SUPPLEMENTARY MATERIAL

SMYD3 promotes the Epithelial-Mesenchymal-Transition in Breast Cancer.

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Supplementary Methods

Supplementary Figures

Supplementary Methods.

The oligonucleotides used in qRT-PCR on NMuMG cells were:

CDH1	Forward: 5'-GTCTACCAAAGTGACGCTGAAGT-3' Reverse: 5'-TCTCGTTTCTGTCTTCTGAGACC-3'
CDH2	Forward: 5'-AATAGACCCCGTGAATGGGCAGATC-3' Reverse: 5'-AGGCGGGATTCCATTGTCAGAAG-3'
CLD6	Forward: 5'-CTCATCTCTGGCATCATCTTTG-3' Reverse: 5'-AGGGGTTGTAGAAGTCCTGGAT-3'
FN1	Forward: 5'-GCTGGATGATGGTGGACTGT-3' Reverse: 5'-CTCGGTTGTCCTTCTTGCTC-3'
SNAI1	Forward: 5'-ACTGGTGAGAAGCCATTCTCCT-3' Reverse: 5'-CTGGTATCTCTTCACATCCGAGT-3'

The oligonucleotides used in qRT-PCR on MCF10 and MDAMB-231 cells were:

CDH2	Forward: 5'-AGGGGACCTTTTCCTCAAGA-3' Reverse: 5'-CTACTGCATGTGCCCTCAAA-3'
CLD6	Forward: 5'-GATGCAGTGCAAGGTGTACG-3' Reverse: 5'-GCCTTGGAATCCTTCTCCTC-3'
FN1	Forward: 5'-TGAAAGACCAGCAGAGGCATAAG-3' Reverse: 5'-CTCATCTCCAACGGCATAATGG-3'
MMP9	Forward: 5'-TGACAGCGACAAGAAGTGGG-3' Reverse: 5'-TTCAGGGCGAGGACCATAGA-3'
OCLN	Forward: 5'-GAAGCCAAACCTCTGTGAGC-3' Reverse: 5'-GAAGACATCGTCTGGGGTGT-3'
SMYD3	Forward: 5'-TGAATGTGACTGTTTCCGTTGC-3' Reverse: 5'-ATTGCTGCTTATGATCGCCTGG-3'
SNAI1	Forward: 5'-ATCGGAAGCCTAACTACAGCGAGC-3' Reverse: 5'-CAGAGTCCCAGATGAGCATTGG-3'
SNAI2	Forward: 5'-CTTTTTCTTGCCCTCACTGC-3' Reverse: 5'-GCTTCGGAGTGAAGAAATGC-3'
SOX4	Forward: 5'-CCAGCAAGAAGGCGAGTTAG-3' Reverse: 5'-CGGAGCCTTCTGTCTTCATC-3'
VIM	Forward: 5'-CCCTCACCTGTGAAGTGGAT-3' Reverse: 5'-GCTTCAACGGCAAAGTTCTC-3'

The oligonucleotides used in ChIP qRT-PCR were:

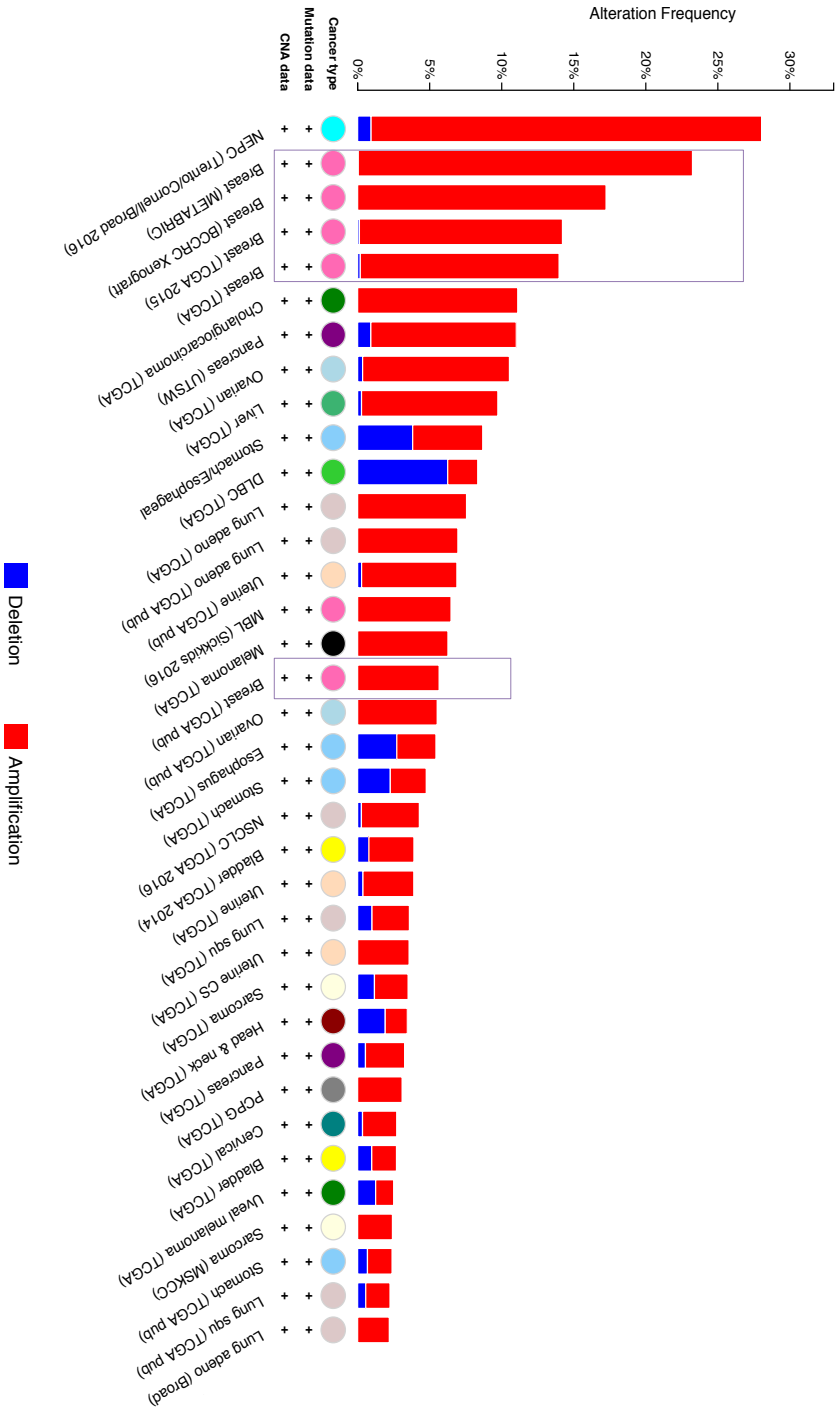
hMMP9	Forward: 5'-GGTGGTGTAAGCCCTTTCTCAT-3' Reverse: 5'-ATGGTGAGGGCAGAGGTGTCT-3'

hSNAI1	Forward: 5'-GAGTGGTTCTTCTGCGCTACTG-3' Reverse: 5'-GCTGTAGTTAGGCTTCCGATTGG-3'
hSOX4	Forward: 5'-ATTGTTTTGTGGCTTTCTCTTCCC-3' Reverse: 5'-TCAGCATTGGAATAAAGAATCAGCC-3'
hVIM	Forward: 5'-CTCTTCTCCGGGAGCCAGTC-3' Reverse: 5'-CGGTAGGAGGACGAGGACAC-3'
mSNAI1	Forward: 5'- CGGAGTTGACTACCGACCTT -3' Reverse: 5'- GACCTAGGTAGTCGGGGTCAC-3'
hUbiquitinB	Forward: 5'-GAAGGAAGAGAAGCGCATAGAGGAGAA-3' Reverse: 5'-CTCATAGCCGTAAGAAAGGCTCCTAAA-3'
beta globin promoter	Forward: 5'-GACAAACATTATTCAGAGGGAGT-3' Reverse: 5'-AAGCAAATGTGAGGAGCAACTGAT-3'

Supplementary Figures

Supplementary Fig. S1. SMYD3 over-expression is present in multiple cancers.
TCGA database was queried for SMYD3 under- or over-expressing SMYD3 tumor types. TCGA data were analyzed using the open-source software cBioPortal for Cancer Genomics (http://www.cbioportal.org/). Breast cancer datasets are in rectangular boxes.

A



Supplementary Fig. S1

Supplementary Fig. S2. SMYD3 knockdown hinders TGFβ-induced EMT in human and mouse breast cancer cells and affects migration.

A, qRT-PCR analysis of epithelial (E-Cadherin, Claudin6) or mesenchymal (N-Cadherin and Fibronectin) markers. shSMYD3 or shScramble NMuMG cells were treated with 10 ng/ml TGFβ for 0, 24, 48 or 96h. GAPDH was used as housekeeping gene. Data represent means±SD. Statistical analysis was performed with 1 way ANOVA, followed by post-hoc Tukey test. $n \geq 3$, * $p \leq 0.05$, ** $p \leq 0.01$

B, Immunoblot of p-ERK, ERK, p-AKT and AKT in NMuMG cells treated with 10 ng/ml TGFβ for 72h. Quantified band intensity of p-ERK and p-AKT is indicated, normalized over total ERK and AKT respectuvely.

C, Brightfield images of siScramble or siSMYD3 MCF10A cells treated with 5 ng/ml TGFβ or vehicle for 24 hours. Scale bar=10μm.

D, NMuMG cells were transduced with pBabe-Empty or pBabe-hSMYD3 retrovirus and depleted of endogenous SMYD3 by siRNA transfection for 24h and treated with TGFβ for 48h. Immunoblot of whole cell extracts using antibodies raised against SMYD3. GAPDH was used as loading control.

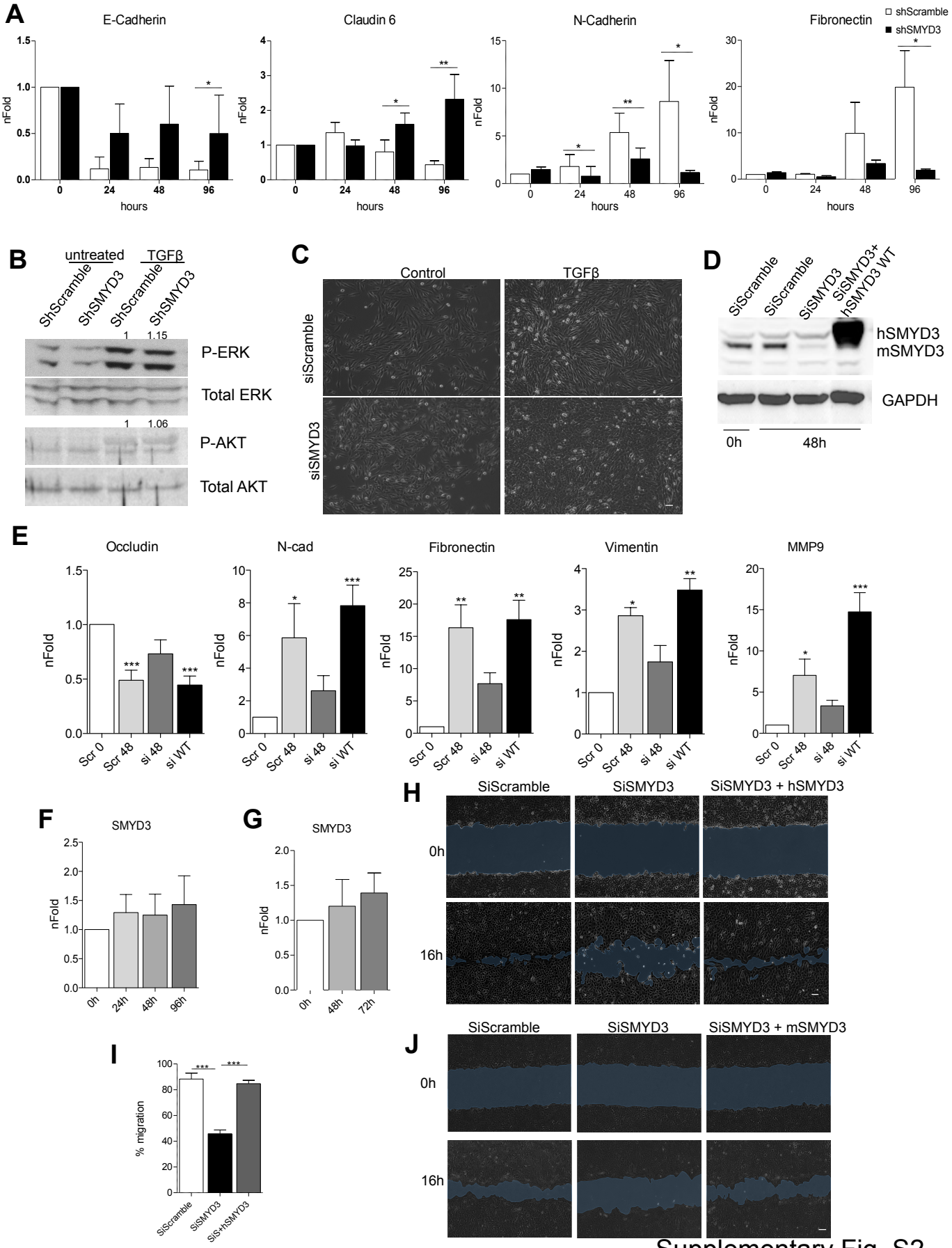
E, Rescue experiments were performed as in D. Total RNA was extracted and epithelial and mesenchymal transcript levels were measured by qRT-PCR. Data represent means±SD. Statistical analysis was performed with 1 way ANOVA, followed by post-hoc Tukey test. $n = 3$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

F, G, SMYD3 mRNA levels were evaluated at different time points in NMuMG (F) and MCF10A (G) cells treated with TGFβ.

H, Wound healing assay on rescue experiment in NMuMG cells treated with 10 ng/ml TGFβ for 0 and 16h. Control and hSMYD3 over-expressing cells were transfected with siSMYD3 for 24h and than treated with TGFβ for 16h.

I, Migration percentage of cells represented in H. Statistical significance was calculated with unpaired Student's t-test. $n = 3$, *** $p \leq 0.001$. Scale bar=10μm.

J, Wound healing assay on a rescue experiment in MCF10A cells treated with 5 ng/ml TGFβ for 0 and 16h. Cells were transfected with siRNA Scramble and siSMYD3. After 16h cells were transfected with pCMV-Empty or pCMV-mSMYD3 vectors. After 24h, cells were treated with TGFβ for 16h.



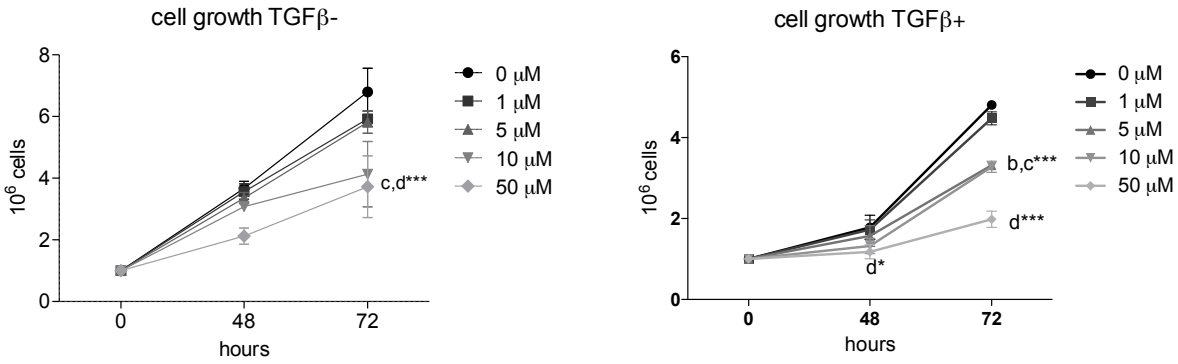
Supplementary Fig. S2

Figure S3. Impact of BCI121 treatment and SMYD3 depletion on NMuMG and MCF10A cells growth, following TGFβ treatment.

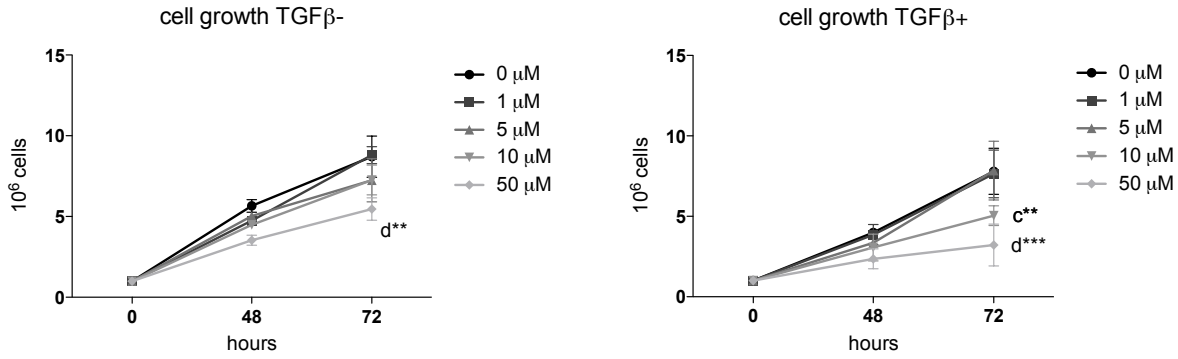
A, B, NMuMG and MCF10A cells growth was evaluated in the presence of TGFβ (right panel) or vehicle (left panel), in cells treated with increasing doses of SMYD3 inhibitor BCI121. Statistical significance was calculated with 1 way Anova, followed Tukey post-test. a,b,c,d refers to statistical significant difference between BCI121-untreated cells and cells treated with 1,5,10,50μM BCI121 respectively.

C, D NMuMG (C) and MCF10A (D) cells growth was evaluated in the presence of TGFβ, in control or SMYD3-depleted cells, obtained by ShRNA infection (C) or siRNA transfection (D). Statistical significance was calculated with Student's t-test. $n = 3$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

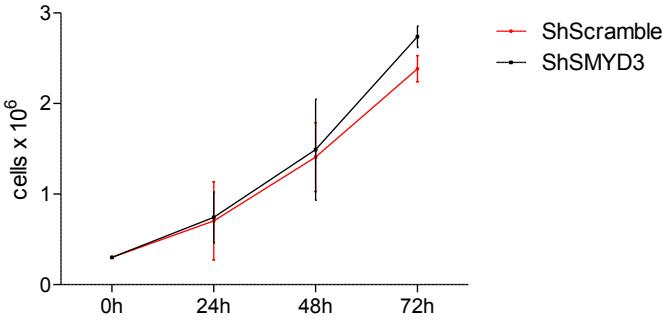
A NMuMG



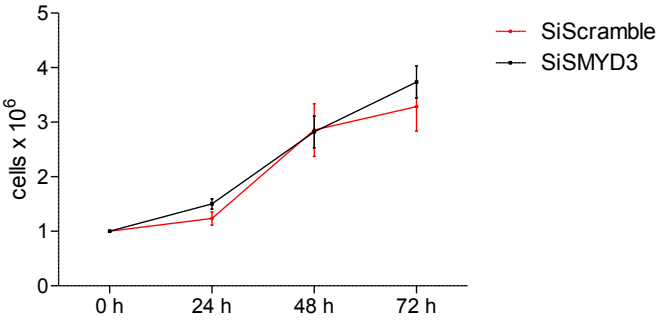
B MCF10A



C NMuMG



D MCF10A



Supplementary Fig. S3

Supplementary Fig. S4. SMYD3 associates with over-expressed SMAD2, 3, 4 and affects EMT through its C-term region, independently of its methylation activity.

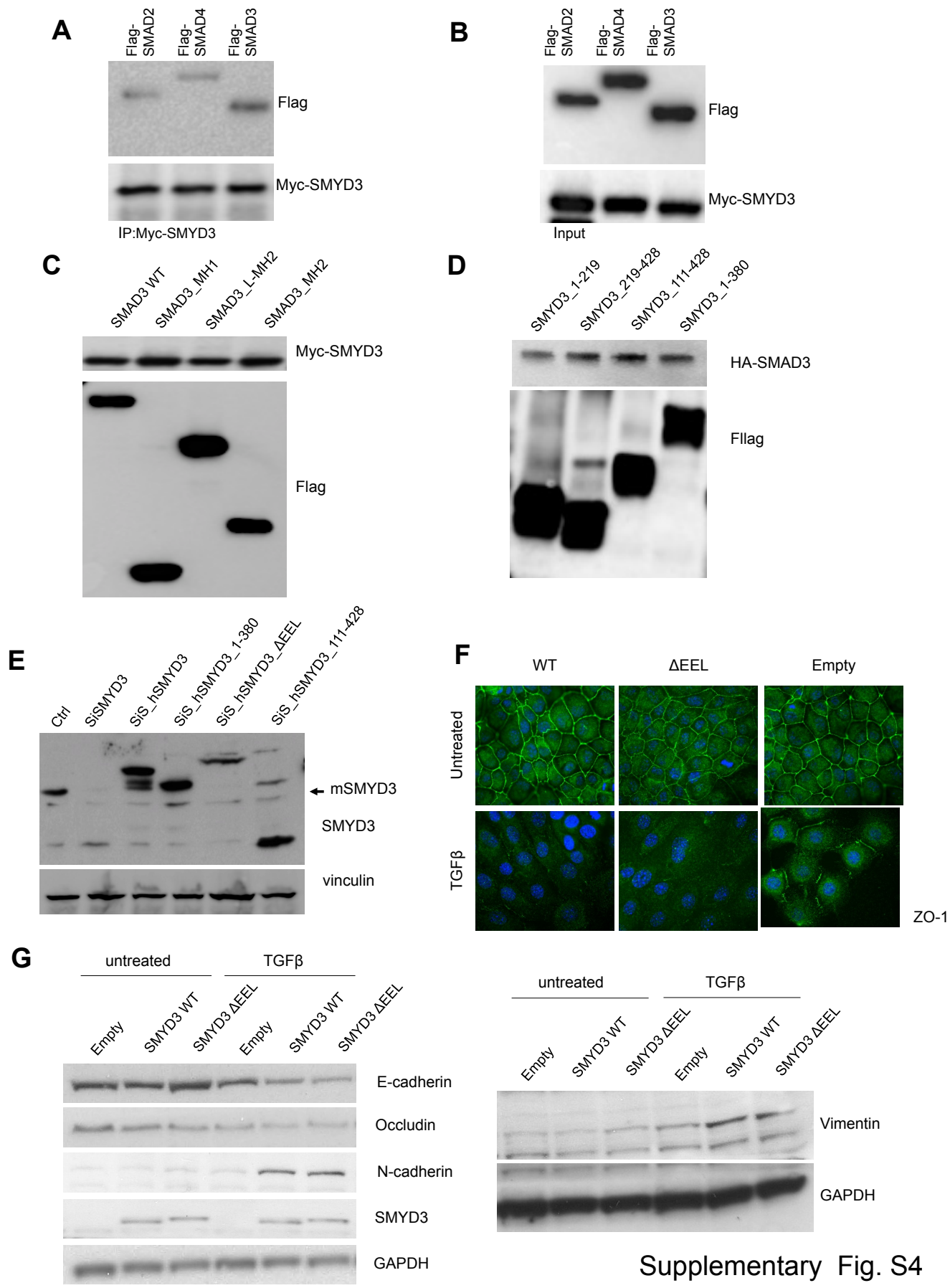
A, B, HEK293T cells were co-transfected with Myc-SMYD3 and Flag- SMAD2, SMAD3 or SMAD4. Extracts were used in IP experiments with Myc-antibodies. Immunoblot was performed with Flag antibodies. Input extracts are shown in B.

C, D Input HEK293T cell extracts used for co-IP assays shown in Fig 3C, D.

E, NMuMG cells were transduced with pBabe-SMYD3, pBabe-SMYD3_ΔEEL and pBabe-EMPTY retroviruses and clones were treated with 10ng/ml TGFβ for 48h. Epithelial (E-Cadherin and Occludin) and mesenchymal (N-Cadherin and Vimentin) protein levels were analyzed by immunoblot.

F, Immunofluorescence microscopy of pBabe-SMYD3, pBabe-SMYD3_ΔEEL and pBabe-EMPTY NMuMG cells treated with TGFβ for 48h, fixed with paraformaldehyde and stained with antibodies against the epithelial marker ZO1.

G, NMuMG cells were transduced with pBabe-Empty**,** pBabe-SMYD3, pBabe-SMYD3_ΔEEL, pBabe-1-380 and pBabe-111-428. Clones were depleted for SMYD3 through siRNA transfection for 24h and than treated with TGF for 48h. Proteins were extracted and analyzed in western blot. SMYD3 depletion (mSMYD3, arrow on the right) and SMYD3 WT/mutants over-expression (arrows within the image) were detected through immunoblot analysis with SMYD3 antibodies. Vinculin was used as a loading control.



Supplementary Fig. S4

Supplementary Fig. S5. SMYD3 interacts with SMAD3 in the nucleus in TGFβ-treated cells and SMYD3 depletion does not disrupt SMAD3/SMAD4 interaction.

A, Nuclear and cytoplasmic extracts from TGFβ-treated NMuMG cells were used in IP experiments. IP was performed with antibodies raised against SMYD3, and immunoblots were assayed with antibodies against SMAD3. GAPDH was used as cytoplasmic marker, histone H4 as nuclear marker.

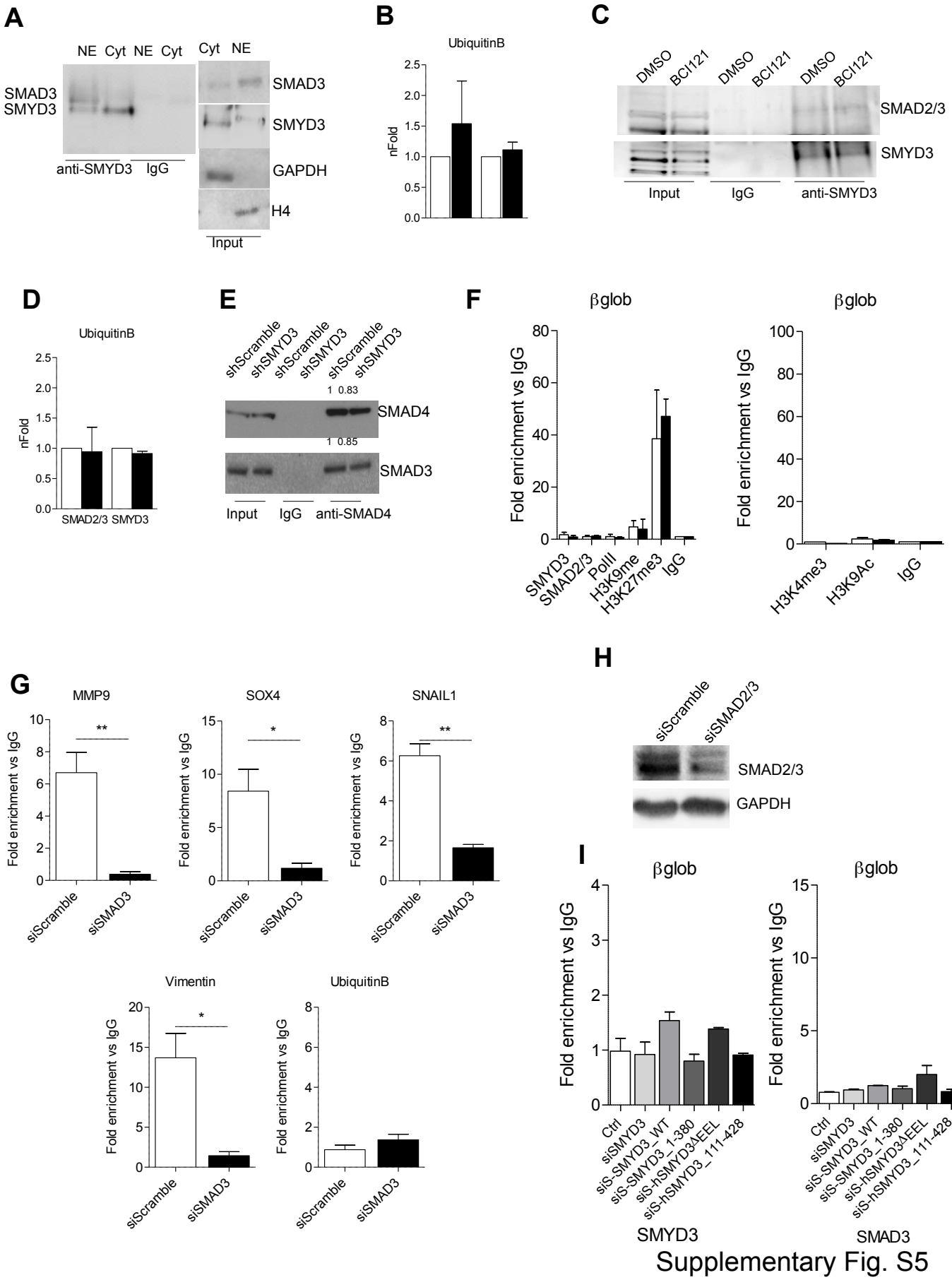
B, D, F, I, UbiquitinB and beta globin promoters were used as control genomic regions for ChIP assays shown in Fig. 4A, B, F, G.

C, MCF10A cells were treated with BCI121 or DMSO for 24h and whole cell extracts were used in immunoprecipitation experiments with antibodies raised against SMYD3. Immunoblot was performed with SMAD2/3 and SMYD3 antibodies.

E, Whole cell extracts from TGFβ-treated Sh-SMYD3 and control NMuMG cells were used in IP experiments with antibodies raised against SMAD4. Immunoblot was performed with antibodies against SMAD3 and SMAD4.

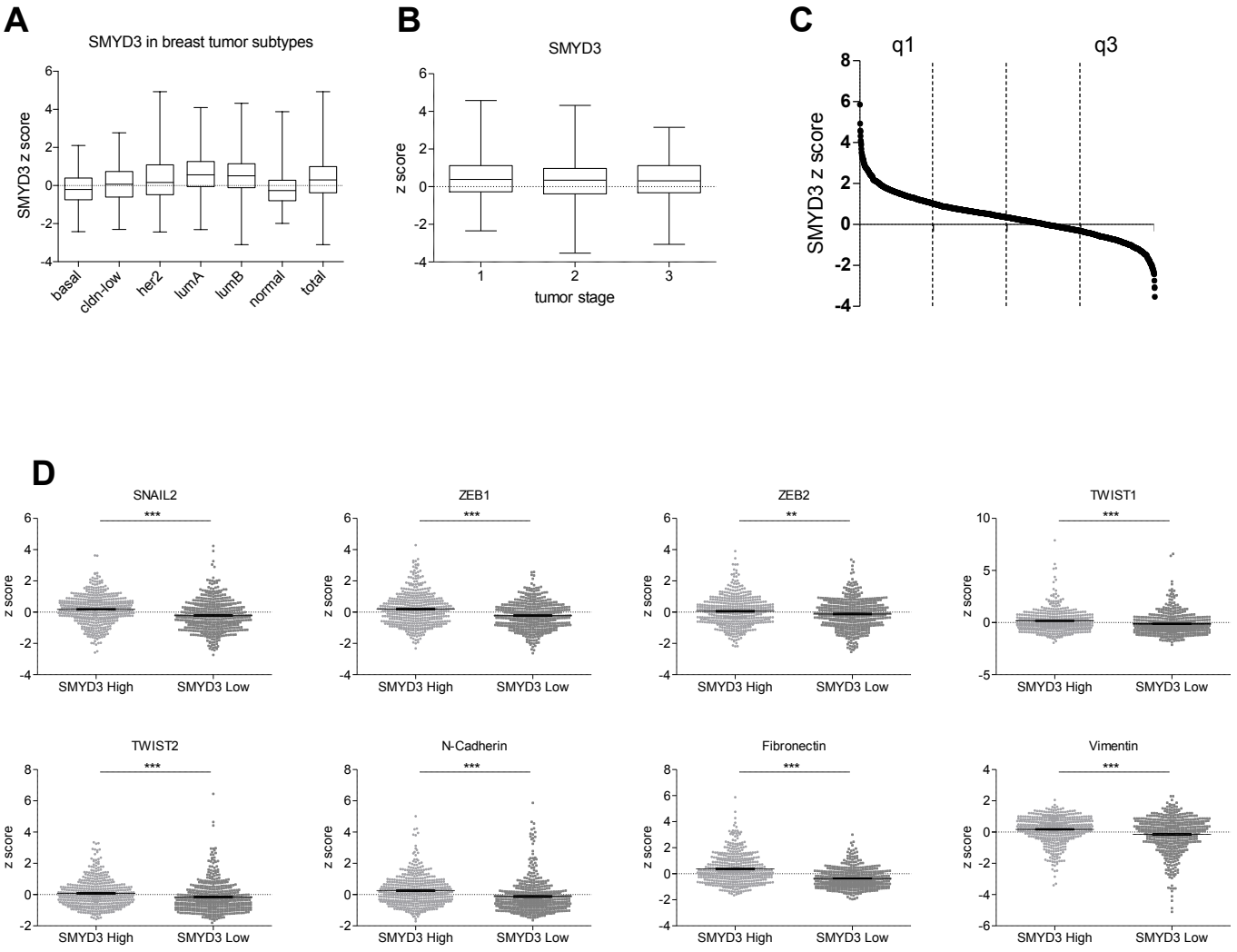
G, SMYD3 association to regulatory regions of EMT genes was analyzed by ChIP qPCR, in siScramble and siSMAD2/3 transfected MCF10A cells. Data are expressed as fold enrichment relative to SiScramble cells and represent means±SD. Statistical analysis was performed with Student's t test. n=3, * $p \leq 0.05$, ** $p \leq 0.01$.

H, MCF10A cells were transfected with siScramble or siSMAD2/3 RNAi for 24h and treated with 5ng/ml TGFβ for 48h. A fraction of the cells used in G, were checked for SMAD2/3 levels in immunoblot.



Supplementary Fig. S5

Supplementary Fig. S6. **Breast cancer clinical dataset analysis.**
A, box plots represent SMYD3 mean z scores across breast tumor subtypes. Data were obtained from the Metabric dataset.
B, Box plots represent SMYD3 mean z scores across tumor stages 1,2,3. Data were obtained from the Metabric dataset.
C, Patients were stratified in two group with "High" (q1) and "Low" (q3) SMYD3 expression levels.
D, Tumors from the whole Metabric dataset were stratified for SMYD3 expression (*high* vs *low*) and Snail2, ZEB1, ZEB2, TWIST1, TWIST2, Vimentin, N-Cadherin, Fibronectin mRNA levels were compared in SMYD3 *low* and SMYD3 *high* tumors. Statistical significance was calculated with unpaired Student's t test, $*p \leq 0.05$, $**p \leq 0.001$, $***p \leq 0.0001$.
E, Correlation between SMYD3 and the mesenchymal markers Snail2, ZEB2, ZEB1, TWIST1, Fibronectin and N-Cadherin expression, calculated on breast cancer tumors from the Metabric dataset, by Pearson correlation analysis.
F, Correlation between SMYD3 expression and the mesenchymal markers Snail2, Fibronectin and N-Cadherin, calculated in the NKI295 breast cancers dataset by Pearson correlation analysis.



E

Metabric

Transcript	R2	Pearson r
SNAIL2	0.02817	0.1678
ZEB1	0.02464	0.0157
ZEB2	0.007805	0.08834
TWIST1	0.015	0.1225
N-caherin	0.02325	0.1525
Fibronectin	0.06851	0.2617

F

NKI295

Transcript	R2	Pearson r
SNAIL2	0.05048	0.2247
N-cadherin	0.0139	0.2792
Fibronectin	0.1112	0.3335

Supplementary Fig. S6

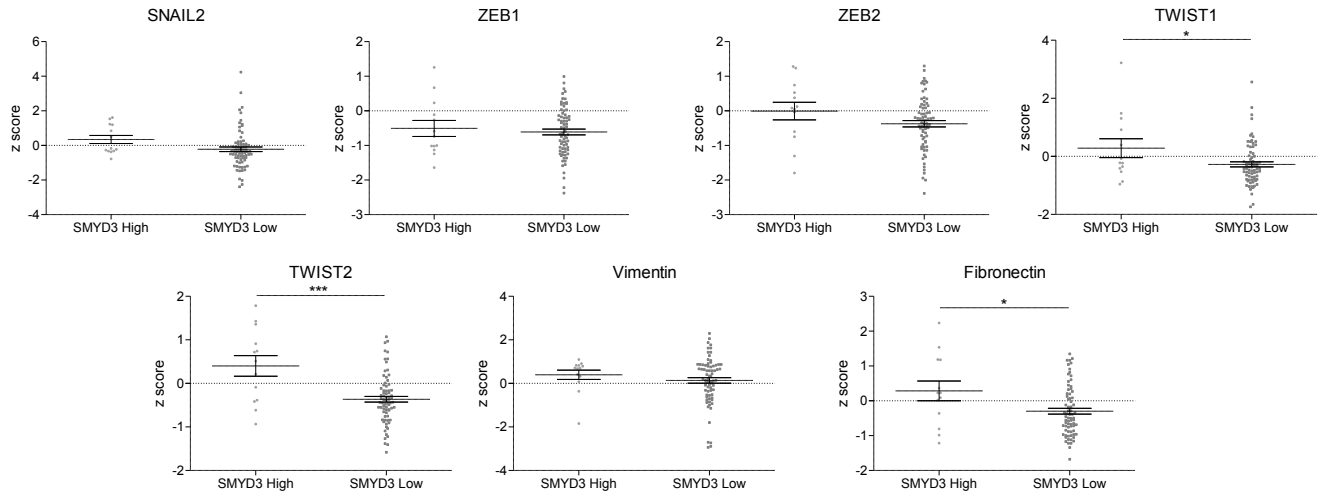
Supplementary Fig. S7. Clinical dataset analysis for the basal subtype breast cancer.
A, Correlation between SMYD3 and the mesenchymal markers Snail2, ZEB2, ZEB1, TWIST1, Fibronectin and N-Cadherin expression, calculated on basal breast cancer tumors from the Metabric dataset, by Pearson correlation analysis.
B, Basal tumors from the Metabric dataset were stratified for SMYD3 expression (*high* vs *low*) and Snail2, ZEB1, ZEB2, TWIST1, TWIST2, Vimentin, Fibronectin mRNA levels were compared in SMYD3 *low* and SMYD3 *high* tumors. Statistical significance was calculated with unpaired Student's t test, $*p \leq 0.05$, $***p \leq 0.0001$.
C, Kaplan-Meier plots of overall survival of patients from the Metabric dataset, stratified by SMYD3 expression levels. *p* value was calculated by log rank test.
D, Kaplan-Meier plots of distant metastasis-free survival of patients with basal tumors (Metabric dataset), stratified by SMYD3 expression levels. *p* value was calculated by log rank test.
E, Kaplan-Meier plots of distant metastasis-free survival of patients with grade 1 breast tumors, stratified by SMYD3 expression levels. *p* value was calculated by log rank test.

A

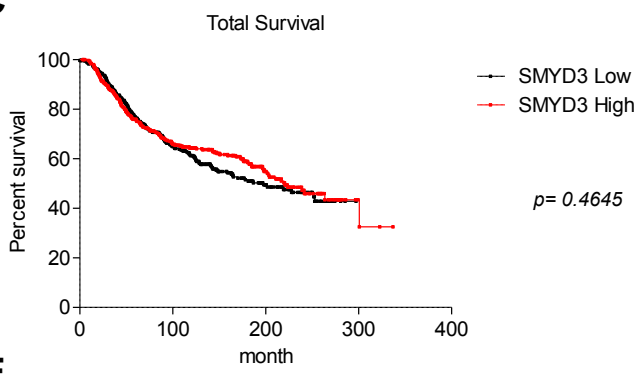
Metabric (Basal subtype)

Transcript	R2	Pearson r
SNAIL2	0.007	0.084
ZEB1	0.002	0.046
ZEB2	0.015	0.125
TWIST1	0.017	0.132
N-cadherin	0.008	0.089
Fibronectin	0.059	0.244

B



C



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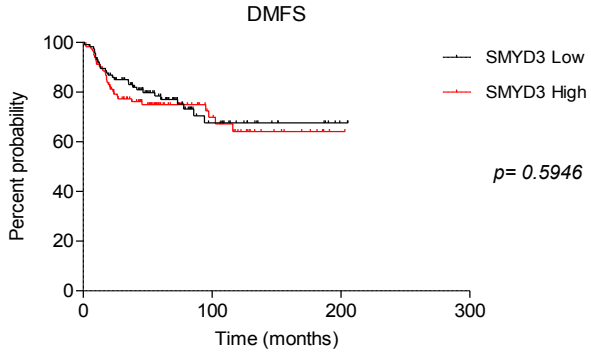

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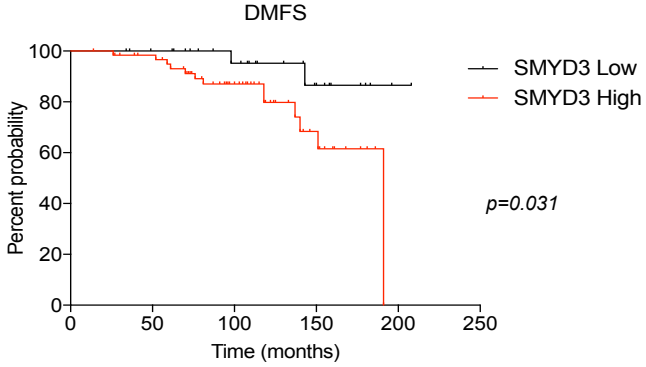

Supplementary Fig. S7