTCTAGATTATGCTTGGTGCAGATTCACC		Cloning
EC-GFP	CCCGGATCCGCCACCATGAGTAAAGGAGAAGAAC TTTTCACTGGAG CCCGAATTCTTATTTGTATATTTCATCCATGCCATG TGT		Cloning
SOX4	CAACGCCAACTCCAGCTC ACCGACCTTGTCTCCCTTC	25	qRT-PCR
AXIN2	CCACACCCTTCTCCAATCC TGCCAGTTTCTTTGGCTCTT	36	qRT-PCR
ESD	TTAGATGGACAGTTACTCCCTGATAA GGTTGCAATGAAGTAGTAGCTATGA	27	qRT-PCR
ZEB1	TTTTTCCTGAGGCACCTGAA AAAATGCATCTGGTGTTCCAT	28	qRT-PCR
GAPDH	GCATCCTGGGCTACACTGAG AGGTGGAGGAGTGGGTGTC	82	qRT-PCR
VIM	TACAGGAAGCTGCTGGAAG ACCAGAGGGAGTGAATCCAG	24	qRT-PCR
DDIT3	CAGAGCTGGAACCTGAGGAG TGGATCAGTCTGGAAAAGCA	9	qRT-PCR
ATF6	CTTTTAGCCCGGGACTCTTT TCAGCAAAGAGAGCAGAATCC	42	qRT-PCR
ATF4	GGTCAGTCCCTCCAACAACA CTATACCCAACAGGGCATCC	88	qRT-PCR
ATF2	TTTGGTCCAGCACGTAATGA CAAACCCACTTCTTCACAGTTTT	5	qRT-PCR
PERK	CCAGCCTTAGCAAACCAGAG TCTTGGTCCCACTGGAAGAG	58	qRT-PCR
IRE1	TGAGGACGAAGGGGACTACA ACGTCCCCAGATTCACTGTC	9	qRT-PCR
GPD2	CGGACAACGAGAAGTCGTC AGTCCTAAAACAGTTGCAAGAGC	25	qRT-PCR
AMPK1	TCTCAGGAGGAGAGCTATTTGATT GAACAGACGCCGACTTTCTTT	42	qRT-PCR

AMPK2	ACCCACTGAAACGAGCAACT AGGAAGGGTCTTCAGGAAATAAG	61	qRT-PCR
LGR5	ACCAGACTATGCCTTTGGAAAC TTCCCAGGGAGTGGATTCTAT	78	qRT-PCR
DDIT3	CCCATTATCCTGCAGATGTGC CTTCCTTCAAGGAAATGAGGAAAGG		genotyping
SOX4 upstream	ACAGATCCAGTCAGATGGCTAC AGTATGTAGGCTGGCAATAGGC		qPCR, ChIP
SOX4 promoter (-800)	TGGGCTATGCAGGATTTACAGG TTGTGCTGTTTGTGGAAGG		qPCR, ChIP
SOX4 promoter (-400)	AGCTGGGGAACAGATTTTGC ATAACAAGGG GCTTGGAACG		qPCR, ChIP
SOX4 exon	ACATCAAGCGACCCATGAAC TTGTCGCTGTCTTTGAGCAG		qPCR, ChIP
SOX4 downstream	TGCCCAATGCCTAAGATTGG AGAGGAAATCTGGCCAAGAGAC		qPCR, ChIP
GAPDH	GGGGCCTGTTTTGTTGTCAT GGGCCGTGTGATTCTTTGTT		qPCR, ChIP
AXIN2[42]	GGAGCAGTAAAAGGCCGTAA CCAAACCATTGAAGCCCTTA		qPCR, ChIP

Supplementary figure legends

Supplementary Figure 1 *SOX4* is a Wnt-inducible gene. (**a-e**) Levels of the indicated mRNA and proteins in the indicated cell lines were monitored by qRT-PCT or WB, respectively. **a,** H1299 cells were treated with: WntCM, epidermal growth factor (EGF, 50 ng/ml), insulin-like growth factor (IGF, 40 ng/ml), basic fibroblast growth factor (bFGF, 1 ng/ml) or insulin (10 μg/ml). Position of unspecific band used as a loading control is indicated by asterisk. Endogenous β-catenin from cytosolic extracts was used as a control of Wnt pathway activation. **b,** H1299 cells were treated with WntCM, as indicated. **c,** Cells were treated with WntCM for 48 h and subjected to siRNA-mediated *β-catenin* knockdown for 76 h. **d,** H1299 cells were transiently transfected with control or *β-catenin*-expressing vectors for 72 h. (**a-d**) Error bars represent mean values ± SD. **e,** Cells were subjected to siRNA-mediated knockdown of *β-catenin* for 48 h.

Supplementary Figure 2 Metformin affects Wnt signaling in lung cancer patients.

Non-linear Spearman's rank correlation analysis of *VIM* or *ZEB1* mRNA expression levels with Wnt signaling (*AXIN2*) in tumor tissue samples from lung cancer patients, non-diabetic or Metformin-taking diabetic. ρ – Spearman's rank coefficient for non-linear correlation: $P \leq 0.05$ (*); ns – not significant; n – number of patients.

Supplementary Figure 3 Metformin's effects on SOX4 could not be imitated by interference with GPD2 or AMPK functions. Levels of the indicated mRNAs and proteins were monitored by qRT-PCR or WB, respectively, in the indicated cell lines, subjected to siRNA-mediated knockdown of *GPD2* (**a**) or *AMPK1/2* (**b**) for 96 h, as indicated. Error bars represent mean values ± SD.

Supplementary Figure 4 DDIT3 is one of the key factors involved in SOX4 repression upon Metformin treatment. (**a-c**) Levels of the indicated mRNA and proteins in the indicated cell lines, treated with WntCM and Metformin, as indicated, were monitored by qRT-PCT or WB, respectively. Asterisk indicates a position of DDIT3 protein band migration. **c,** DLD1 cells were pre-treated with Metformin for 72 h and later transferred into fresh media for the indicated time period. **d,** TOPFLASH luciferase reporter assay in HEK293T cells: Wnt signaling was activated by co-transfection of *LRP6* with *wnt8-frizzled5* fusion constructs (*LRP6/W/Fz*) or by *β-catenin*, in presence of or without *DDIT3* expression construct. Experiment was done in triplicates, error bars correspond to mean values ± SD; RLA – relative luciferase activity. **e,** Confirmation of DDIT3 knockout in H1975 cell line generated with CRISPR/Cas9 genome editing system. Top: Genotyping result showed a single nucleotide insertion mutation (marked by asterisk) in a selected clone that caused translation frame shift. No wildtype alleles were detected for the selected clone after multiple sequencing. Bottom: H1975 wildtype cells and the selected mutant clone were treated with 5 µg/ml Tunicamycin, 60 nM Nigericin or 20 nM Bortezomib for 48 h, and DDIT3 protein induction was monitored by WB.

Supplementary Figure 5 Efficiency of siRNA-mediated knockdown experiments performed in this study was controlled by qRT-PCR. **a,** Samples from experiment presented in **Fig. 3a**. **d**, Samples from experiment presented in **Fig. 3f**.

Supplementary Figure 6 The UPR-inducing drugs induce DDIT3 and reduce protein levels of SOX4. (**a-b**) Levels of the indicated proteins in the indicated cell lines treated with WntCM and different drugs were monitored by WB. Asterisk indicates position of unspecific band used as a loading control. **b,** H1299 cells were transfected with *β-catenin* expression construct for 72 h and treated with Metformin or Tunicamycin, as indicated. **c,** 3D cell invasion assay done with H1299 cells treated with WntCM and the indicated drugs (6 mM Metformin or 5 nM Bortezomib) for 96 h. Cells were fixed and imaged using confocal microscopy. Bottom: Representative images of 3D cell invasion assay. Experiment was done with 5 biological replicates, error bars correspond to mean values ± SD.

$P < 0.0001$ (****). Scale bar, 150 μm.

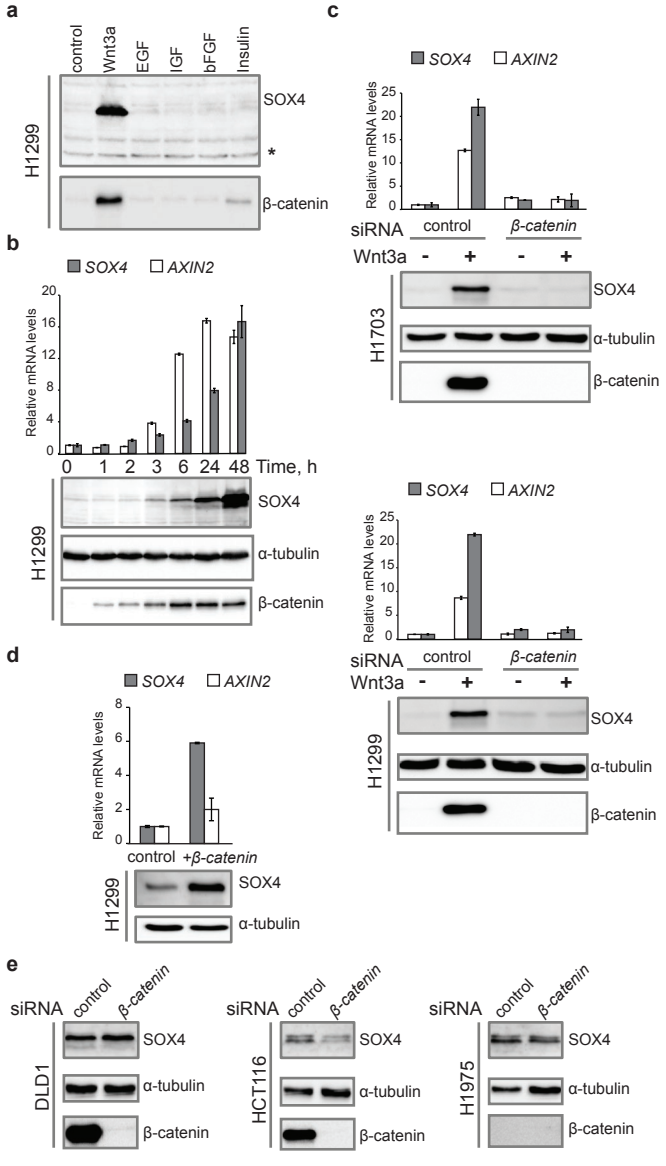
Supplementary Figure 7 Mitochondrial complex I inhibitors cause intracellular acidification. (**a-c**) Fluorometric measurements of pHi changes (EC-GFP/mCherry) in DLD1$^{EC\text{-}GFP/mCHERRY}$ cells upon treatment with increasing concentrations of the indicated MCI inhibitor drugs for 72 h. **b,** pHi calibration curve built for conversion of EC-GFP/mCherry ratio values to pHi. DLD1$^{EC\text{-}GFP/mCHERRY}$ cells were exposed to standard calibrating solutions with pH range from 6.2 to 8.8, for 0.5 h, before fluorometry. **c,** Treatment was done as in **a**, using increasing concentrations of MCII inhibitor, Lonidamine, in presence or without of 5 nM Monensin. Error bars correspond to mean values ± SD for 6 biological replicates. (**a, c**) Positions of black lines on the right indicate a range of pHi changes caused by the treatments. The pHi values were converted from EC-GFP/mCherry ratios using the calibration curve presented in **b**.

Supplementary Figure 8 Only when drug treatment causes intracellular acidification, it blocks Wnt signaling. **a,** Fluorometric measurements of pHi changes (EC-GFP/mCherry) in DLD1$^{EC\text{-}GFP/mCHERRY}$ cells upon treatment with increasing concentrations of the indicated monocarboxylate transporter (MCT) inhibitors: CHC (2-Cyano-3-(4-hydroxyphenyl)-2-propenoic acid) and AZD3965, or carbonic anhydrases (CA) inhibitor (Azetazolamide) for 72 h, as indicated. **b,** Levels of indicated proteins were monitored by WB in H1299 cells treated with indicated drugs (as in **a,** for 72 h) and induced with WntCM for 48 h.

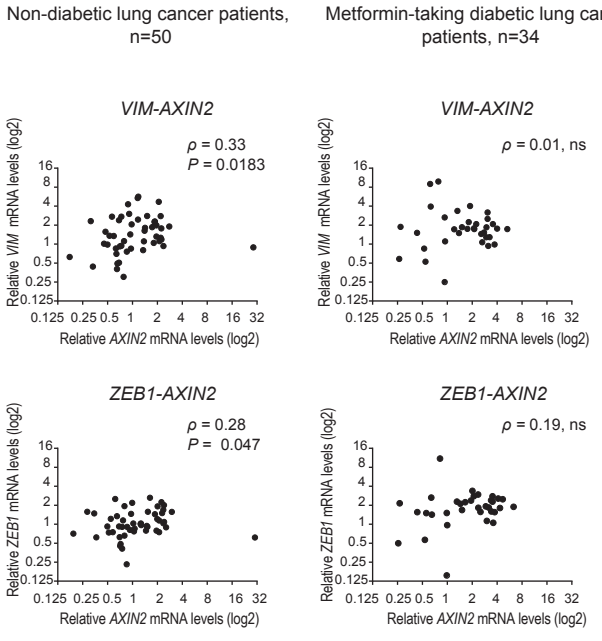
Supplementary Figure 9 Drugs, selected according to the 'Warburg Trap' model, cooperatively drop pHi and inhibit Wnt signaling. **a,** Confocal microscopy in H1975$^{EC\text{-}GFP}$ cells treated with combinations of Rotenone and Nigericin, or Papaverine and Salinomycin, as indicated. Live cells were imaged at 405 nm and 488 nm excitations to record control and pH-sensitive GFP emissions, respectively. Scale bar, 150 μm. (**b-c)** Levels of the indicated proteins in the indicated cell lines treated as in **Fig. 5b** (H1975 and MDA-MB-231, **b**), or as indicated (MRC5, **b**; or H1299, **c**), were evaluated by WB. **d,** qRT-PCR analysis of the indicated mRNAs expression levels in the samples from the experiment presented in **Fig. 5c**. **e,** ATP concentration measurements in the indicated cell lines treated with Rotenone/Nigericin, as indicated. Error bars correspond to mean values ± SD for 6 biological replicates; $P = 0.0042$ (**); ns – not significant.

Supplementary Figure 10 Drugs, selected according to the 'Warburg Trap' model, cooperatively affect cancer cells viability and inhibit SOX4 protein accumulation, also in presence of ROS scavengers. **a,** Cell viability measured in the indicated cell lines with the indicated drug combinations. Assay was done as in **Fig. 5f**. Error bars correspond to mean values ± SD for 6 biological replicates. **b,** The indicated cell lines were treated with different drug combinations: 10 nM Nigericin (Nig), 5nM

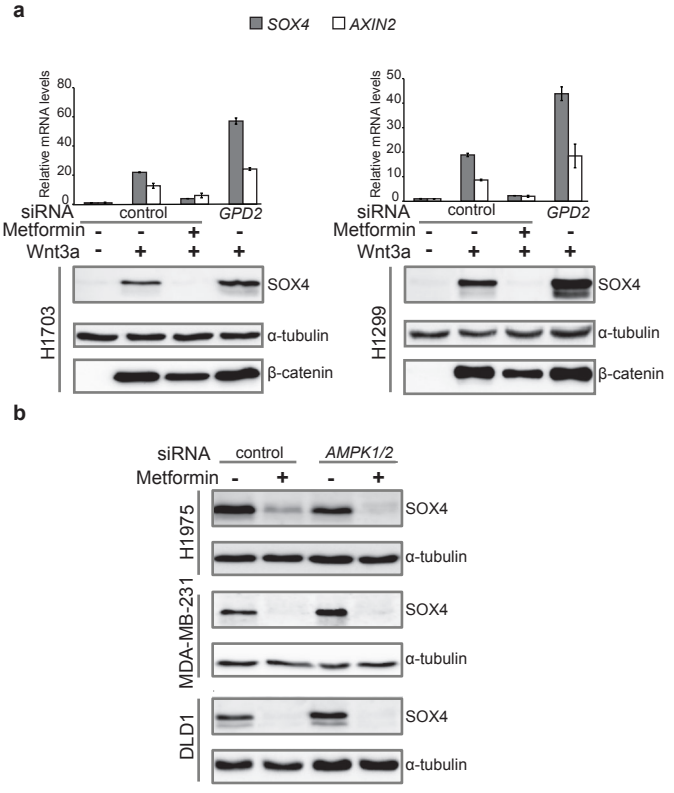
Rotenone (Rot), 1 μM Papaverine-HCl (Pap), 10 nM Salinomycin (Sal), in presence or without of 1mM NAC (N-acetyl cysteine), 20 μM Doxycycline, 20 μM Minocycline, 100μM Trolox (soluble form of vitamin E), as indicated, and levels of SOX4 protein were evaluated with WB.

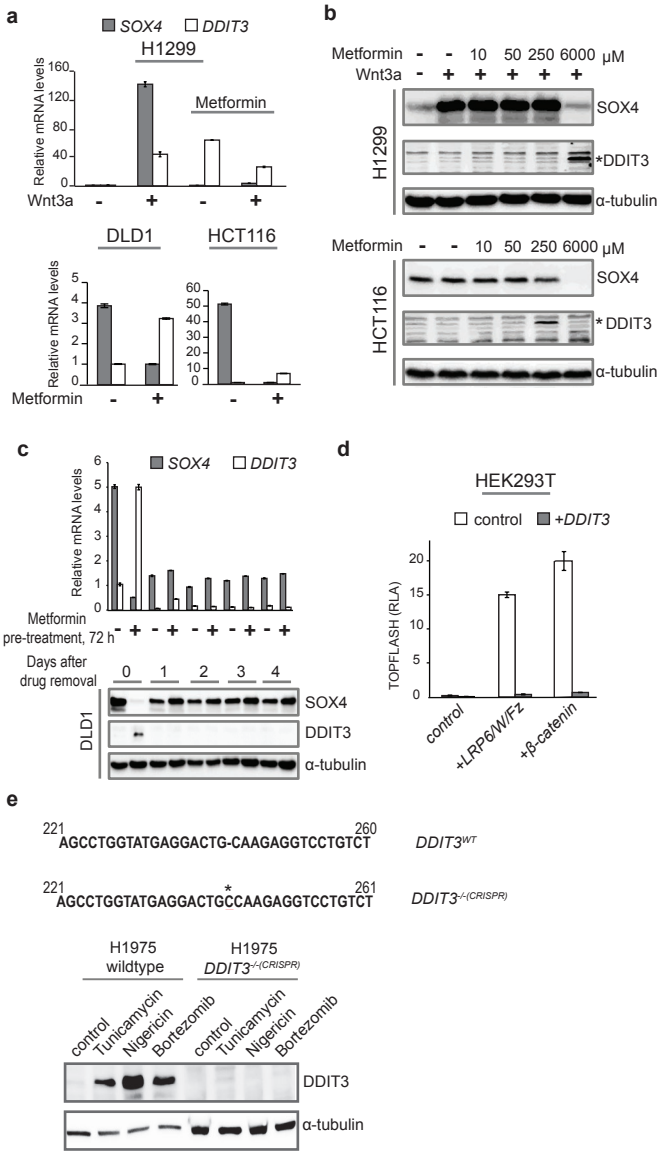

a
control
Wnt3a
EGF
IGF
bFGF
Insulin
H1299
SOX4
*
β-catenin
b
SOX4
AXIN2
Relative mRNA levels
Time, h
0 1 2 3 6 24 48
H1299
SOX4
α-tubulin
β-catenin
c
SOX4
AXIN2
Relative mRNA levels
siRNA
control
β-catenin
Wnt3a
H1703
SOX4
α-tubulin
β-catenin
H1299
SOX4
α-tubulin
β-catenin
d
SOX4
AXIN2
Relative mRNA levels
control
+β-catenin
H1299
SOX4
α-tubulin
e
siRNA
control
β-catenin
DLD1
HCT116
H1975
SOX4
α-tubulin
β-catenin

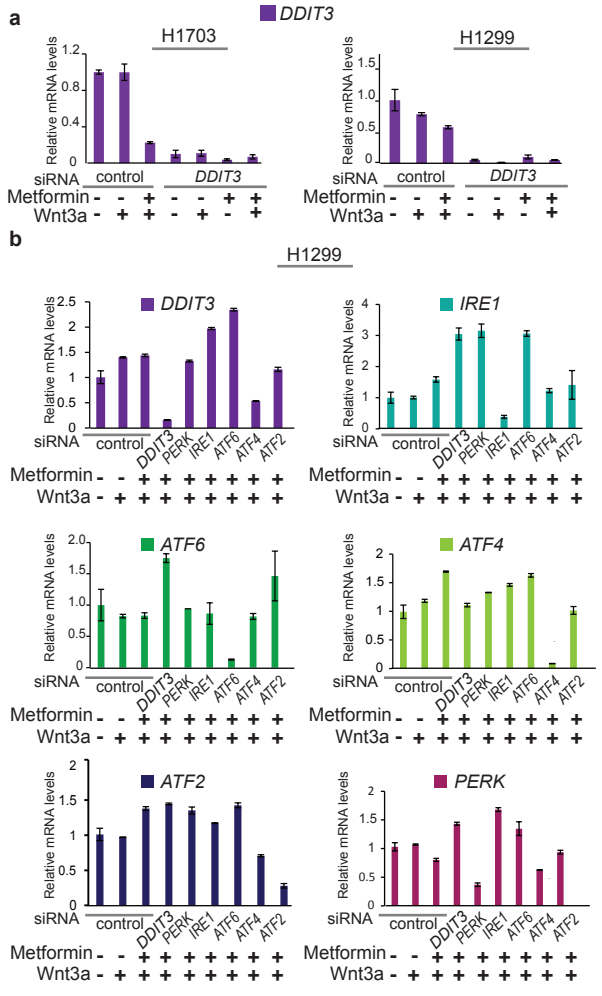
Melnik *et al.,* Supplementary Figure 2



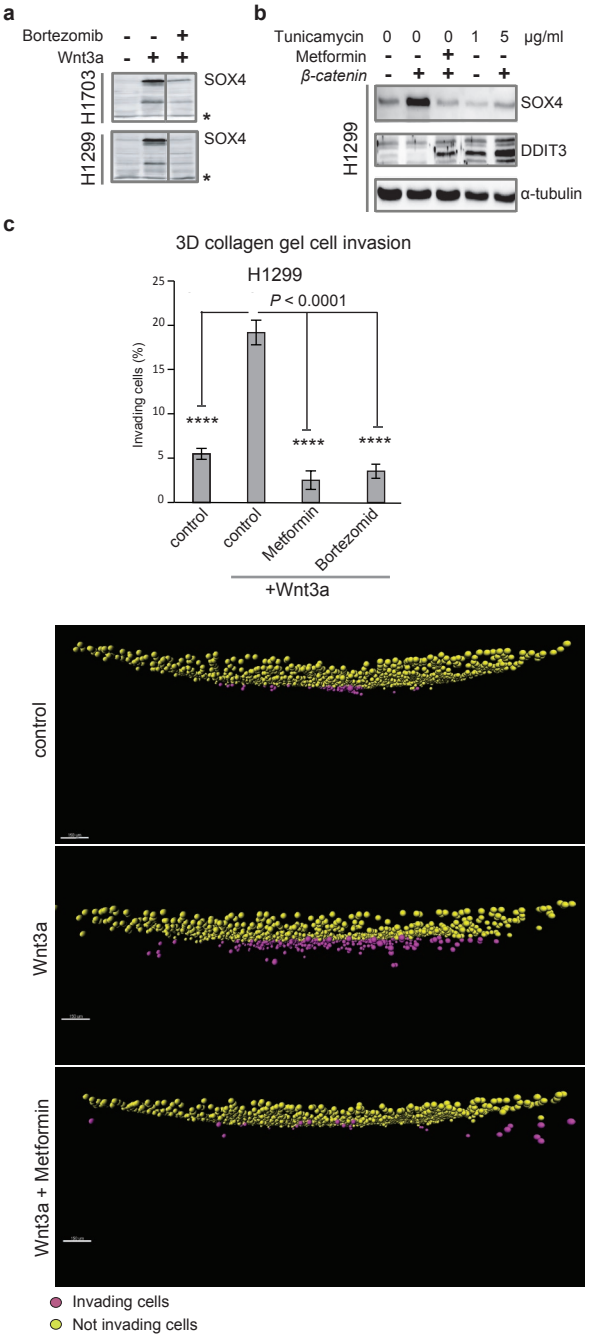
Melnik *et al.*, Supplementary Figure 3



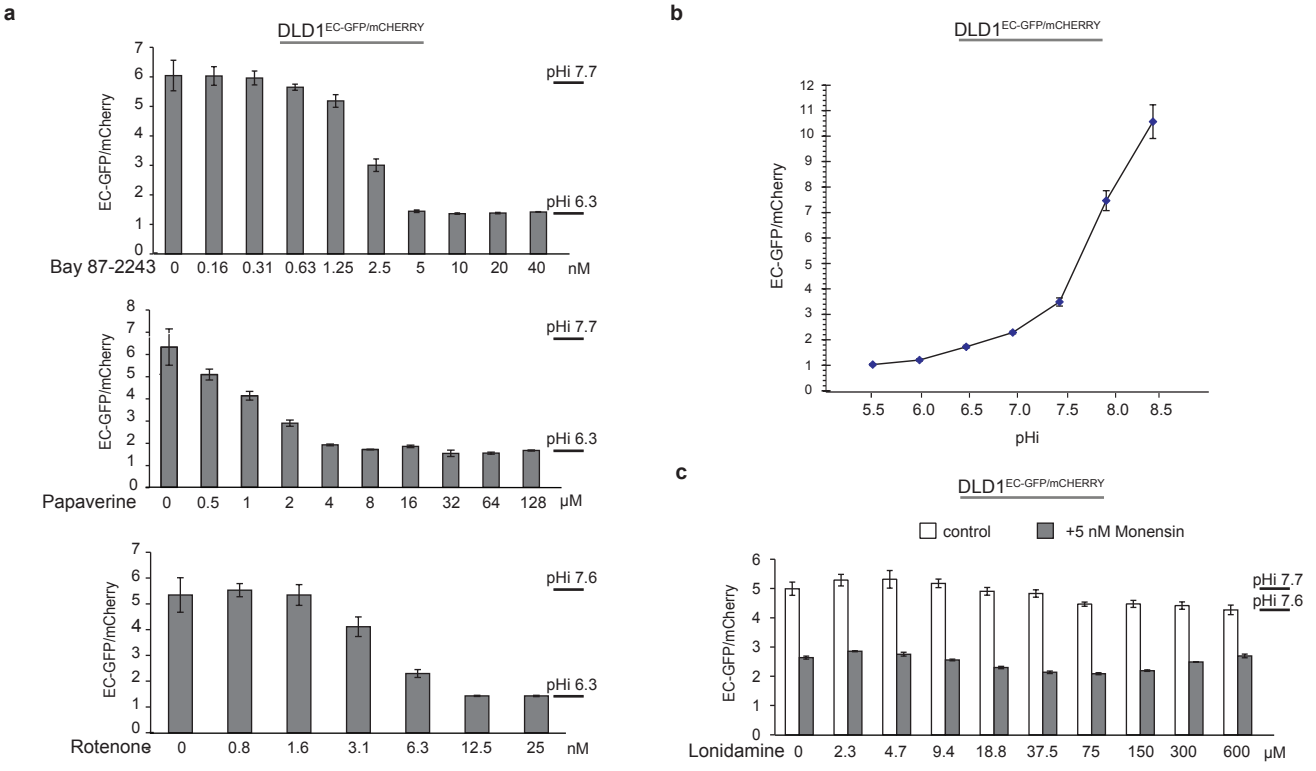

a
SOX4
DDIT3
H1299
Relative mRNA levels
Metformin
Wnt3a
DLD1
HCT116
Metformin
b
Metformin - - 10 50 250 6000 μM
Wnt3a - + + + + +
H1299
SOX4
*DDIT3
α-tubulin
Metformin - - 10 50 250 6000 μM
HCT116
SOX4
* DDIT3
α-tubulin
c
SOX4
DDIT3
Relative mRNA levels
Metformin
pre-treatment, 72 h
Days after
drug removal
0 1 2 3 4
DLD1
SOX4
DDIT3
α-tubulin
d
HEK293T
control
+DDIT3
TOPFLASH (RLA)
control
+LRP6/W/Fz
+β-catenin
e
221 AGCCTGGTATGAGGACTG-CAAGAGGTCCTGTCT 260
DDIT3WT
221 AGCCTGGTATGAGGACTGCCAAGAGGTCCTGTCT 261
DDIT3-/-(CRISPR)
H1975
wildtype
H1975
DDIT3-/-(CRISPR)
control
Tunicamycin
Nigericin
Bortezomib
control
Tunicamycin
Nigericin
Bortezomib
DDIT3
α-tubulin


a
DDIT3
H1703
H1299
Relative mRNA levels
siRNA
control
DDIT3
Metformin
Wnt3a
b
H1299
DDIT3
IRE1
ATF6
ATF4
ATF2
PERK
DDIT3
PERK
IRE1
ATF6
ATF4
ATF2

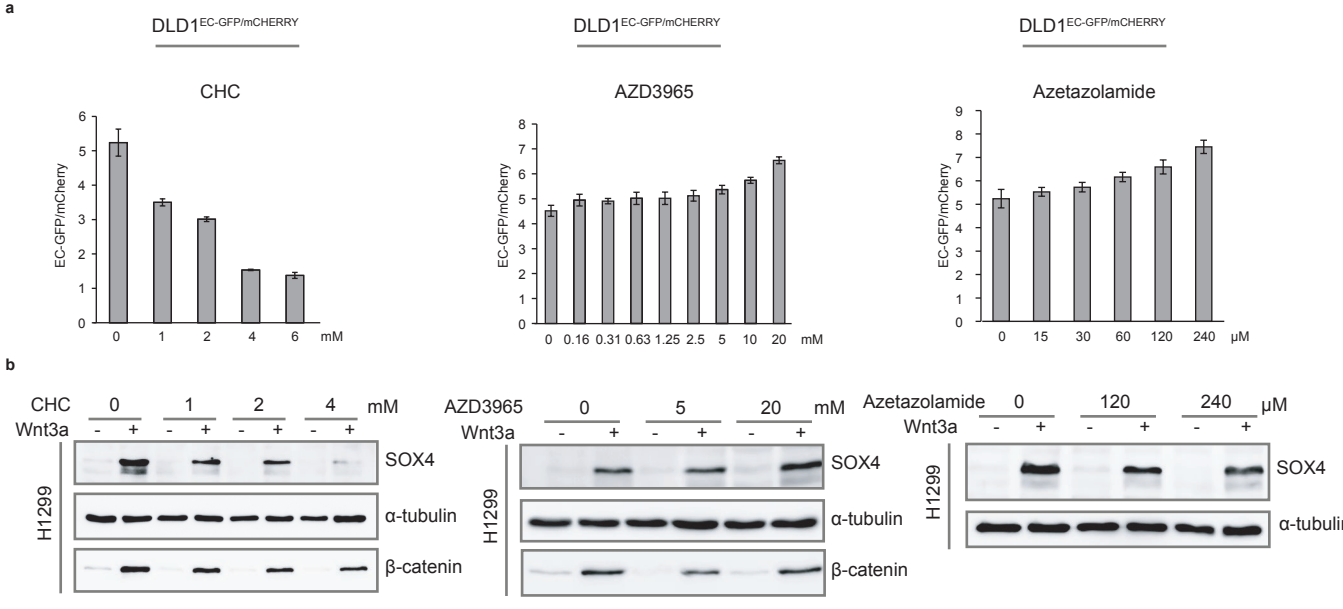
Melnik *et al.*, Supplementary Figure 6

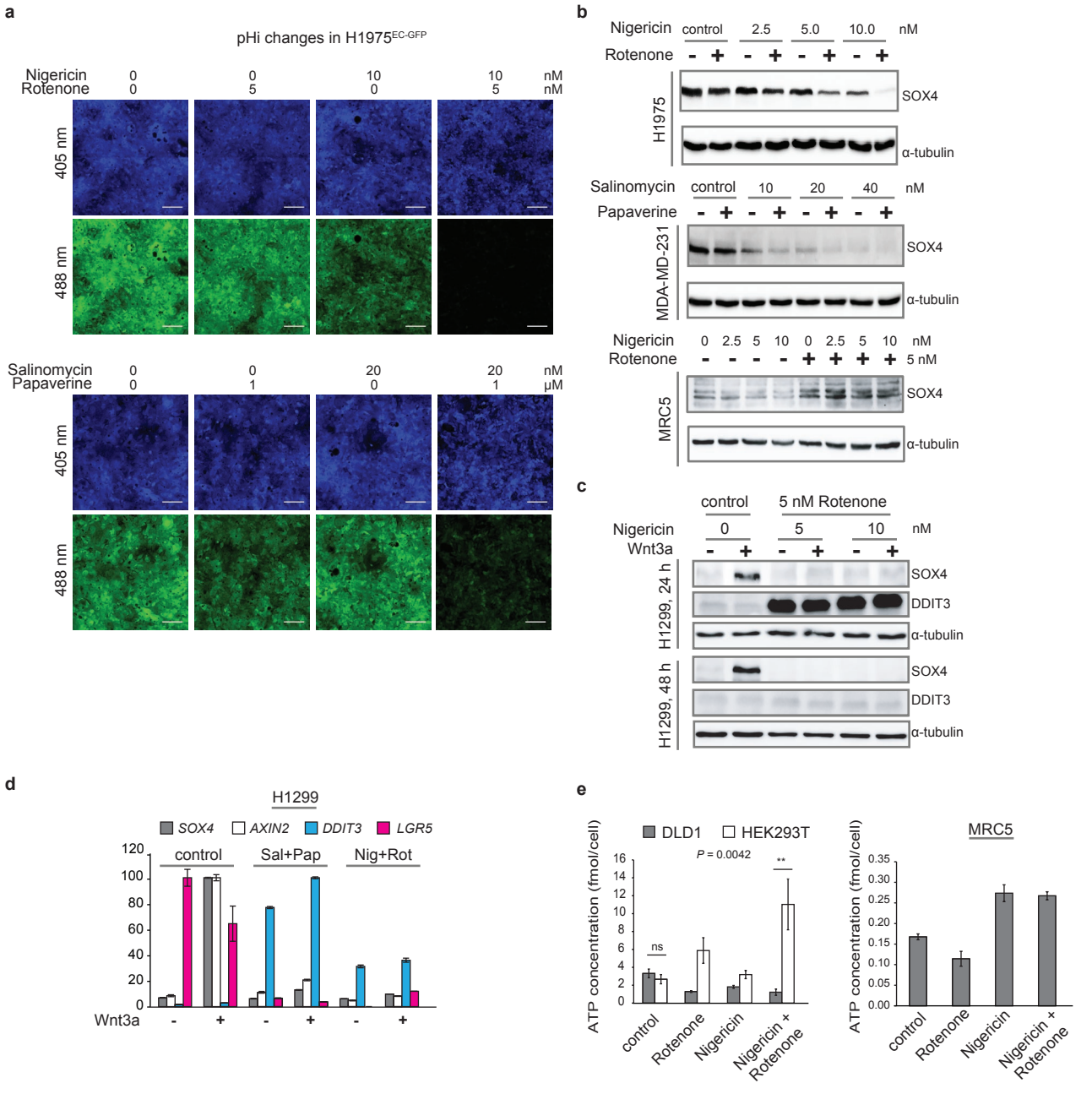


Melnik *et al.*, Supplementary Figure 7



Melnik *et al.,* Supplementary Figure 8




a
pHi changes in H1975EC-GFP
Nigericin 0 0 10 10 nM
Rotenone 0 5 0 5 nM
405 nm
488 nm
Salinomycin 0 0 20 20 nM
Papaverine 0 1 0 1 µM
405 nm
488 nm
b
Nigericin control 2.5 5.0 10.0 nM
Rotenone - + - + - + - +
H1975
SOX4
α-tubulin
Salinomycin control 10 20 40 nM
Papaverine - + - + - + - +
MDA-MD-231
SOX4
α-tubulin
Nigericin 0 2.5 5 10 0 2.5 5 10 nM
Rotenone - - - - + + + + 5 nM
MRC5
SOX4
α-tubulin
c
control
5 nM Rotenone
Nigericin 0 5 10 nM
Wnt3a - + - + - +
H1299, 24 h
SOX4
DDIT3
α-tubulin
H1299, 48 h
SOX4
DDIT3
α-tubulin
d
H1299
SOX4
AXIN2
DDIT3
LGR5
control
Sal+Pap
Nig+Rot
120
100
80
60
40
20
0
Wnt3a - + - + - +
e
DLD1
HEK293T
P = 0.0042
ns
**
ATP concentration (fmol/cell)
16
14
12
10
8
6
4
2
0
control
Rotenone
Nigericin
Nigericin + Rotenone
MRC5
ATP concentration (fmol/cell)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
control
Rotenone
Nigericin
Nigericin + Rotenone

Melnik *et al.*, Supplementary Figure 10