Figure S1

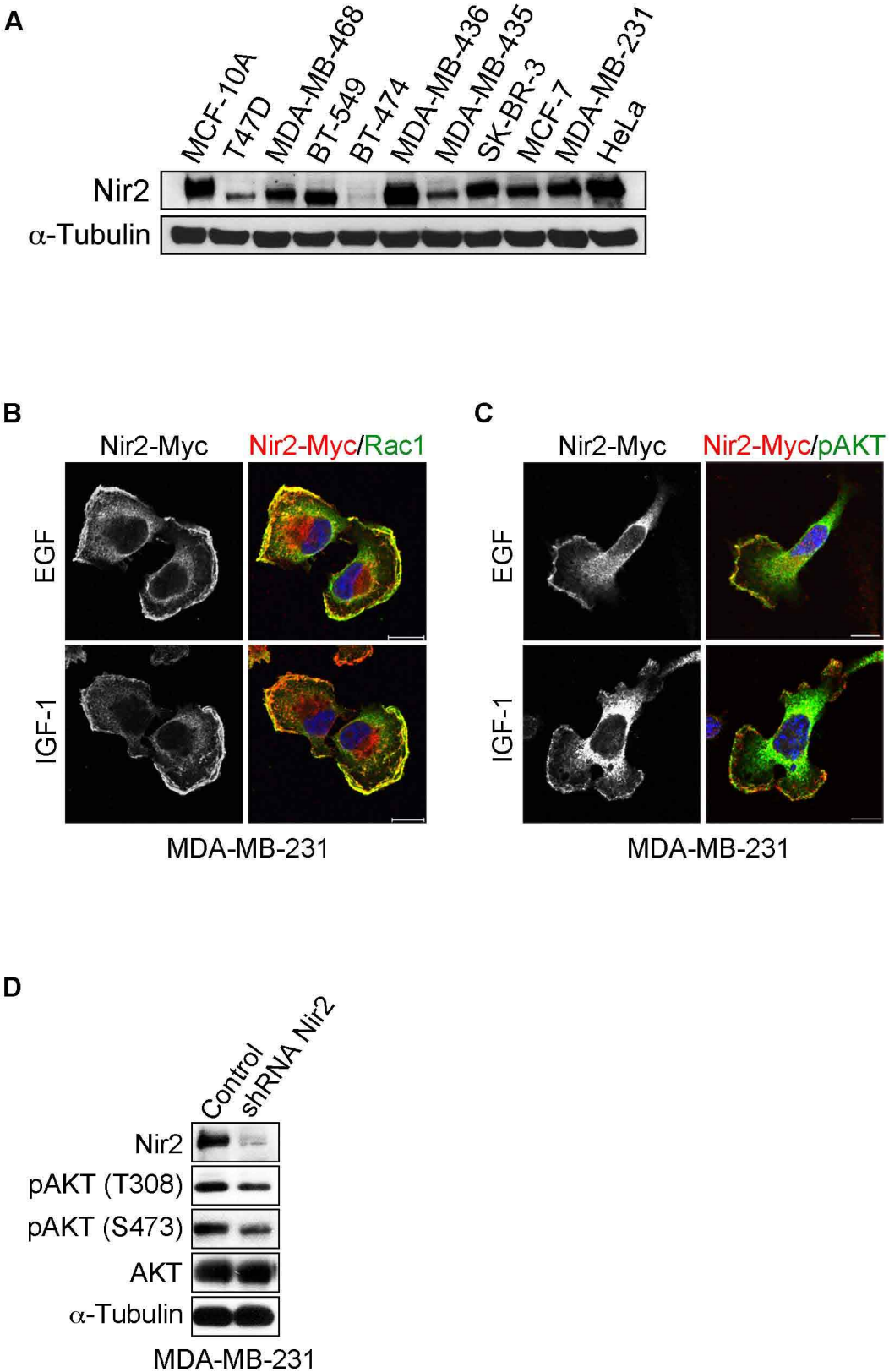

A
MCF-10A
T47D
MDA-MB-468
BT-549
BT-474
MDA-MB-436
MDA-MB-435
SK-BR-3
MCF-7
MDA-MB-231
HeLa
Nir2
α-Tubulin
B
Nir2-Myc
Nir2-Myc/Rac1
EGF
IGF-1
MDA-MB-231
C
Nir2-Myc
Nir2-Myc/pAKT
EGF
IGF-1
MDA-MB-231
D
Control
shRNA Nir2
Nir2
pAKT (T308)
pAKT (S473)
AKT
α-Tubulin
MDA-MB-231

Figure S2

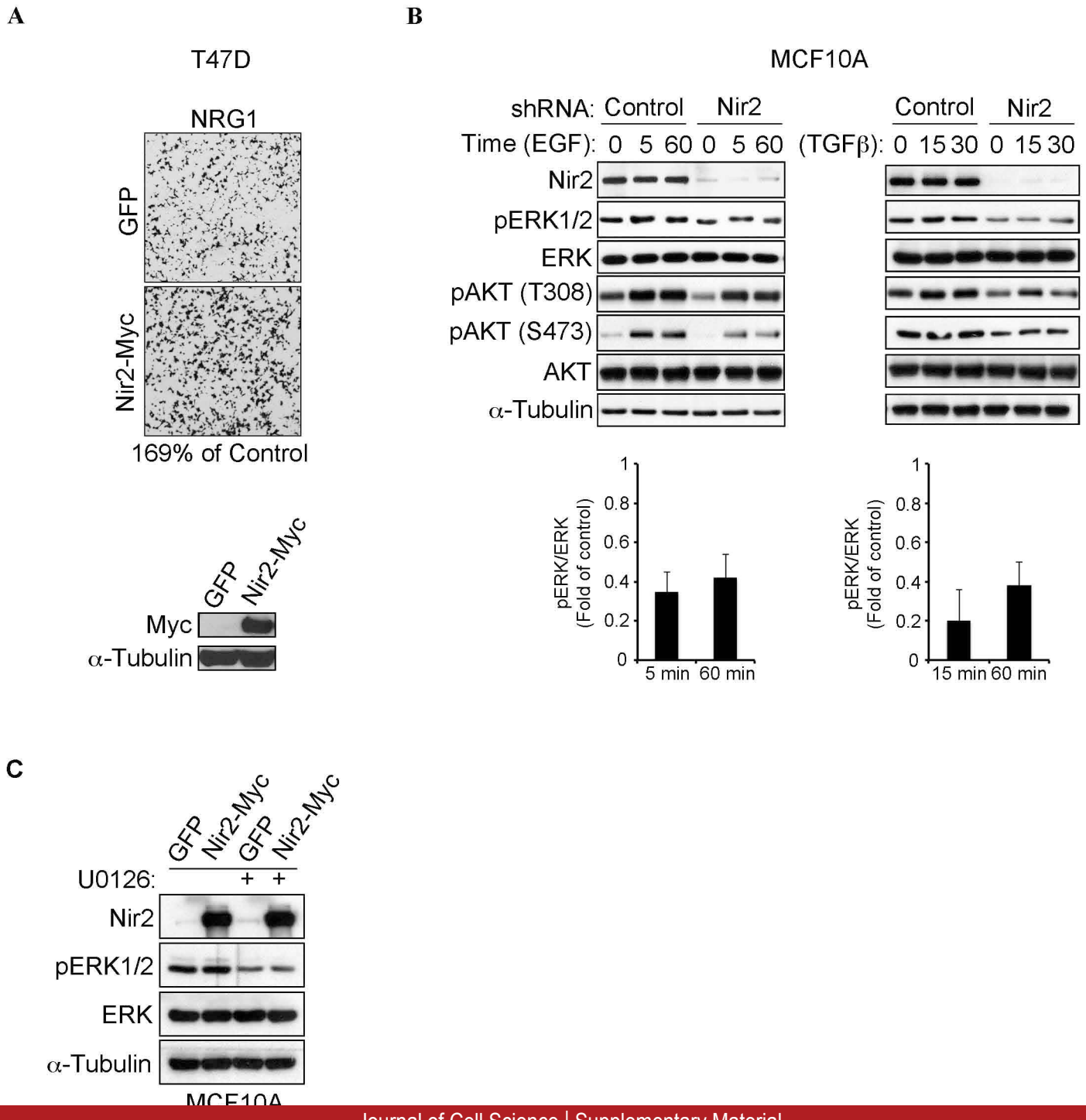

A
T47D
NRG1
GFP
Nir2-Myc
169% of Control
GFP
Nir2-Myc
Myc
α-Tubulin
B
MCF10A
shRNA: Control Nir2
Time (EGF): 0 5 60 0 5 60
Control Nir2
(TGFβ): 0 15 30 0 15 30
Nir2
pERK1/2
ERK
pAKT (T308)
pAKT (S473)
AKT
α-Tubulin
pERK/ERK (Fold of control)
5 min 60 min
15 min 60 min
C
GFP
Nir2-Myc
GFP
Nir2-Myc
U0126: + +
Nir2
pERK1/2
ERK
α-Tubulin
MCF10A

Figure S3

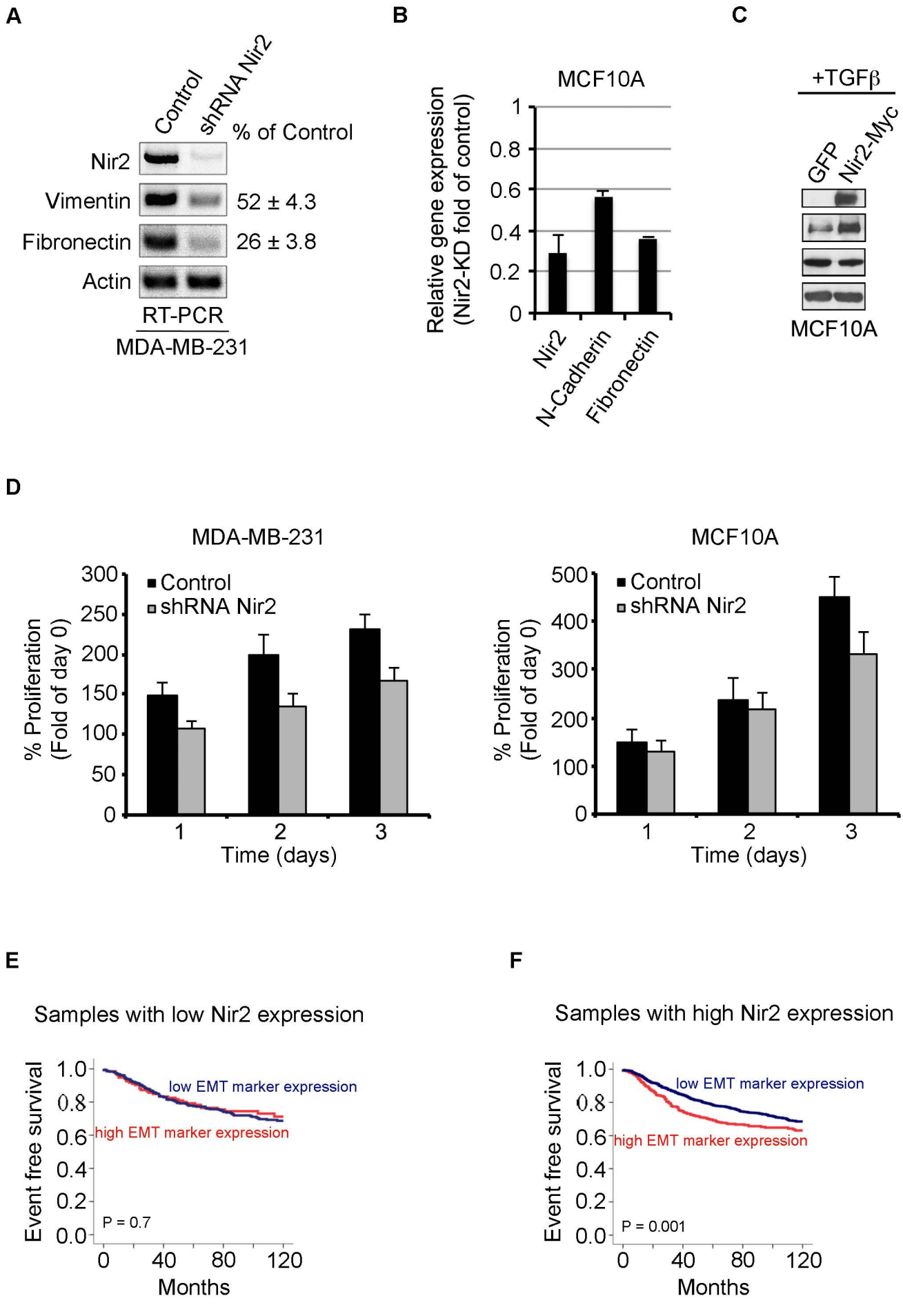
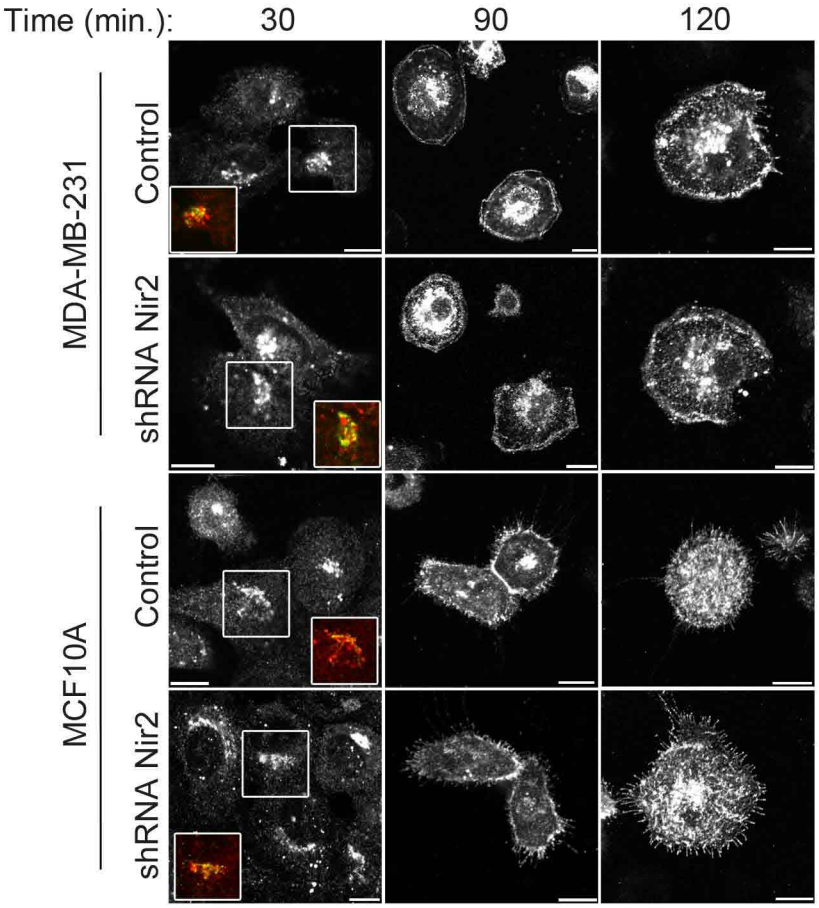
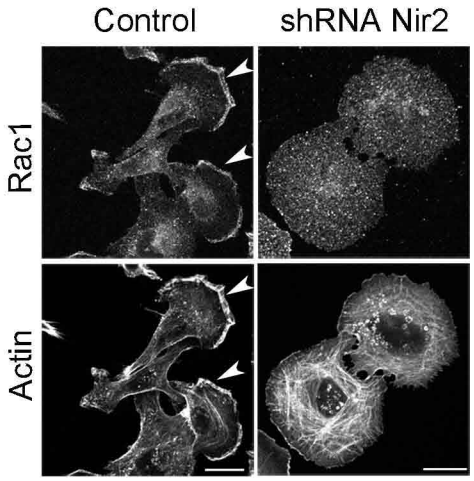

A
Control
shRNA Nir2
% of Control
Nir2
Vimentin
52 ± 4.3
Fibronectin
26 ± 3.8
Actin
RT-PCR
MDA-MB-231
B
MCF10A
Relative gene expression
(Nir2-KD fold of control)
1
0.8
0.6
0.4
0.2
0
Nir2
N-Cadherin
Fibronectin
C
+TGFβ
GFP
Nir2-Myc
MCF10A
D
MDA-MB-231
% Proliferation
(Fold of day 0)
300
250
200
150
100
50
0
Control
shRNA Nir2
1
2
3
Time (days)
MCF10A
% Proliferation
(Fold of day 0)
500
400
300
200
100
0
Control
shRNA Nir2
1
2
3
Time (days)
E
Samples with low Nir2 expression
Event free survival
1.0
0.8
0.6
0.4
0.2
0.0
low EMT marker expression
high EMT marker expression
P = 0.7
0
40
80
120
Months
F
Samples with high Nir2 expression
Event free survival
1.0
0.8
0.6
0.4
0.2
0.0
low EMT marker expression
high EMT marker expression
P = 0.001
0
40
80
120
Months

Figure S4

A


Time (min.):
30
90
120
MDA-MB-231
Control
shRNA Nir2
MCF10A
Control
shRNA Nir2

B


Control
shRNA Nir2
Rac1
Actin

Supplementary Information

Figure S1: **Nir2 is differentially expressed in human breast cancer cell lines, affects the activation of AKT, and localizes to the PM following GF treatment.** (A) The steady-state expression level of Nir2 in the indicated human cell lines was assessed by Western blotting (WB) of total cell lysates using anti-Nir2 antibody. WB with α-tubulin was used as a loading control. (B,C) MDA-MB-231 expressing Nir2-Myc were serum-starved for 16 hrs and then treated with EGF (50 ng/ml) or IGF-I (20 ng/ml) for 15 min at 37°C. The cells were then fixed and processed for immunofluorescence analysis using anti-Myc and either anti-Rac1 (A) or anti-pAKT (S473) (B) antibodies. Shown are representative confocal images. Co-localization of Nir2 and Rac1 or pAKT appears in yellow. Scale bar, 10 μm. (D) Control and Nir2-depleted MDA-MB-231 cells were grown in full medium. Total cell lysates were prepared and analyzed for AKT phosphorylation using the corresponding antibodies and Western blot analysis. Reproducible results were obtained in three independent experiments.

Figure S2: **Nir2 overexpression facilitates the migration of T47D cells, while Nir2 depletion attenuates ERK activation in MCF10A cells.** (A) T47D cells expressing either GFP or Nir2-Myc were plated in Boyden chambers and stimulated with NRG1 (20 ng/ml) for 24 hr. Cell migration was assessed as described in materials and methods. The mean values ±s.d. of three independent experiments are shown. (B) Nir2-depleted MCF10A cells were serum-starved for 24 hrs, and then stimulated with EGF (10 ng/ml) or TGFβ (5 ng/ml) for the indicated time periods. Total cell lysates were prepared and analyzed for ERK1/2 and AKT phosphorylation using Western blotting. The mean values ±s.d. of four independent experiments are shown. (C) MCF10A cells expressing either GFP or Nir2-Myc were grown in the absence or presence of the MEK1/2 inhibitor U0126 (1 μM) for 24 hours. Total cell lysates were prepared and analyzed for ERK1/2 and AKT phosphorylation using the corresponding antibodies and Western blotting.

Figure S3: **Nir2 influences proliferation and mRNA expression levels of EMT markers in MDA-MB-231 and MCF10A cells as well as the prognostic values of EMT markers in clinical breast cancer.** The mRNA levels of the indicated EMT markers were assessed in control and Nir2-depleted MDA-MB-231 cells (A) and MCF10A cells (B) by RT-PCR and real-time PCR, respectively. The mean values ±s.d. of four independent experiments are shown. (C) The influence of Nir2 overexpression on TGFβ (5 ng/ml for 3 days)-induced EMT markers was examined by WB using the indicated antibodies. (D) Control and Nir2-depleted MCF10A ($3*10^3$ cells/well) or MDA-MB-231 ($5*10^3$ cells/well) cells were seeded in 96 well plates and grown for the indicated time periods in a complete media. Cell viability was assessed by MTT assay. The relative MTT values obtained compared to zero time are shown. The mean values ±s.d. of three independent experiments are shown. (E,F) The prognostic value of an EMT marker score was calculated for 4,467 breast cancer samples with Affymetrix gene expression data that has been described previously (Hanker et al., 2013). The EMT marker score was based on the mean expression of 21 Affymetrix probesets representing a set of genes previously described to be upregulated during EMT (Table S1, (Hatami et al., 2013). Samples were then stratified using the highest quartile of this score as either high or low EMT marker expression. Two separate Kaplan-Meier analyses of event free survival of patients are shown either for those samples with low Nir2 expression (Affymetrix probeset 203826_s_at, lower quartile) in (E) or high Nir2 expression in (F).

Figure S4: Nir2 expression affects the spreading and polarization of MDA-MB-231 but not Golgi-to-plasma membrane transport of VSVG. (A) Control and Nir2-depleted MDA-MB-231 cells were plated on fibronectin coated coverslips and immunostained with anti-Rac1 antibody and TRITC-Phalloidin. Scale bar, 10 μm. (B) The VSVG transport assay was carried out as previously described (Kim et al., 2013). Shown are representative images of control and Nir2-depleted MDA-MB-231 and MCF10A cells at the indicated time points. Insert shows colocalization of VSVG and the Golgi marker GRASP65. Scale bar, 10μm.

References

Hanker, L. C., Rody, A., Holtrich, U., Pusztai, L., Ruckhaeberle, E., Liedtke, C., Ahr, A., Heinrich, T. M., Sanger, N., Becker, S. et al. (2013). Prognostic evaluation of the B cell/IL-8 metagene in different intrinsic breast cancer subtypes. *Breast cancer research and treatment* **137**, 407-416.

Hatami, R., Sieuwerts, A. M., Izadmehr, S., Yao, Z., Qiao, R. F., Papa, L., Look, M. P., Smid, M., Ohlssen, J., Levine, A. C. et al. (2013). KLF6-SV1 drives breast cancer metastasis and is associated with poor survival. *Science translational medicine* **5**, 169ra112.

Kim, S., Kedan, A., Marom, M., Gavert, N., Keinan, O., Selitrennik, M., Laufman, O. and Lev, S. (2013). The phosphatidylinositol-transfer protein Nir2 binds phosphatidic acid and positively regulates phosphoinositide signalling. *EMBO Rep* **14**, 891-899.

Table S1. The 21 probesets of marker genes that are upregulated during EMT.

Gene symbol	Affymetrix probeset
BMP1	202701_at
CDH2	203440_at
COL1A2	202403_s_at
FN1	210495_x_at
FOXC1	213260_at
GNG11	204115_at
ITGA5	201389_at
ITGAV	202351_at
MMP2	201069_at
MMP3	205828_at
MMP9	203936_s_at
MSN	200600_at
SNAI1	219480_at
SNAI2	213139_at
TCF4	213891_s_at
TGFB1	203085_s_at
TWIST1	213943_at
VCAN	221731_x_at
VIM	201426_s_at
ZEB1	212758_s_at
ZEB2	203603_s_at

A set of marker genes that are upregulated during EMT as previously described (Hatami et al., 2013) and for which probesets are available on Affymetrix U133A Gene-Chips. These 21 probesets were used to develop a robust EMT marker score. The relationship of Nir2 expression and the EMT marker score was assessed as described in Fig. S7.

Table S2. Correlation of Nir2 expression with clinical/pathologic parameters and other biomarkers.

	Nir2 expression			
Parameters/markers	*Total*	*Positive (%)*	*Negative (%)*	*P-value*
Age (years)				
<50	17	12 (71)	5 (29)	0.47
>50	39	31 (79)	8 (21)	
Tumor size				
<2 cm (T1)	27	18 (67)	9 (33)	0.096
>2 cm (T2,3,4)	28	24 (86)	4 (14)	
Lymph node status				
Negative	40	29 (72.5)	11 (27.5)	0.25
Positive	16	14 (87.5)	2 (12.5)	
Distant metastasis				
Positive	0			N/A
Negative	53	40 (75)	13 (25)	
ER status				
Positive	27	19 (70)	8 (30)	0.27
Negative	29	24 (83)	5 (17)	
PR status				
Positive	22	16 (73)	6 (27)	0.42
Negative	33	27 (82)	6 (18)	
HER-2/neu status				
Positive	9	8 (89)	1 (11)	0.33
Negative	46	34 (74)	12 (26)	
Subtype				
TNBC	23	18 (78)	5 (22)	0.83
Luminal/Her2+	33	25 (76)	8 (24)	

Correlation of Nir2 expression with clinical and pathologic parameters and other biomarkers. We found no statistical correlation between patient age, tumor size, lymph node status or biomarkers. However there was a statistically significant correlation between Nir2 expression and tumor grade (see Fig. 7D).