Supplementary Data, including:

- **Supplementary Figure Legends;**
- **Supplementary Figures;**
- **Supplementary Table.**

Supplementary Figure Legends

Figure S1. Representative images of immunohistochemistry controls for HSA tissues. Representative images are shown for: **(A)** WNT6 stainings in skin tissues, used as both negative and positive control, for its specific WNT6 expression patterns, where WNT6 expression might be found in the epidermis and its derivative appendages (i.e. hair follicle) and not in the other subsequent layers, to ensure for the specificity of the primary antibody; **(B)** Negative controls without primary antibody incubation; **(C)** Hematoxylin and Eosin stainings to confirm the presence of tumor; **(D)** Nestin and **(E)** GFAP stainings were used as typical GBM markers.

Figure S2. *WNT6* expression is present in all 4 molecular subtypes of GBM. Distribution of WNT6-high patients among GBM molecular subtypes from the TCGA (n = 201), Freije (n = 59), Gravendeel (n = 159) and Vital (n = 26) datasets.

Figure S3. WNT6 overexpression promotes GBM aggressiveness *in vitro* and *in vivo*. **(A)** The efficiency of WNT6 overexpression in U87 glioblastoma cells was analyzed by qRT-PCR (top) and WB (bottom). (Top) *WNT6* expression levels were normalized to *TBP*. (Bottom); α-tubulin was used as reference protein. **(B and C)** Cell viability was measured by trypan blue **(B)** and MTS **(C)** assays in Ctrl and WNT6 cells. **(D and E)** Matrigel invasion

assays were used to assess the cells' invasion capacity. Representative images (**D**; cell nuclei stained with DAPI; scale bar = 300 µm) and quantification **(E)** of invasive U87 Ctrl and WNT6 cells. **(F)** Representative images of U87 Ctrl/WNT6 neurospheres at day 10 after plating (40x magnification; scale bar = 200 µm). **(G)** Quantification of neurospheres formation. **(H)** Limiting dilution assays in U87-Ctrl (black) and U87-WNT6 (red) cells to assess their sphere-forming capacity. The trend lines represent the estimated active cell frequency (n = 3 independent assays; p = 0.004, likelihood ratio test). **(I-L)** U87 transfected cells were orthotopically injected in the brain of NSG mice (n = 6 per group). **(I)** Mice weight curves after tumor implantation. **(J)** Kaplan-Meier overall survival curves of mice (p = 0.006; Log-rank test). **(K and L)** Post-mortem brain histological and molecular analyses. H&E **(K)** and anti-WNT6 IHC staining **(L)** were used as controls for GBM formation and successful long-term WNT6 overexpression, respectively. Results represent data from at least 3 independent experiments (mean ± SD). *, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.005$ (otherwise stated, a two-sided unpaired t-test with Welch's correction being applied when homoscedasticity was not verified).

Figure S4. WNT6 does not affect sensitivity of GBM cells to radiotherapy.

U373 shCtrl and shWNT6 cells were treated with increasing doses of radiation (0, 2, and 4 Gy) and cell survival was analyzed by colony formation assays. ns = non-statistically significant (two-way ANOVA post-hoc Sidak's test).

Figure S5. *WNT6* correlates with stem cell genes.

Correlation graphs between *WNT6* expression (x-axis) and the expression of stem cell genes selected based on the heatmap in Figure 4 (y-axis; Pearson's correlation test *r* and *p* values are indicated).

Figure S6. WNT6 activates the WNT canonical signaling pathway.

TCF/LEF reporter assays in U373 shCtrl/shWNT6 **(A and B)** and U87 Ctrl/WNT6 cells **(C and D)**. **(A and C)** Representative images are displayed (100x magnification; scale bar = 100 µm). **(B and D)** GFP expression was used as a measure of TCF/LEF promoter activity and was normalized against negative and positive controls. (n = 3 independent assays; mean ± SD; *, $p < 0.05$ and **, $p < 0.01$, two-sided unpaired *t*-test).

Figure S7. Expression levels of WNT6 protein are prognostically valuable in GBM patients.

Kaplan-Meier survival curves of WNT6-low and WNT6-high GBM patients derived from IHC (protein) data from a Brazilian dataset (n = 87; median OS 6.5 vs 3.8 months, low vs high WNT6 expression, respectively; $p = 0.069$, Log-rank test). Representative IHC microphotographs showing different levels of WNT6 protein expression in particular tumors are shown.

Figure S8. Manipulation of WNT6 expression levels does not concomitantly affect levels of *WNT1* or *WNT3a*.

WNT1 and *WNT3a* expression was evaluated by qRT-PCR in U373 shCtrl/shWNT6, SNB19 shCtrl/shWNT6 and U87 shCtrl/shWNT6 cells (n = 3 independent assays; mean ± SD; *, $p < 0.05$, two-sided unpaired *t*-test).

Figure S1

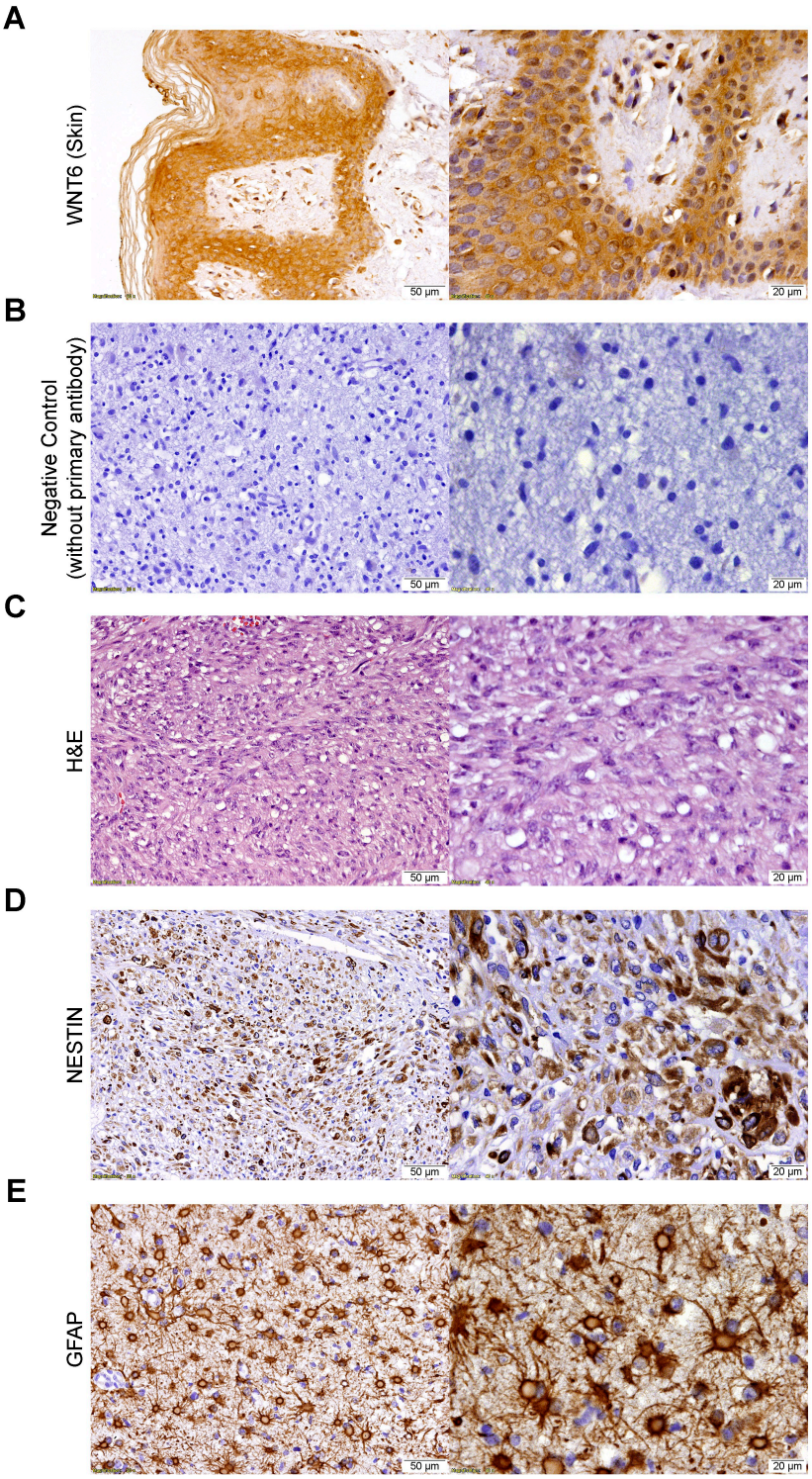
A
WNT6 (Skin)
50 µm
20 µm
B
Negative Control
(without primary antibody)
50 µm
20 µm
C
H&E
50 µm
20 µm
D
NESTIN
50 µm
20 µm
E
GFAP
50 µm
20 µm

Figure S2

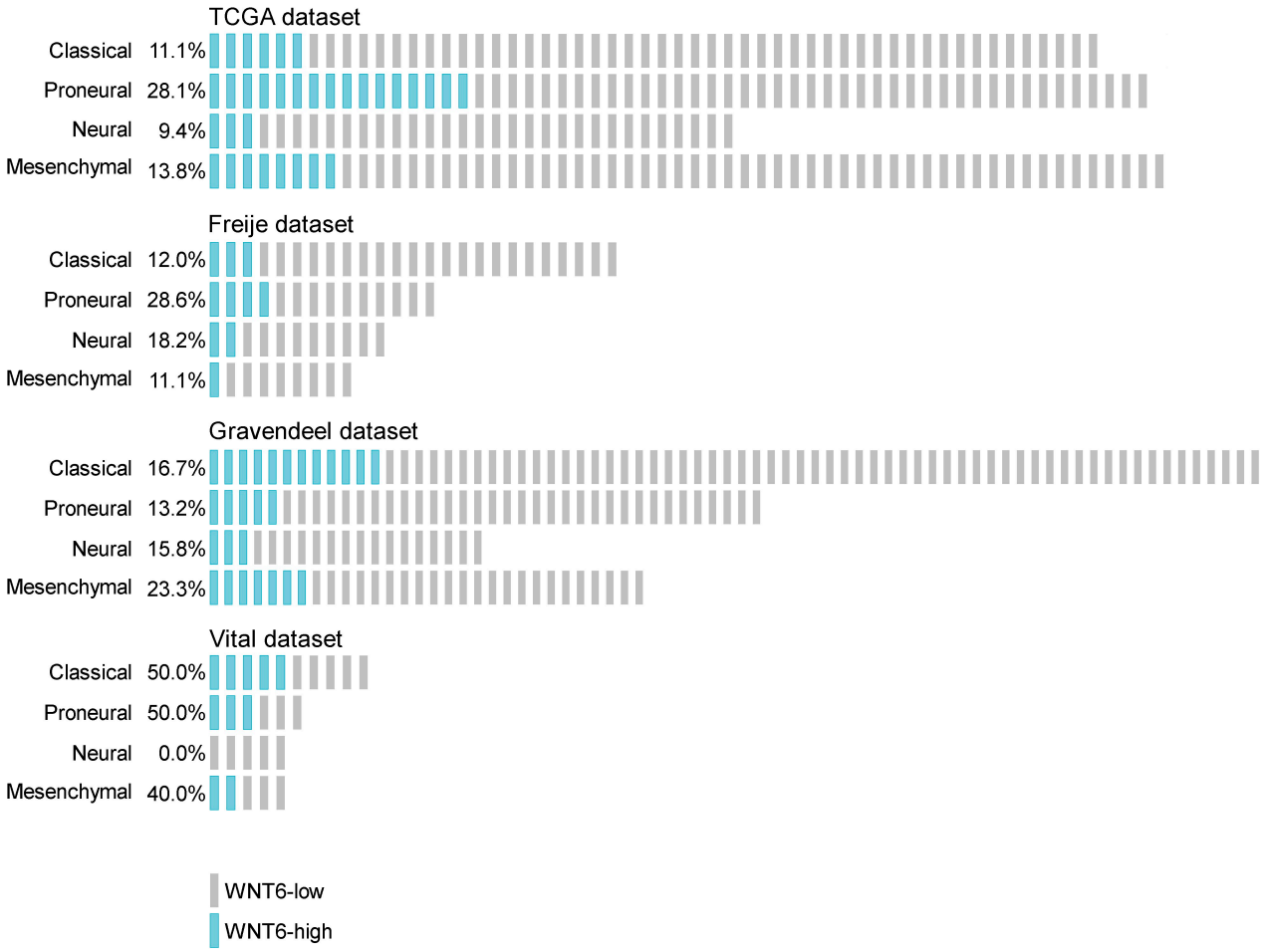
TCGA dataset
Classical 11.1%
Proneural 28.1%
Neural 9.4%
Mesenchymal 13.8%
Freije dataset
Classical 12.0%
Proneural 28.6%
Neural 18.2%
Mesenchymal 11.1%
Gravendeel dataset
Classical 16.7%
Proneural 13.2%
Neural 15.8%
Mesenchymal 23.3%
Vital dataset
Classical 50.0%
Proneural 50.0%
Neural 0.0%
Mesenchymal 40.0%
WNT6-low
WNT6-high

Figure S3

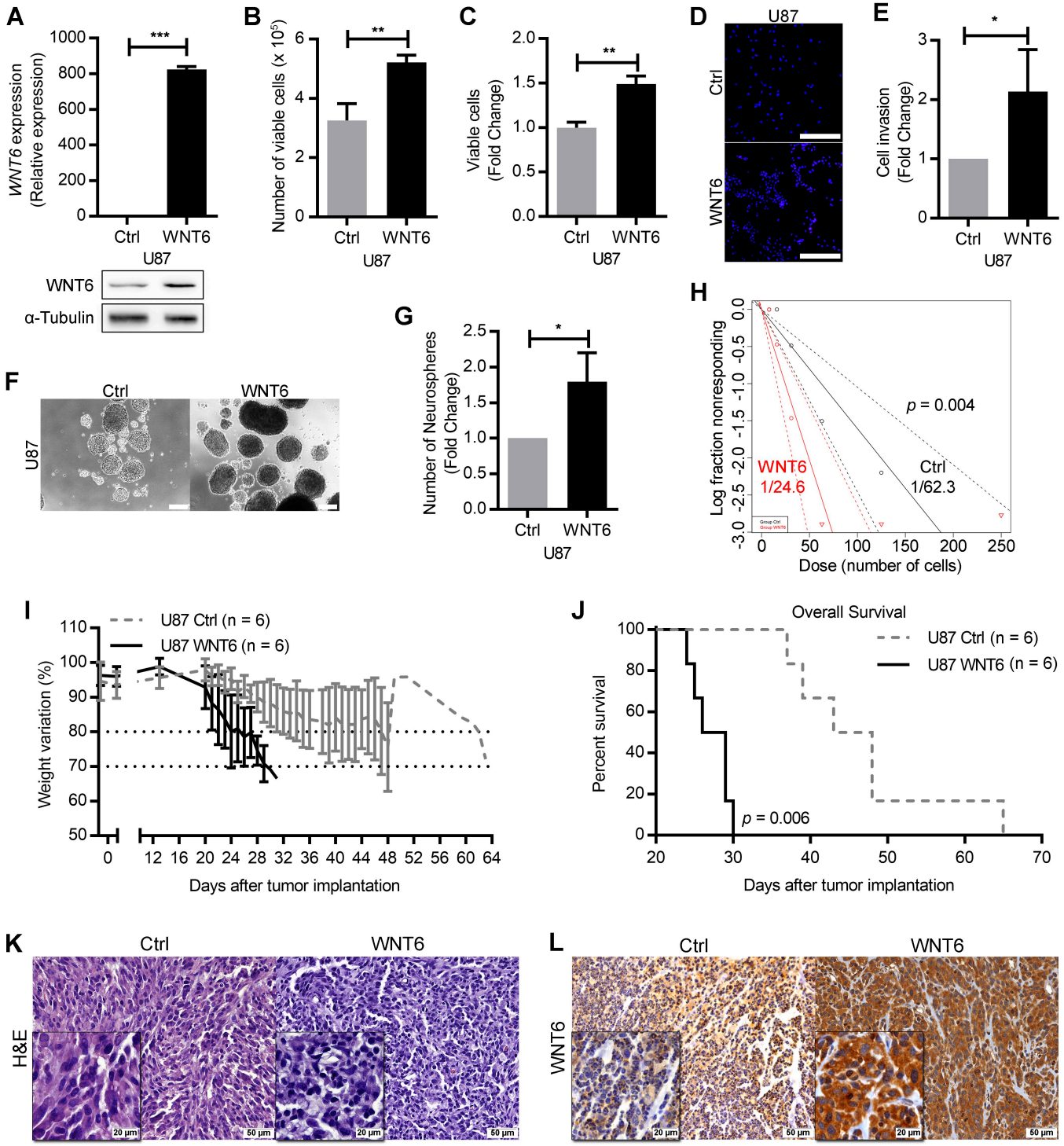
A
WNT6 expression (Relative expression)
1000
800
600
400
200
0
***
Ctrl
WNT6
U87
WNT6
α-Tubulin
B
Number of viable cells (x 10^5)
6
4
2
0
**
Ctrl
WNT6
U87
C
Viable cells (Fold Change)
2.0
1.5
1.0
0.5
0.0
**
Ctrl
WNT6
U87
D
U87
Ctrl
WNT6
E
Cell invasion (Fold Change)
3
2
1
0
*
Ctrl
WNT6
U87
F
Ctrl
WNT6
U87
G
Number of Neurospheres (Fold Change)
2.5
2.0
1.5
1.0
0.5
0.0
*
Ctrl
WNT6
U87
H
Log fraction nonresponding
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
p = 0.004
WNT6
1/24.6
Ctrl
1/62.3
0
50
100
150
200
250
Dose (number of cells)
I
U87 Ctrl (n = 6)
U87 WNT6 (n = 6)
Weight variation (%)
110
100
90
80
70
60
50
0
12 16 20 24 28 32 36 40 44 48 52 56 60 64
Days after tumor implantation
J
Overall Survival
U87 Ctrl (n = 6)
U87 WNT6 (n = 6)
Percent survival
100
80
60
40
20
0
p = 0.006
20
30
40
50
60
70
Days after tumor implantation
K
Ctrl
WNT6
H&E
20 µm
50 µm
20 µm
50 µm
L
Ctrl
WNT6
WNT6
20 µm
50 µm
20 µm
50 µm

Figure S4

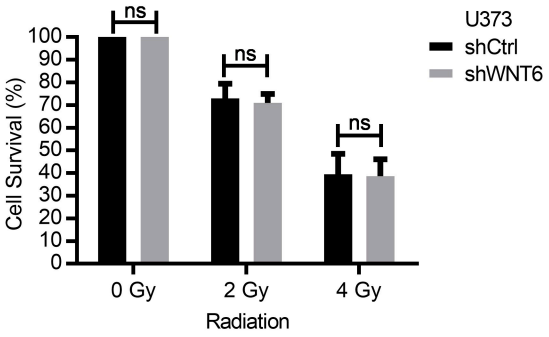
ns
ns
ns
100
90
80
70
60
50
40
30
20
10
0
Cell Survival (%)
0 Gy
2 Gy
4 Gy
Radiation
U373
shCtrl
shWNT6

Figure S5

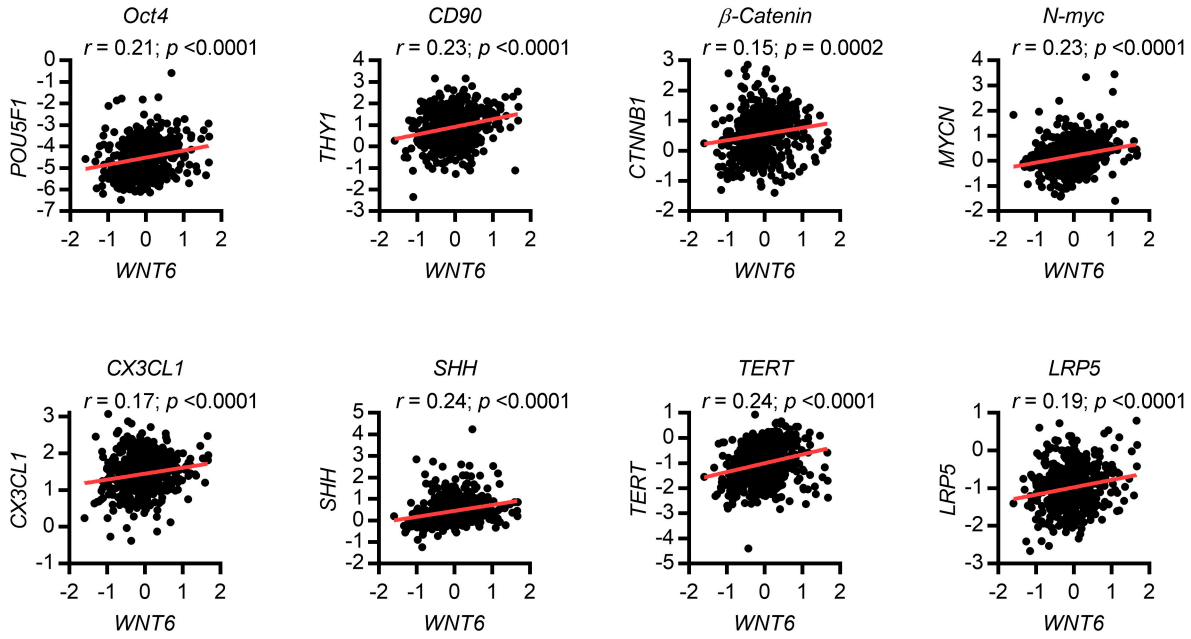
Oct4
r = 0.21; p <0.0001
POU5F1
WNT6
CD90
r = 0.23; p <0.0001
THY1
WNT6
β-Catenin
r = 0.15; p = 0.0002
CTNNB1
WNT6
N-myc
r = 0.23; p <0.0001
MYCN
WNT6
CX3CL1
r = 0.17; p <0.0001
CX3CL1
WNT6
SHH
r = 0.24; p <0.0001
SHH
WNT6
TERT
r = 0.24; p <0.0001
TERT
WNT6
LRP5
r = 0.19; p <0.0001
LRP5
WNT6

Figure S6

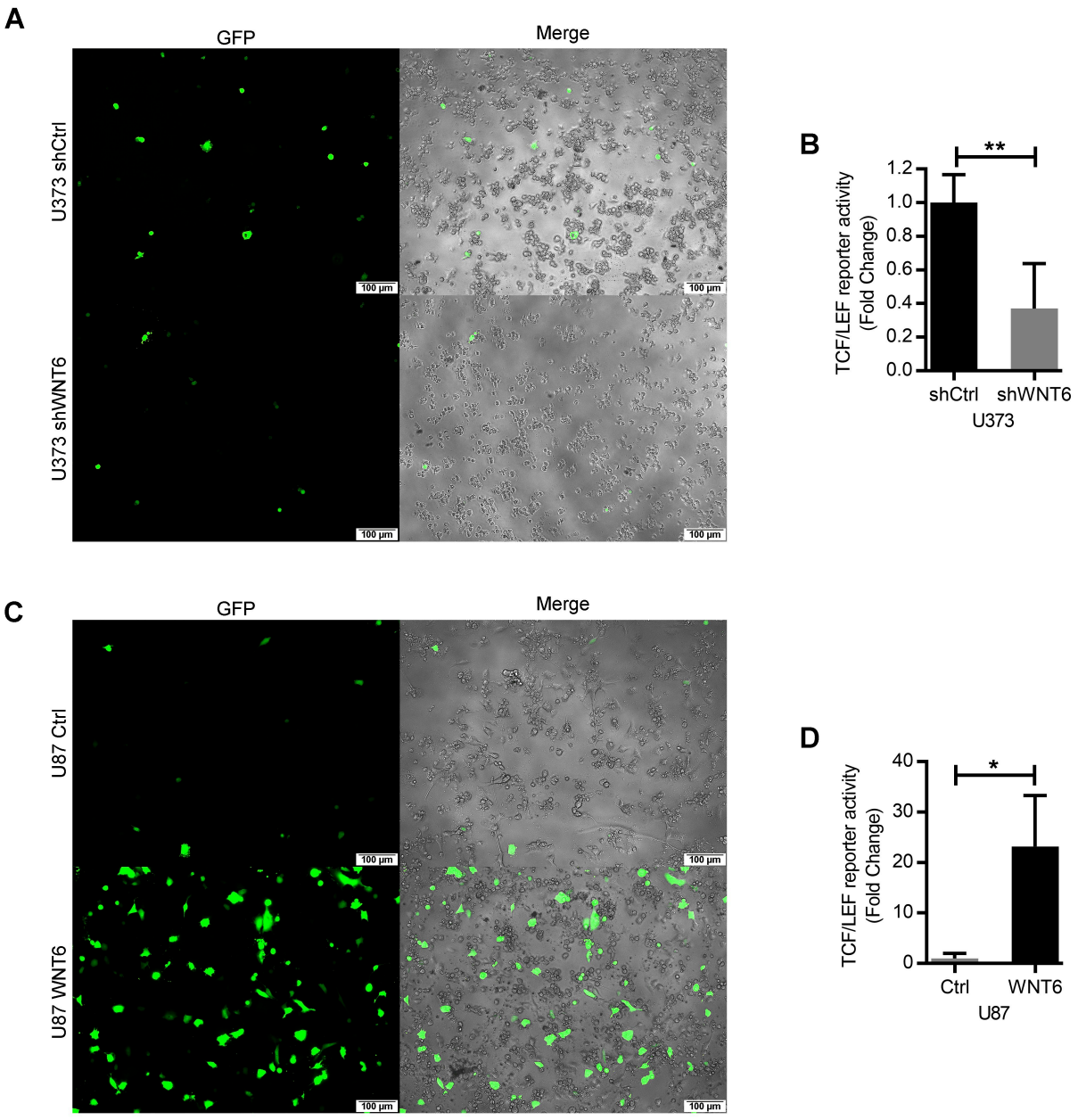
A
GFP
Merge
U373 shCtrl
U373 shWNT6
100 µm
B
TCF/LEF reporter activity
(Fold Change)
**
1.2
1.0
0.8
0.6
0.4
0.2
0.0
shCtrl
shWNT6
U373
C
GFP
Merge
U87 Ctrl
U87 WNT6
100 µm
D
TCF/LEF reporter activity
(Fold Change)
*
40
30
20
10
0
Ctrl
WNT6
U87

Figure S7

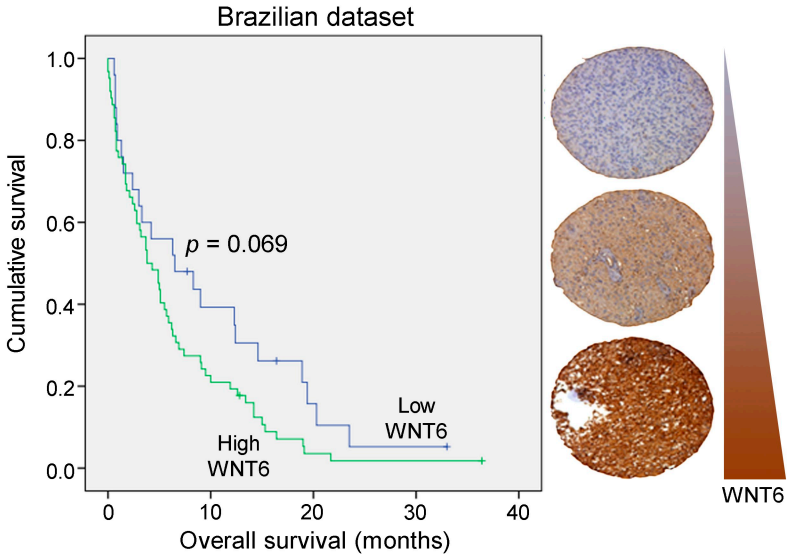
Brazilian dataset
Cumulative survival
1.0
0.8
0.6
0.4
0.2
0.0
p = 0.069
Low
WNT6
High
WNT6
0
10
20
30
40
Overall survival (months)
WNT6

Figure S8

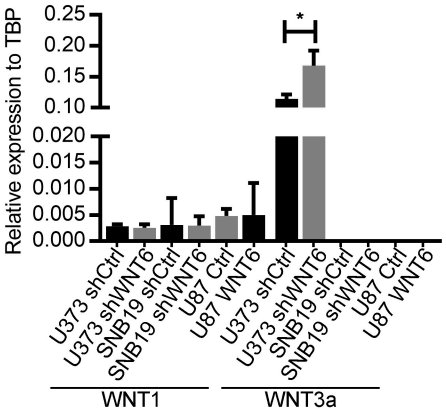
Relative expression to TBP
0.25
0.20
0.15
0.10
0.020
0.015
0.010
0.005
0.000
*
U373 shCtrl
U373 shWNT6
SNB19 shCtrl
SNB19 shWNT6
U87 Ctrl
U87 WNT6
U373 shCtrl
U373 shWNT6
SNB19 shCtrl
SNB19 shWNT6
U87 Ctrl
U87 WNT6
WNT1
WNT3a

Table S1 - List of antibodies used for immunohistochemistry, immunofluorescence, and Western blot.

Protein	Reference	Source	Technique	Dilution	Incubation
WNT6	ab50030	Abcam	IHC	1:450	O/N at 4˚C
			IF	1:1 000	O/N at 4˚C
	ab154144		WB	1:500	O/N at 4˚C
Ki-67	550609	BD Biosciences	IHC	1:200	O/N at 4˚C
Cyclin D1	2978	Cell Signaling	IHC	1:100	O/N at 4˚C
SOX2	AB5603	EMD Millipore	IHC	1:500	O/N at 4˚C
			IF	1:300	O/N at 4˚C
BCL2	2870	Cell Signaling	IHC	1:200	O/N at 4˚C
NESTIN	MAB5326	EMD Millipore	IF	1:100	1h at RT
rabbit IgG (H+L) – Alexa fluor® 594	A-11012	ThermoFisher Scientific	IF	1:1 000	1h at RT in the dark
mouse IgG (H+L) – Alexa fluor® 488	A-11001	ThermoFisher Scientific	IF	1:1 000	1h at RT in the dark
p-STAT3 Y705	9145	Cell Signaling	WB	1:2 000	O/N at 4˚C
STAT3	9132	Cell Signaling	WB	1:1 000	O/N at 4˚C
non-p-β-catenin S33/S37/T41	4270	Cell Signaling	WB	1:5 000	O/N at 4˚C
β-catenin	610153	BD Bioscience	WB	1:2 000	O/N at 4˚C
p-AKT S473	4051	Cell Signaling	WB	1:1 000	O/N at 4˚C
AKT	2920	Cell Signaling	WB	1:2 000	O/N at 4˚C
α-tubulin	sc-23948	Santa Cruz Biotechnology	WB	1:1 000	1h at RT
mouse IgG – HRP conjugated	sc-2031	Santa Cruz Biotechnology	WB	1:1 000	1h at RT
rabbit IgG – HRP conjugated	sc-2004	Santa Cruz Biotechnology	WB	1:1 000	1h at RT

IHC: Immunohistochemistry; IF: Immunofluorescence; WB: Western Blot; O/N: Overnight; RT: Room Temperature