Supplementary Materials

TGFβ Governs the Pleiotropic Activity of NDRG1 in Triple-Negative Breast Cancer Progression.

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Supplementary figures.



Fig. S1. NDRG1 expression and patient survival in other breast cancer subtypes. A Kaplan-Meier curves with the KM plotter and **B** GOBO databases of high *NDRG1* expression and relapse-free survival (RFS) in luminal A (LA), luminal B (LB), and HER2⁺ breast cancer subtypes. **C** Correlation of NDRG1 expression with cumulative survival of TNBC patients by Kaplan-Meier according to the subcellular localization.

Supplementary Figure S2



Fig. S2. Association of NDRG1 and p-NDRG1 (Thr346) with TGF β , p-GSK3 β (Tyr216), and survival of patients with TNBC. A Western blot analysis of NDRG1 and p-NDRG1 (Thr346) in SUM159 and MDA-MB-231 cell lines after treatment with TGF β 1 at indicated time points. B Number of MDA-MB-231 cells that express NDRG1 and p-NDRG1 with or without TGF β 1 stimulation. C Impact of nuclear p-NDRG1 (Thr346) staining score (<7.5 and \geq 7.5) of TNBC patients' tumor tissue (n=77). D Representative images of negative, 1+, 2+, and 3+ TGF β 1 staining intensity in TNBC patients' tumor tissue (original optical objective: 40×). E Representative images of negative, 1+, 2+, and 3+ p-GSK3 β (Tyr216) staining intensity in TNBC patients'

tumor tissue (original optical objective: 40×). **F** Kaplan-Meier analysis of p-NDRG1(N) and NDRG1(N) staining status as risk factors of shorter cumulative survival after diagnosis of TNBC patients. No Risk Factor (RF) (p-NDRG1(N)⁺/NDRG1(N)⁻), 1RF⁻ (p-NDRG1(N)⁻/NDRG1(N)⁻), 1RF⁺ (p-NDRG1(N)⁺/NDRG1(N)⁺), and 2RF (p-NDRG1(N)⁻/NDRG1(N)⁺), and paired comparison by Chi-square test (n=74). * Indicates differences between -TGF β 1 and +TGF β 1. ** p<0.01.



Fig. S3. NDRG1 and TGFβ-induced CSCs. A Migration assay in Hs578T and MDA-MB-468, derived from primary tumor and pleural effusion, respectively, treated with TGFβ1 and siNDRG1 (8+48 protocol). **B** Mammosphere-forming efficiency (MSFE) in secondary mammospheres of SUM159 cells by *NDRG1* knockdown treated with TGFβ1 (8+48 protocol). **C** Primary mammospheres (1MS) in SUM159, BT549 (14-day protocol), MDA-MB-231, and MDA-MB-436 cells (8+48 protocol) after *NDRG1* knockdown,

with/without treatment with TGF β 1. **D** Tertiary mammospheres (3MS) of SUM159 (14-day protocol) and MDA-MB-231 cells (8 +48 protocol) after NDRG1 knockdown, with/without TGF β 1 treatment. * Indicates differences between siNDRG1 and SCR. \$ Indicates differences between SCR with and without TGF β 1. * p<0.05; ** p<0.01; *** p<0.01; \$ p<0.05; \$\$ p<0.01; \$\$ p<0.01; \$\$



Fig. S4. Short-term protocol of NDRG1+TGFβ1 in primary-tumor-derived cell lines and flow cytometry plots. A Flow cytometric analysis of ALDH1⁺ cell population after *NDRG1* knockdown and TGFβ1 in SUM159 and BT549 cell lines. **B** Dot plots of flow cytometric analysis of aldefluor-positive (ALDH1⁺)

population and DEAB control in the four cell lines tested after *NDRG1* knockdown and TGFβ1 treatment. C Dot plots of flow cytometric analysis of CD44^{high}/CD24⁻ population in SUM159, MDA-MB-231, and MDA-MB-436 cell lines after *NDRG1* knockdown, with/without TGFβ1. **D** Dot plots of side population in SUM159 and MDA-MB-231 cell lines after *NDRG1* knockdown, with/without TGFβ1.



Fig. S5. MDR1 expression, and elucidation of the potential pathway of TGFβ-induced NDRG1 and its targeting. A Western blot of MDR1 in both SUM159 and MDA-MB-231 cells transfected with siNDRG1 treated or not with TGFβ1. **B** Protein expression of NDRG1, p-NDRG1, p-p65, and p65 after the treatment with inhibitors of PI3K, Akt, mTOR, SGK1/2, GSK3β, and TGFβ in SUM159 and MDA-MB-231 cells for 24, 48, and 72h upon TGFβ1 stimulation. **C** Western-blot of NDRG1 and p-NDRG1 after treatment with

rapamycin (Rap), LY2157299 (LY), CHIR99021(CHIR), Rap+CHIR (R+C), Rap+LY (R+L), LY+CHIR (L+C) in SUM159 and MDA-MB-231 cells stimulated with TGF β 1 for 24h. The vehicle is depicted as V. **D** Western-blot of p-p65 and p65 after treatment with LY, CHIR, and LY+CHIR (L+C) in SUM159 and MDA-MB-231 cells, upon stimulation with TGF β 1, for 24 and 72h, respectively, compared with vehicle (V). **E** Primary mammospheres of SUM159, MDA-MB-231, and BT549 cells stimulated or not with TGF β 1 and treated with LY, CHIR, and LY+CHIR (Combo). * p<0.05; ** p<0.01; *** p<0.001.



Supplementary Figure S6

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Fig. S6. A Flow cytometric dot plots of aldefluor-positive (ALDH1 ⁺) population and DEAB control in
secondary mammospheres of SUM159, MDA-MB-231, and BT549 cell lines after treatment with TGF β 1, LY,
CHIR, and LY+CHIR (Combo). B Flow cytometric dot plots of CD44 ^{high} /CD24 ⁻ in 2MS of SUM159 and
MDA-MB-2312 cell lines after treatment with TGF β 1, LY, CHIR, and LY+CHIR (Combo).

Supplementary Tables.

Supplementary Table S1. Univariate analysis of the effect of high/low expression of p-NDRG1 (Thr346) over patient survival.

Variable	Number of patients	% of total	Estimated mean survival (years)	p-value (Log- rank)	HR	Cox regression (95% CI)	p-value	
Total p-NDRG1								
(Score according to								
$ROC: \geq 65$)								
(Total $n = 83$)								
≥ 65	22	26.5	16.82	0 107	1.00	Ref.	0.127	
< 65	61	73.5	11.16	0.107	0.50	0.2-1.21		
Nuclear p-NDRG1								
(Score according to								
$ROC: \geq 7.5$)								
(Total $n = 77$)								
≥ 7.5	23	29.9	18.75	0.004*	1.00	Ref.	0.009*	
< 7.5	54	70.1	10.08	0.004	0.24	0.08-0.7		
Cytoplasmic								
p-NDRG1								
(Score according to								
$ROC: \geq 12.5$)								
(Total $n = 83$)								
≥ 12.5	36	43.4	15.45	0.152	1.00	Ref.	0.171	
< 12.5	47	56.6	10.97		0.61	0.3-1.23		
Membrane								
p-NDRG1								
(Score according to								
$ROC: \ge 120)$								
(Total $n = 77$)								
≥ 120	1	1.3	ND	NΛ	NA	NA	NA	
< 120	76	98.7	13.51					
ND: Not Deceased. NA: Not Available								

Variable	Number of patients	% of total	Estimated mean survival (years)	<i>p</i> -value (Log- rank)	HR	Cox regression (95% CI)	p-value		
$TGF\beta l$ (total $n = 69$)									
Positive	57	82.6	13.84	0.146	2.7	0.64-11.5	0.174		
Negative	12	17.4	10.99	0.140	1.00	Ref.			
Missing	14								
Nuclear									
Positive	6	8.8	10.16	0.210	1.89	0.65-5.52	0.239		
Negative	62	91.2	13.08	0.218	1.00	Ref.			
Missing	1								
Cytoplasmic									
Positive	57	83.8	13.84	0 107	2.44	0.57-10.34	0.225		
Negative	11	16.2	10.79	0.197	1.00	Ref.			
Missing	1								
Membrane									
Positive	14	20.6	10.10	0.824	1.10	0.44-2.75	0.829		
Negative	54	79.4	14.66	0.024	1.00	Ref.			
Missing	1								
p -GSK3 β (total $n = 72$)									
Positive	56	7.8	14.73	0 500	1.00	Ref.	0.597		
Negative	16	22.2	11.37	0.388	1.26	0.53-2.96			
Nuclear									
Positive	29	40.3	14.25	0.010	1.04	0.49-2.20	0.912		
Negative	43	59.7	12.62	0.910	1.00	Ref.			
Cytoplasmic									
Positive	49	68.1	15.59	0.129	1.00	Ref.	0.144		
Negative	23	31.9	10.47	0.120	1.74	0.82-3.69			
Membrane									
Positive	0	0	NA	NA	NA	NA	NA		
Negative	72	100	14.379			INA			
NA: Not Available. HR: Hazard Ratio									

Supplementary Table S2. Clinical evolution, p-GSK3 β (Tyr216) and TGF β 1 staining, and univariate analysis.