Supplemental Material. Hannigan et al., Epigenetic downregulation of human Disabled-2 switches TGF-β from a tumor suppressor to a tumor promoter.

Supplemental Methods.

Plasmids and Reagents

A *DAB2* cDNA expression plasmid was purchased from origene (pCMV6-XL5-*DAB2*). Sequencing revealed the presence of the S634N mutation previously described as a protein stability mutant (1). The Quickchange II® site-directed mutagenesis kit (Stratagene) was used to convert the point mutation to the originally described sequence (NCBI; NM001343) as per the manufacturer's instructions, to generate the 'N634S' or wildtype *DAB2*. The primers used for the site-directed mutagenesis reaction were designed using the Quickchange II® primer design program (Stratagene). *Not*I digestion generated the linear N634S *DAB2* sequence which was ligated into a *Not*I digested and linearised pTRE2pur plasmid (Clontech) to generate pTRE2pur-*DAB2*. The desired orientation was validated by restriction analysis and sequencing

Cell lines

MDAMB231 cells were cultured in DMEM supplemented with 10% fetal calf serum, 2mM L-glutamine and 100 U/ml penicillin and streptomycin. SNU-16 cells were cultured in RPMI-1640 supplemented with 10% fetal calf serum, 2mM L-glutamine and 100 U/ml penicillin and streptomycin. Both cell lines were purchased from ATCC.

5-azacytidine and Trichostatin A treatments

Cells were treated with DMSO control or 2μM 5-azacytidine (Sigma, UK) for 5 days with refeeding in media containing 5-azacytidine or DMSO, with or without the addition of 100ng/ml trichostatin A (Sigma, UK) for the last 24 hours of treatment.

Immunofluorescence

Immunofluorescence was performed as previously described (2) and slides were analysed by confocal microscopy utilising a Leica SP2 confocal microscope.

Retroviral Infections

pBabe puro and pBabe Cre retroviruses were generated as described (3). Stable cell pools were generated after outgrowth in medium containing 0.5 μg/ml puromycin.

Generation of inducible DAB2 and stable DAB2 cell lines

The A431 TetOn cell line was generated by transfection with pTetOn (Clontech) using lipofectamine and selection with G418. Isolated TetOn clones were tested for induction by transfection with pTRE2hyg-Luc (Clontech) followed by luciferase assay. A clone (A431 TetOn) was selected with low level expression in the absence of doxycycline (dox) and high fold induction in the presence of dox (>30-fold). This clone was transfected with pTRE2pur-*DAB2* and selected with puromycin to generate double-stable clones. Levels of DAB2 induction were tested by the addition of 1μg/ml dox for 24hrs followed by western blotting. Two clones were selected for further analysis (A431 TD2#1, A431 TD2#2). A431 and SKOV3 cells were co-transfected with either pSG5 or pSG5FlagDAB2 plasmids and pTk-neo in a ratio of 10:1. Stable clones were selected in G418 and assessed for DAB2 expression by western blotting.

One vector control (A431V, SKOV3V) and two DAB2 stable cell lines from each transfection (A431D2#1, A431D2#2, SKOV3D2#1, SKOV3D2#2), were selected for further analysis.

Proliferation Studies

$5x10^3$ cells per well were seeded into twelve well plates in 1ml of culture medium in triplicate overnight. The following day 0.5mls of media was added +/- TGF-β as appropriate. Cells were either harvested by trypsinisation and cell number determined using a Casy cell counter (Casy) or re-fed with 0.5mls of media +/- 1ng/ml TGF-β every 2 days.

Bisulphite sequencing

To map methylation of the *DAB2* CpG island in detail, genomic DNAwas subjected to modification with sodium bisulphite using the EZ DNA methylation kit (Zymo, California) according to the manufacturer's instruction. and was used in PCR amplification reactions with the following primer pairs.

F1; 5'TAGTTTTTTGTTTAAAGGGTTTTAA,

R1; 5'TCCCCAAAAATATACCAAAATATTTT,

F2; 5'TTTTGGGGAGTTTTTAGTTGTTAGTAT,

R2; 5'TACTAATTTCCCCAAACACAAAATC:

F3; 5'GATTTTGTGTTTGGGGAAATTAGTA;

R3; 5'CAACCACCTCAATATCCTAAAAAAA.

Reaction conditions for PCR were as follows: an initial incubation at 95ºC for 15 min was followed by 40 cycles of 95ºC for 30 s, 55ºC for 30 s, and 72ºC for 30 s and a

final extension at 72ºC for 5 min. PCR products were purified with a PCR purification kit (Qiagen), ligated into a TA cloning vector (Invitrogen), and transformed into top 10 Escherichia coli –competent cells (Invitrogen). Plasmid DNA was isolated using the Templiphi system (GE Healthcare) and used for sequencing with the BigDye Terminator Cycle kit (PE Applied Biosystems). For each sample, a minimum of 12 colonies was sequenced.

Methylation Sensitive PCR

Methylation PCR was performed utilising the following primers and conditions:

Unmethylated Forward (UF1); 5' - ACCAACCAAAAACTTCAAAACCACACAA,

Unmethylated Reverse (UR1); 5' - GTGGGGTTTTTTGTGTTGTTGTAGTGT,

Methylated Forward (MF1); 5' - GACCGAAAACTTCGAAACCGCGCGA,

Methylated Reverse (MR1); 5' - GGGGTTTTTTGCGTCGTTGTAGCGC.

PCR conditions were as follows: 8 cycles of 95ºC for 2 min, 60ºC for 30 s, and 72ºC for 30 s were followed by 32 cycles of 95ºC for 30 s, 60ºC for 30 s, and 72ºC for 30 s and then a final extension at 72ºC for 5 min. The PCR products were electrophoresed through 2% agarose gels, stained with ethidium bromide, and visualized using a transilluminator.

DAB2 pyrosequencing

Methylation in the CpG island of the *DAB2* gene was analyzed with Pyrosequencing technology, which allows simultaneous quantification of the degree of methylation at several CpGs in close proximity with high quantitative resolution. After bisulfite treatment and PCR, the degree of methylation at each CpG position in a sequence is

determined from the ratio of T and C. Biotage Sample Prep kit and primers designed for amplifying a 196 bp fragment across the CpG island of *DAB2* gene and optimized for software dedicated to methylation analysis were used.

Primer sequences:

PCR F 5'- ATGGAGTTAGAGGGAAGAAGGGT – 3'

PCR R 5'- AAAATCCTCAACTACCAACATCT – 3'

Sequencing 5'- TTAGAGGGAAGAAGGGT – 3'

After the Pyrosequencing run, analysis was performed using Pyro Q-CpG Software (Biotage).

DAB2 qRT-PCR primers

DAB2 exon 10 Forward primer 5'-CCAACAGAAAGCAAAGATATCC-3'

DAB2 exon 9 to10 reverse primer 5'-GTTGGTCGAGGAAGAGAAC-3'

siRNA oligos

DAB2#1: Silencer™ pre-designed siRNA 121309 (Ambion)

Sense CCGGGCAUUUGGUUACGUGtt

Antisense CACGUAACCAAAUGCCCGGtt

DAB2#2: Silencer™ pre-designed siRNA 121311 (Ambion)

Sense GCAAAGAUAUCCUGUUAGUtt

Antisense ACUAACAGGAUAUCUUUGCtt

Chromatin immunoprecipitation (ChIP) assays

Protein/DNA complexes were cross-linked by the addition of 1% formaldehyde to cells in 15cm plates and incubated at 37^{o}C for 10mins. Glycine (125mM final concentration) was added to stop the cross-linking. Cells were washed twice with ice-cold PBS and then scraped in ice-cold PBS (with protease inhibitors). Cell scrapings from plates of the same cells/treatment were combined and centrifuged at 700g, 5mins at 4^{o}C. The resulting pellet was resuspended in SDS lysis buffer (1% SDS, 10mM EDTA, 50mM Tris pH8) at a concentration of 2x10^{7} cells/ml. Cell lysates were sonicated for 60-80secs at 10% amplitude, (5secs on/5secs off) on ice. Sonicated lysates were centrifuged at 13,000rpm, 10mins at 4^{o}C in a bench top centrifuge. Each sample was diluted 10-fold (equivalent to 2x10^{6} cells/ml) with ChIP dilution buffer (0.01% SDS, 1.1% tritonX-100, 1.2mM EDTA, 16.7mM Tris-HCl pH8, 167mM NaCl). The lysates were pre-cleared by rotation at 4^{o}C with protein G agarose / salmon sperm DNA 50% slurry for 30mins. 1ml of the pre-cleared supernatant (= 2x10^{6} cells) was incubated with 5µl of antibody, rotating at 4^{o}C o/n. 40µl of protein G agarose / salmon sperm DNA 50% slurry was added to each sample and rotated at 4^{o}C for 1hr. The supernatant was removed after centrifugation at 5000rpm for 2mins at 4^{o}C. The beads were washed successively with 1ml of low salt immune complex buffer (2mM EDTA, 20mM Tris-HCl pH8, 150mM NaCl, 0.1% SDS, 1% tritonX-100), 1ml high salt immune complex buffer (2mM EDTA, 20mM Tris-HCl pH8, 500mM NaCl, 0.1% SDS, 1% tritonX-100), 1ml LiCl salt immune complex buffer (1mM EDTA, 10mM Tris-HCl pH8, 250mM LiCl, 1% NP40, 1% deoxycholate) and twice with 1ml of TE pH8. Immunoprecipitated protein/DNA complexes were eluted from the beads by the addition of 250µl of elution buffer (0.1M $NaHCO_3$, 1% SDS)

and shaking at room temperature for 15mins. The beads were centrifuged at 2000rpm for 1min at RT, the supernatant collected and the elution step repeated with a further 250µl of elution buffer. Cross-links were reversed by the addition of 20µl of 5M NaCl and incubation at 65^{o}C for 4hrs. To each sample was added 10µl of 0.5M EDTA, 20µl of Tris-HCl pH8 and 2µl of proteinase K and incubated at 45^{o}C for 1hr. This was followed by the addition of 500µl of phenol/$CHCl_3$/isoamyl alcohol, inverted for 2mins and centrifuged at 13,000rpm for 2mins. The aqueous layer was transferred to fresh tubes to which was added 1µl of glycogen and 1ml of 100% EtOH and left to precipitate at -20^{o}C o/n. Samples were centrifuged at 13,000rpm for 10mins at 4^{o}C, the supernatant removed and the pellet washed with 500µl of 70% ethanol. The DNA/glycogen pellets were resuspended in 50µl of dH_2O and then purified using a PCR clean up kit as per the manufacturer's instructions (Qiagen). The DNA was then used directly in quantitative PCR reactions (as previously described). Antibodies used for ChIP; Rabbit IgG control (#PD46B), anti-acetyl Histone H3 (#06-599), anti-acetyl Histone H4 (#06-866), anti-trimethyl-Histone H3(Lys27) (#17-622) all from upstate.

ChIP Primers

Region A - *DAB2* promoter

For: 5'-GGAACCGTTGTCCCTTGTC-3'

Rev: 5'-GAGCTTCGTTTCACAGATCCG-3'

Region B - *DAB2* promoter

For: 5'-CTTCTCCCCGAACACATTTCC-3'

Rev: 5'-CCTCAACGTGTCTGCAGAGTC-3'

Gamma-globin promoter

For: 5' AGAATAAAAGGAAGCACCCTTCAG-3'

Rev: 5'-TGGCGTCTGGACTAGGAGCTTA-3'

Other reagents

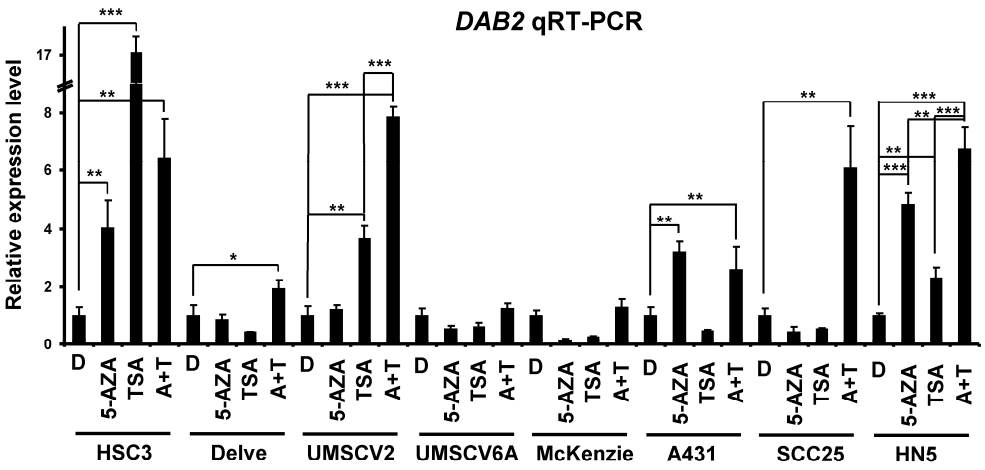
Anti-EZH2 rabbit polyclonal antibody (Active Motif).

Supplemental References

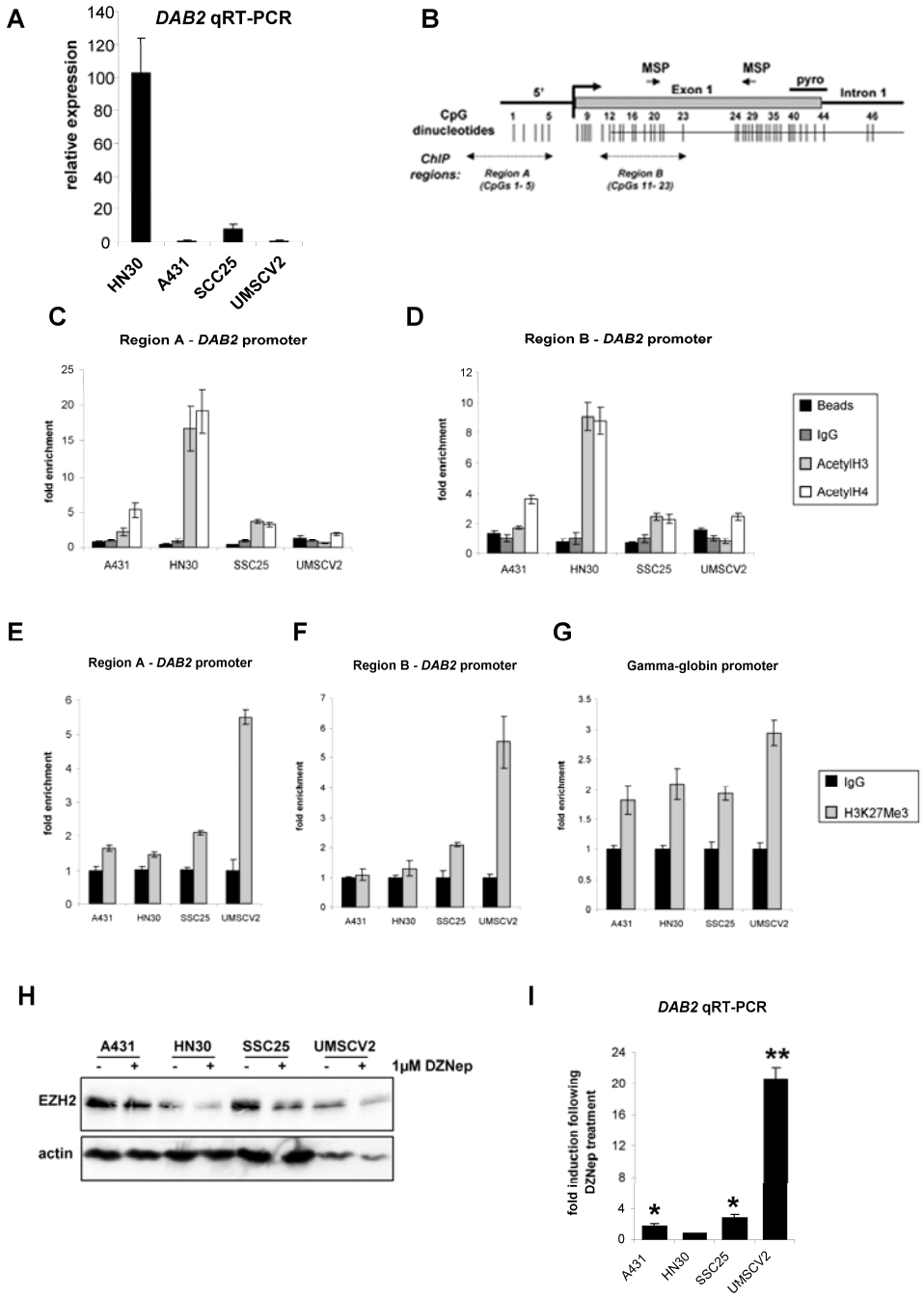
1 Hocevar, B.A., Smine, A., Xu, X.X., and Howe, P.H. 2001. The adaptor molecule Disabled-2 links the transforming growth factor beta receptors to the Smad pathway. *Embo J* 20:2789-2801.

2 Inman, G.J., Nicolas, F.J. and Hill, C.S. 2002. Nucleocytoplasmic shuttling of Smads 2,3 and 4 permits sensing of TGF-β receptor activity. *Mol. Cell*, 10:283-294.

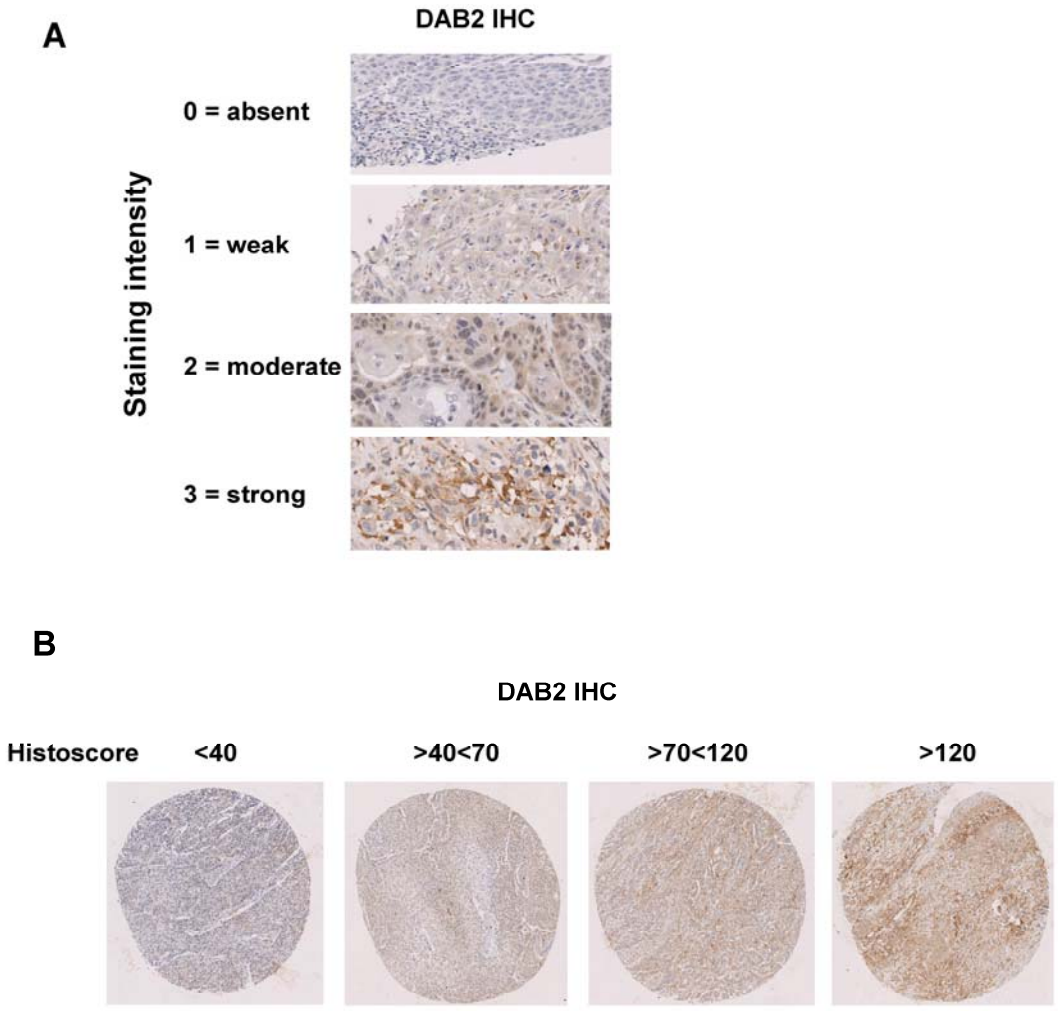
3 Spender LC, O'Brien DI, Simpson D, et al. TGF-beta induces apoptosis in human B cells by transcriptional regulation of BIK and BCL-XL. *Cell Death Differ* 2009; 16: 593-602.



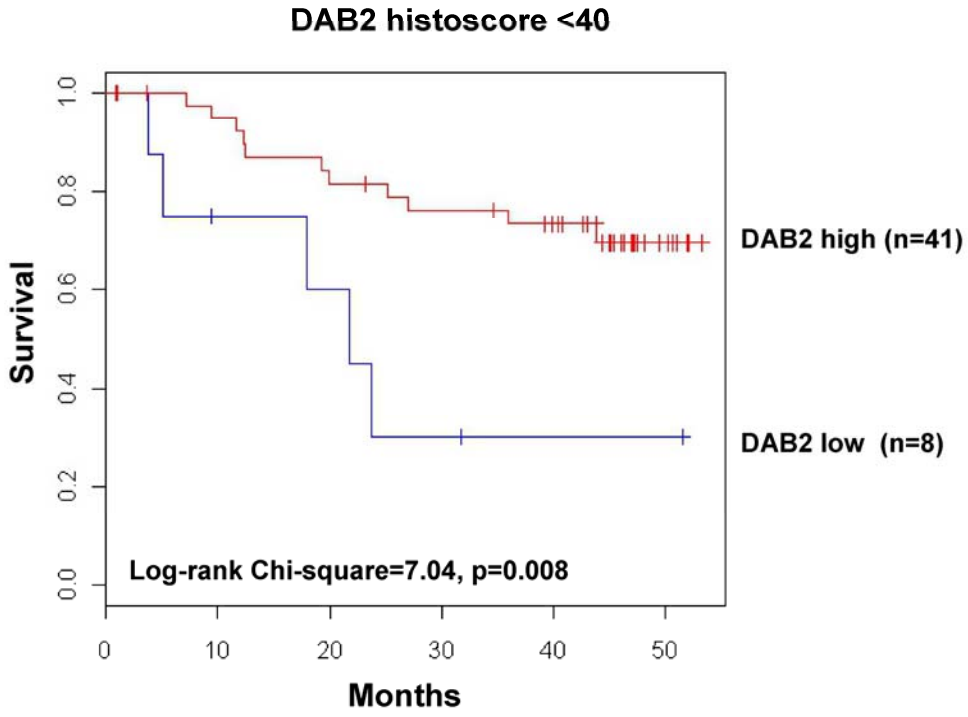
Supplemental Figure 1. *DAB2* is epigenetically silenced in SCC cell lines. The indicated cell lines were treated with 5-azacytidine (5-AZA) and/or Trichostatin A (TSA), RNA was harvested and *DAB2* mRNA levels were measured by qRT-PCR. The expression level of cells treated with carrier control DMSO alone (D) was assigned the arbitary value of 1. Data represent the means $\pm$ SD, n=3. Statistical analyses were performed with paired 2 tailed t-tests, *=$p<0.05$, **=$p<0.01$, *** $p<0.001$.



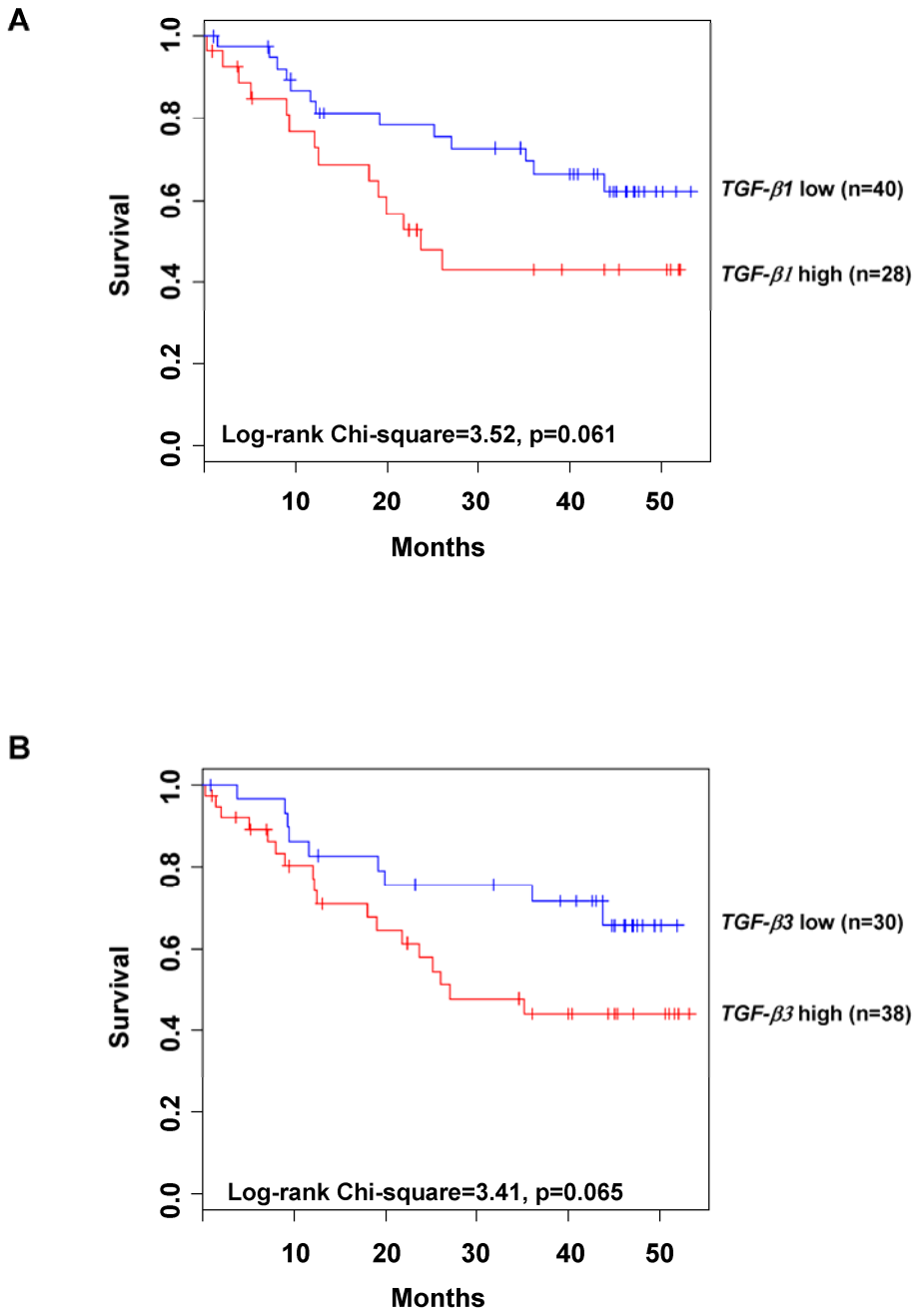
Supplemental Figure 2. Polycomb-mediated repression of the *DAB2* promoter accounts for loss of *DAB2* expression in some SCC cell lines. (A) *DAB2* expression assessed by qRT-PCR analysis in A431, SCC25 and UMSCV2 cell lines relative to the high level *DAB2* expressing cell line HN30. (B) Schematic indicating the position of the two *DAB2* promoter regions (Regions A and B) interrogated by quantitative ChIP. (C, D) Quantitative ChIP analysis of the *DAB2* promoter regions A and B immunoprecipitated with beads only, IgG control, acetylated Histone H3 and acetylated Histone H4 antibodies in the indicated cell lines. (E, F, G) Quantitative ChIP analysis of the *DAB2* promoter regions (A and B) and the gamma-globin promoter immunoprecipitated with an IgG control or with anti-trimethyl-Histone H3 K27 (H3K27Me3) antibody. (E,F,G) Values are expressed as fold enrichment relative to the IgG control of each cell line. (H) Western blots of cells treated for 24hrs +/- 1μM DZNep and probed with anti-EZH2 or anti-actin as a loading control. (I) qRT-PCR analysis of *DAB2* expression in cells treated +/- 1μM DZNep for 24hrs expressed as fold change relative to untreated controls. Statistical analyses were performed with paired 2 tailed t-tests, *=$p<0.05$, **=$p<0.01$.



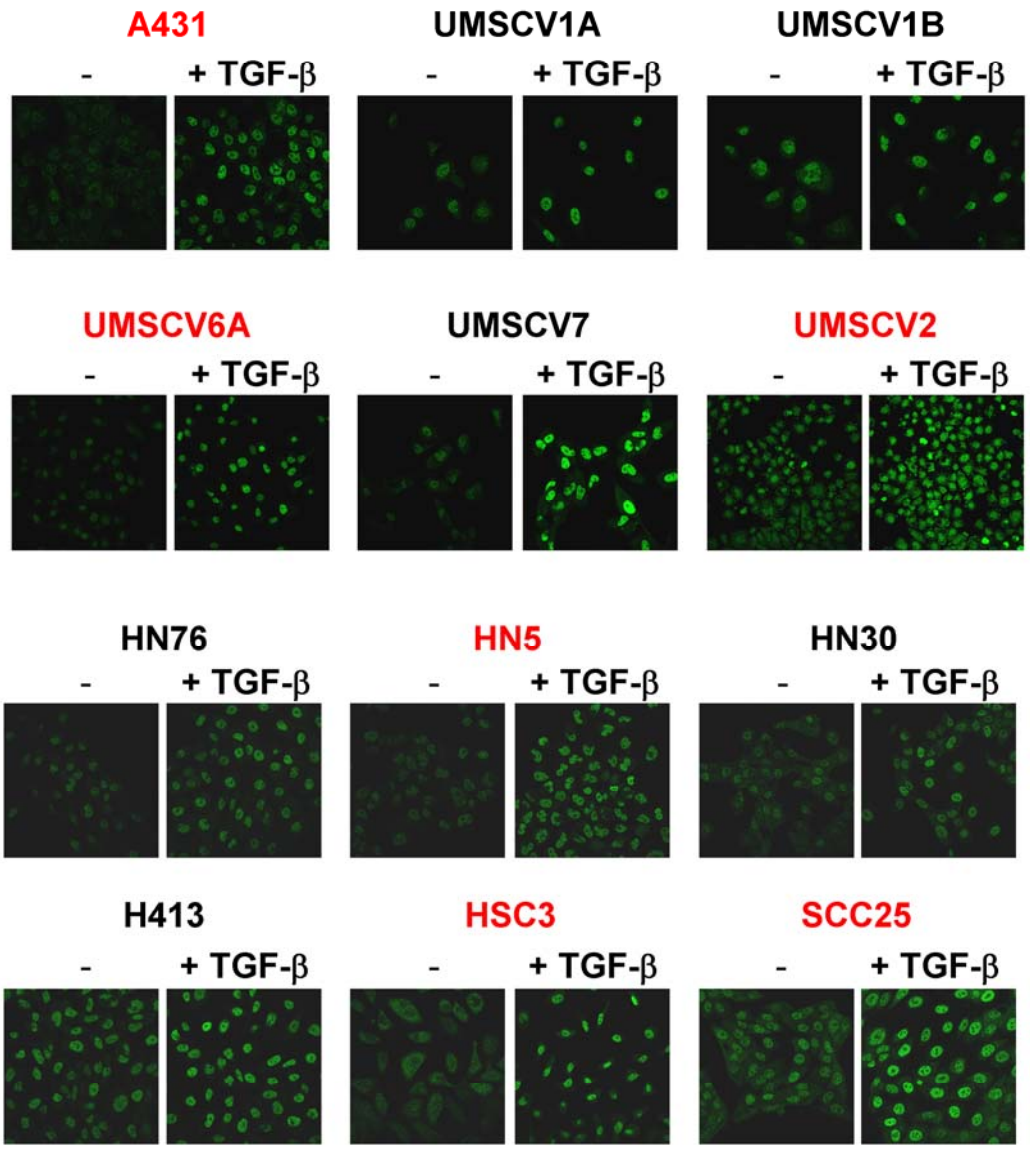
Supplemental Figure 3. **DAB2 immunohistochemistry.** (A) TMAs were stained with an anti-DAB2 antibody. (A) Examples of high magnification regions of tumor cores exhibiting tumor cells with absent (0), weak (1), moderate (2) or strong (3) staining. (B) Example images of tumor cores showing the indicated tumor cell histoscore.



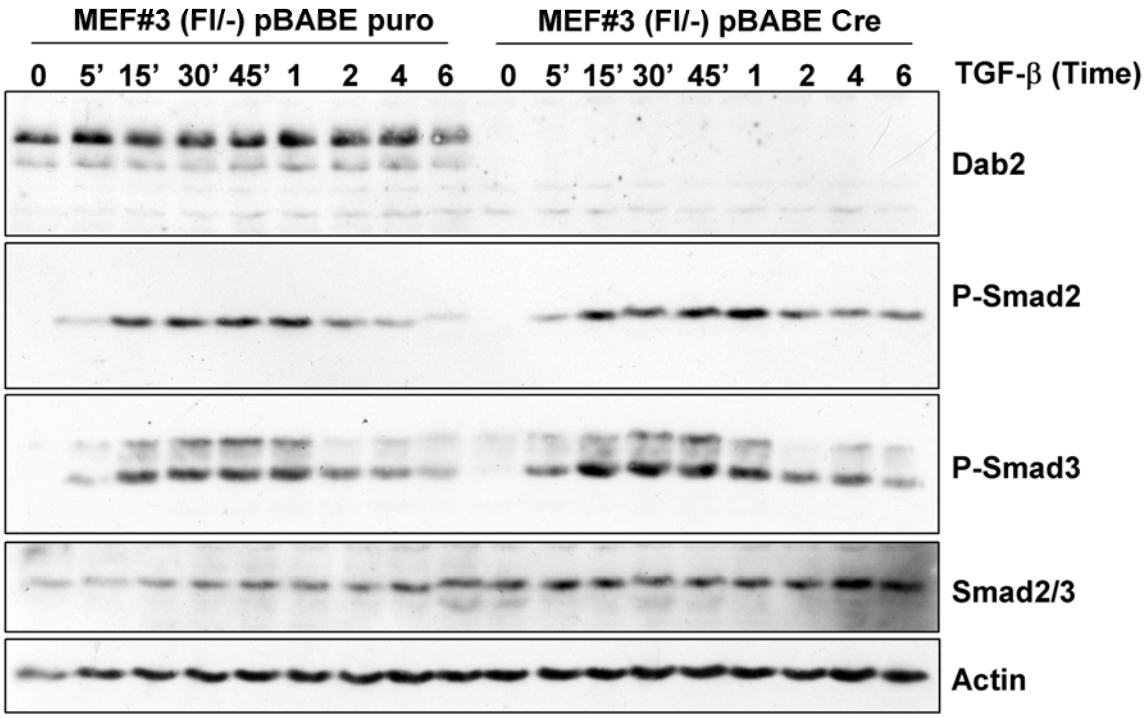
Supplemental Figure 4. Low level DAB2 expression correlates with poor survival. A HNSCC TMA was stained with an anti-DAB2 antibody and Dab2 protein levels were measured by histoscore analysis. Patients were separated into high (DAB2 histscore >40) and DAB2 low level groups and overall patient survival was assessed using univariate Cox and Kaplan-Meier survival analysis. Low level DAB2 protein expression correlates with poor survival (p=0.008)



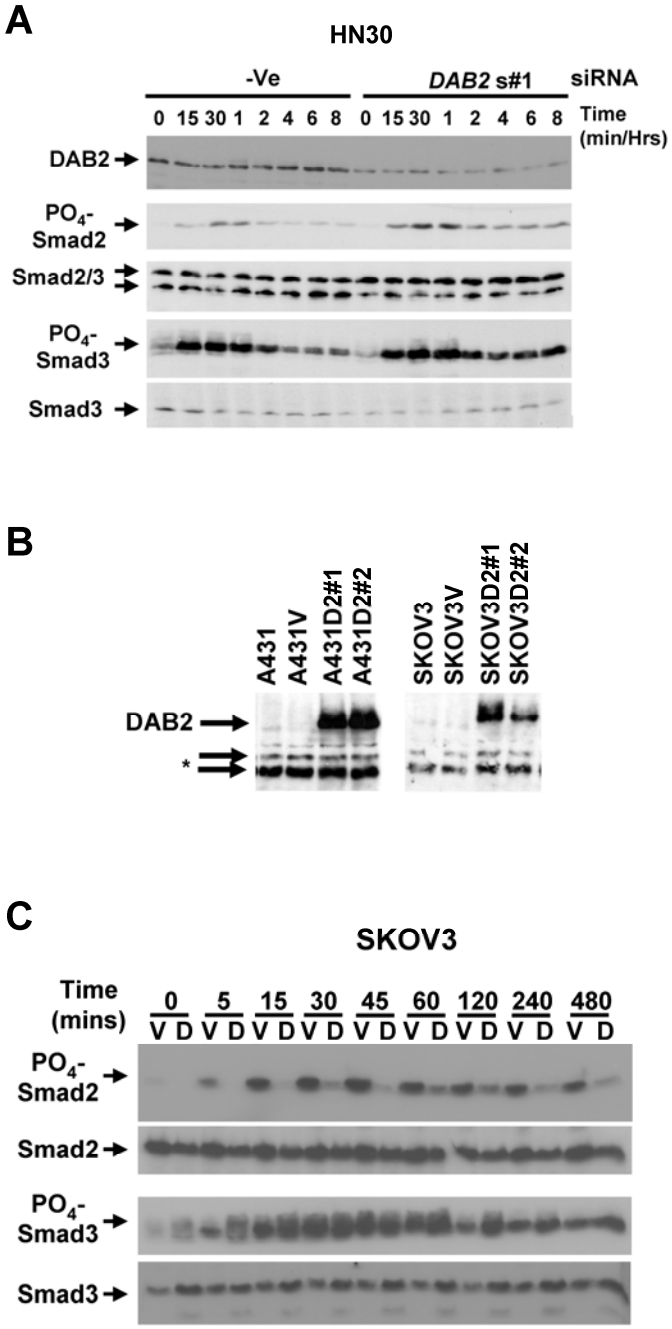
Supplemental Figure 5. Survival analysis of HNSCC patients. Microarray analyses of 68 primary HNSCC tumor samples were assessed for *TGF-β1*, (A) and *TGF-β3* (B) expression. Tumor samples were separated after automated discretisation into high and low level groups and overall patient survival was assessed using univariate cox and Kaplan-Meier survival analysis. High level expression of *TGF-β* isoforms indicates potential poorer survival (*TGF-β1* high average survival = 23.3 months, *TGF-β1* low average survival = 31.8 months, *TGF-β3* high average survival = 24.72 months, *TGF-β3* low average survival = 32.9 months) although this fails to reach statistical significance (Log-rank Chi-square=3.52, p=0.061 (A); Log-rank Chi-square=3.41, p=0.065 (B).



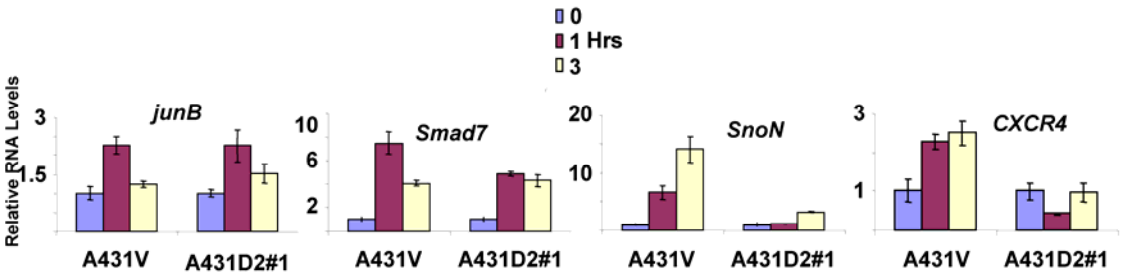
Supplemental Figure 6. Low level DAB2 expression does not preclude Smad2/3 nuclear accumulation. SCC cell lines were treated or not with 1ng/ml TGF-β for 1 hour prior to indirect immunofluorescence and confocal microscopy analysis utilising a Smad2/3 specific antibody. Low level DAB2 expressing cell lines are labelled in red.



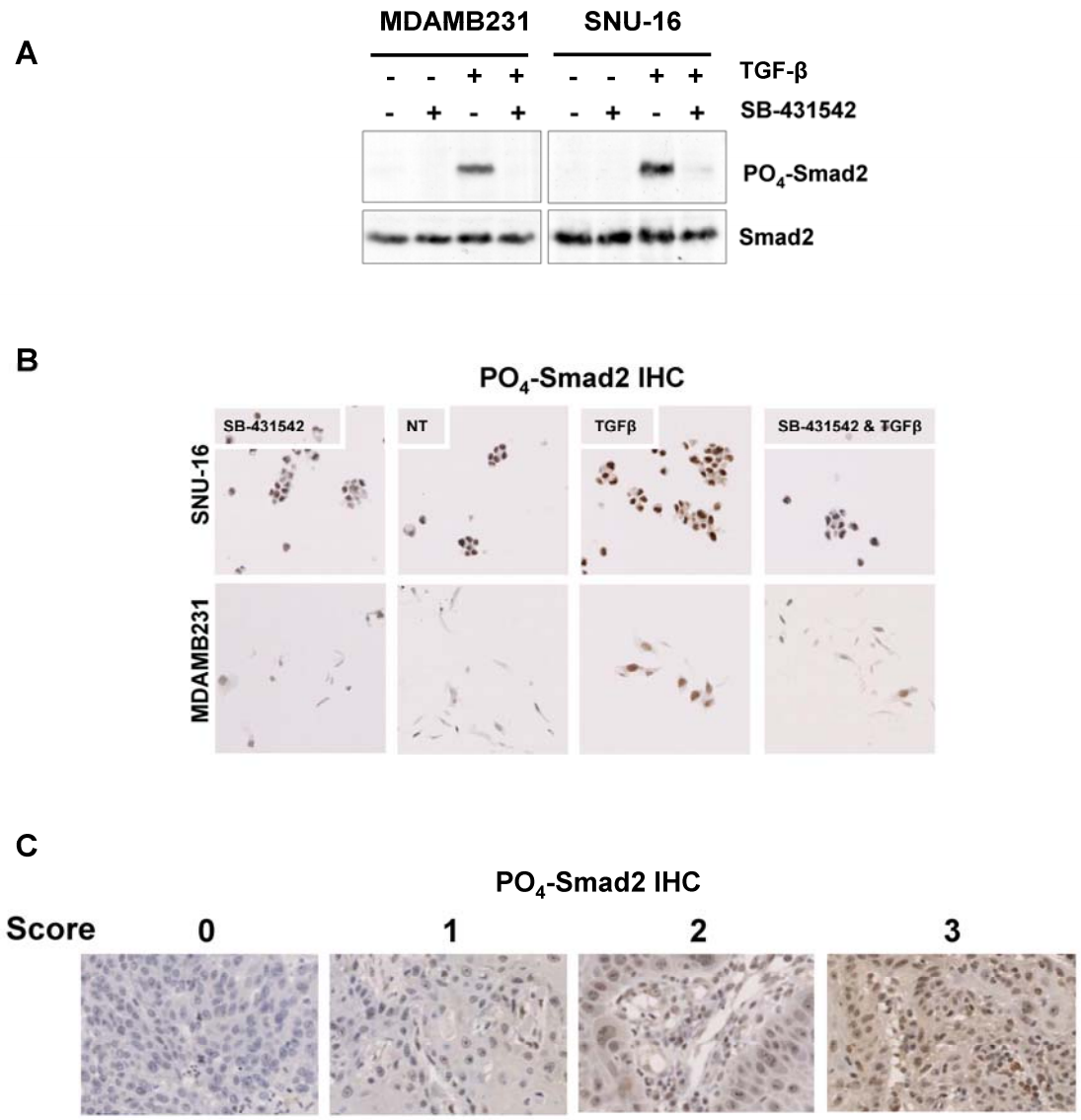
Supplemental Figure 7. Dab2 is not required for Smad2 and Smad3 activation. MEFs isolated from *Dab2*$^{(Fl/-)}$ knockout mice were infected with control (pBABEpuro) or Cre recombinase expressing (pBABE Cre) retroviruses. Cells were selected in puromycin for 3 days and then treated for the indicated time points with 1ng/ml TGF-β1 prior to harvesting and analysis by western blotting for Dab2, PO_4-Smad2 (P-Smad2), PO_4-Smad3 (P-Smad3), Smad2/3 and actin as a loading control.



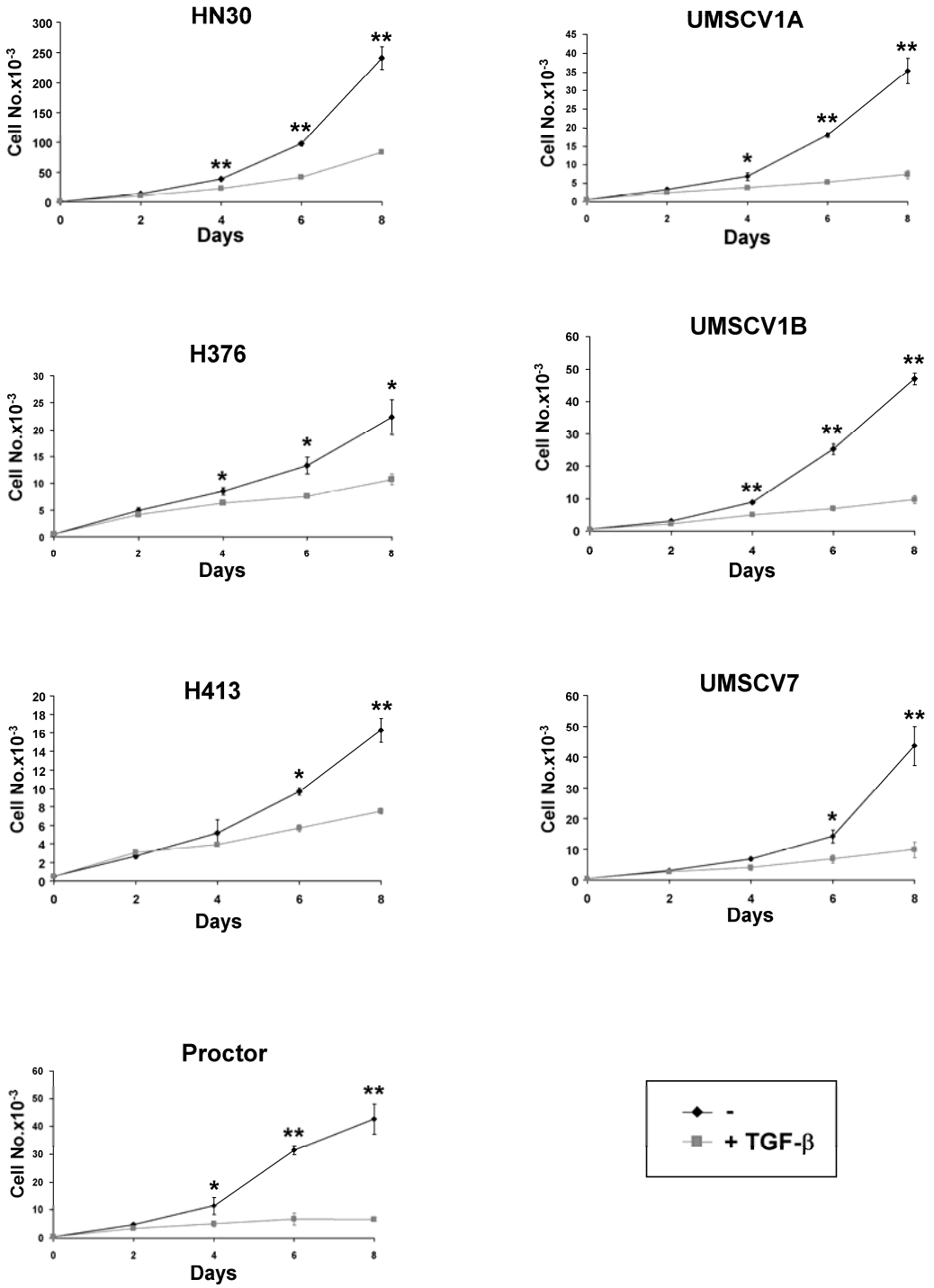
Supplemental Figure 8. DAB2 represses Smad2 activation. (A) siRNA mediated knockdown of DAB2 enhances TGF-β mediated Smad2 activation. Western blotting analysis of TGF-β mediated activation of Smad2 and Smad3 phosphorylation in HN30 cells following transient siRNA transfection with non-silencing control (-Ve) and *DAB2* specific siRNA (*DAB2*s#1) and TGF-β treatment (1ng/ml) for the indicated time points. Knockdown of Dab2 was assessed by Dab2 western blotting and analysis of phospho-Smad2 (PO_4-Smad2), phospho-Smad3 (PO_4-Smad3), total Smad2 and Smad3 levels are shown. (B) Stable re-expression of DAB2. A431 and SKOV3 cells were co-transfected with either pSG5 or pSG5FlagDAB2 plasmids and pTk-neo in a ratio of 10:1. Stable clones were selected in G418 and assessed for DAB2 expression by western blotting. One vector control (A431V, SKOV3V) and two DAB2 stable cell lines from each transfection (A431D2#1, A431D2#2, SKOV3D2#1, SKOV3D2#2), were selected for further analysis. (C) DAB2 stable re-expression inhibits TGF-β mediated Smad2 activation. Western blotting analysis of SKOV3 vector control (V) and DAB2 stable cell lines (SKOV3D2#1, (D)) analysed as in (A).



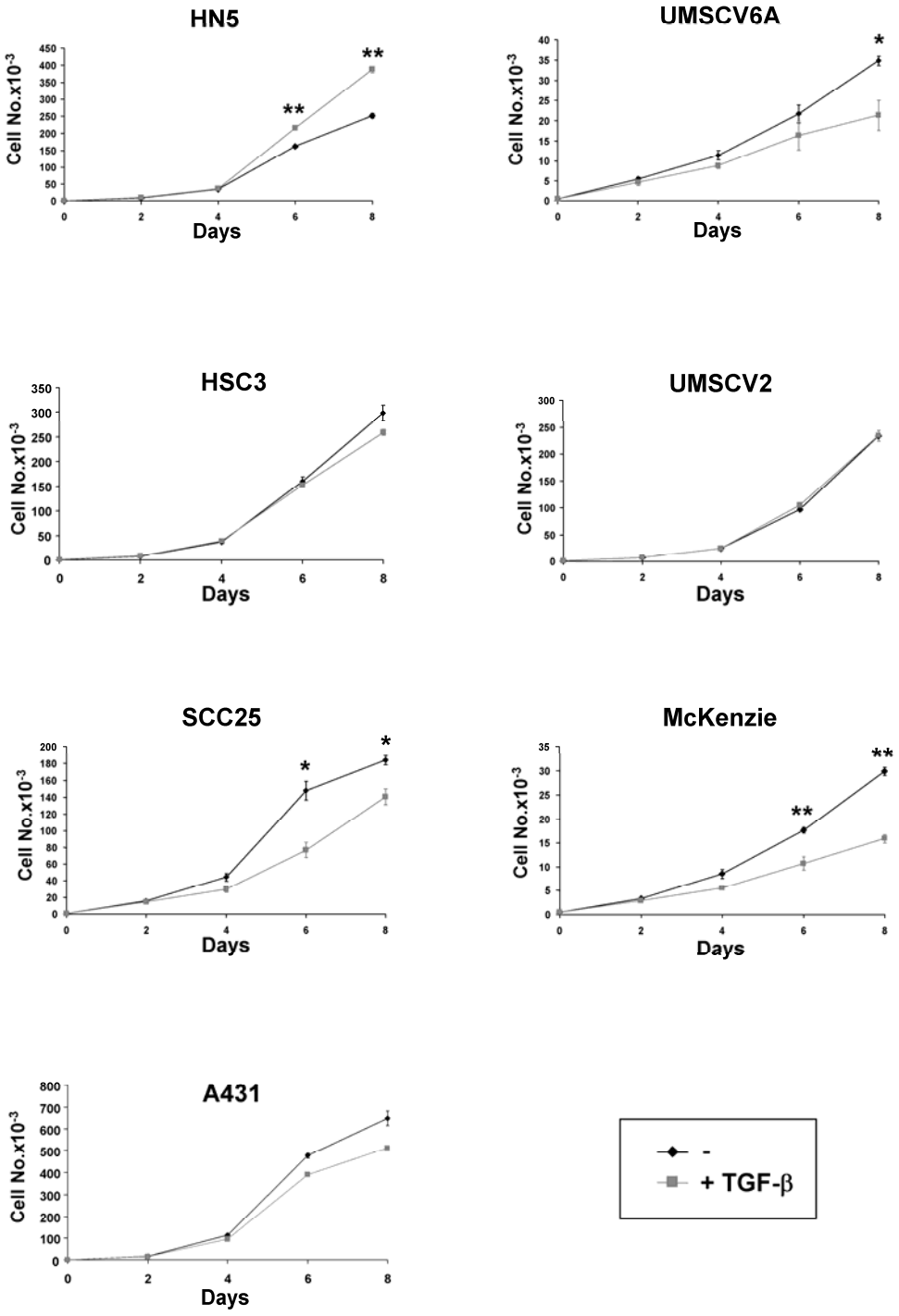
Supplemental Figure 9. DAB2 re-expression differentially regulates TGF-β mediated activation of target gene expression. qRT-PCR analysis of *junB*, *Smad7*, *SnoN* and *CXCR4* expression of A431 vector control and DAB2 stable cell lines treated for the indicated time points with 1ng/ml TGF-β. Data represent the means ± SD of triplicate determinations (n=2).



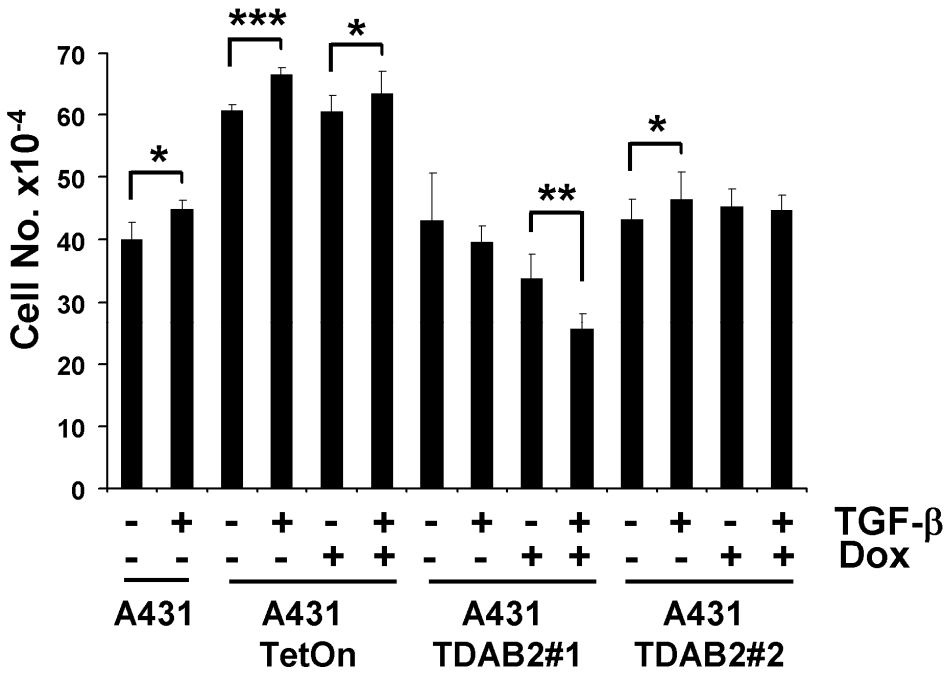
Supplemental Figure 10. **phospho-Smad2 immunohistochemistry.** (A) Western blot analysis of extracts of the indicated cell lines treated for 1 hour with or without 1ng/ml TGF-β without or without prior incubation for 15 minutes with 10μM SB-431542. Membranes were incubated with PO_4-Smad2 and Smad2 antibodies as shown. (B) Cells were treated as in (A), fixed in formalin and then were harvested by trypsinisation and centrifugation (MDAMB231) or centrifugation alone (SNU-11) and then embedded in paraffin. Sections of embedded cells were then incubated with the anti-phospho-Smad2 antibody and analysed by immunohistochemistry. Cells treated with TGF-β show brown nuclear staining whilst those treated with SB-431542 do not. (C) A HNSCC TMA was stained as in (B). Examples of high magnification regions of tumor cores exhibiting tumor cells with absent (0), weak (1), moderate (2) or strong (3) staining are shown.



Supplemental Figure 11. TGF-β induces proliferation arrest in cell lines which express "normal levels" of DAB2. Cell proliferation curves of the indicated cell lines treated or not as indicated with 1ng/ml TGF-β. Data represent the means + SD, n=3. Statistical analyses were performed with paired 2 tailed t-tests, *=p<0.05, **=p<0.01.



Supplemental Figure 12. Cell lines which express "low levels" of DAB2 are largely refractory to TGF-β mediated proliferation arrest. Cell proliferation curves of the indicated cell lines treated or not as indicated with 1ng/ml TGF-β. Data represent the means + SD, n=3. Statistical analyses were performed with paired 2 tailed t-tests, *=p<0.05, **=p<0.01.



Supplemental Figure 13. High level DAB2 expression partially restores TGF-β mediated inhibition of proliferation. Cell lines described in Figure 7 were seeded at $4x10^3$ per well and then treated or not with 0.1ng/ml TGF-β and/or 1μg/ml doxycycline for 6 days followed by cell counting. Data represent the means + SD, n=3. Statistical analyses were performed with paired 2 tailed t-tests, *=p<0.05, **=p<0.01, ***=p<0.001.

	DAB2 expression		*DAB2* promoter methylation		*DAB2* re-expression		
	Western blot	qRT-PCR	sequencing	MSP	5-AZA	TSA	5-AZA + TSA
HSC3	-	low	high	M/M	↑↑	↑↑↑↑	↑↑↑
Delve	-	nd	high	nd	-	↓	↑
A431	-	v.low	v.low	nd	↑↑	-	↑↑
UMSCV2	-	v.low	low	nd	-	↑↑	↑↑↑
HN5	+/-	low	nd	U/M	↑↑	↑	↑↑↑
SCC25	+/-	low	none	nd	↓	↓	↑↑↑
UMSCV6A	+	low	high	U/M	-	-	-
McKenzie	+	low	med	U/M	↓	↓	-
H376	++	nd	nd	U/U	nd	nd	nd
H413	++	med	nd	nd	nd	nd	nd
Proctor	++	nd	nd	nd	nd	nd	nd
UMSCV1A	+++	nd	none	U/U	nd	nd	nd
HN76	+++	nd	nd	U/U	nd	nd	nd
UMSCV7	+++	med	none	U/U	nd	nd	nd
UMSCV1B	+++	med	none	U/U	nd	nd	nd
HN30	+++	high	v.low	U/U	nd	nd	nd

Supplemental Table 1. *DAB2* is epigenetically downregulated in SCC cell lines. Assessment of DAB2 expression by western blotting and qRT-PCR. Med = medium, v.low = very low. *DAB2* promoter methylation was determined by bisulphite sequencing (sequencing), high = high level of methylation. *DAB2* promoter methylation was also assessed by MSP (methylation sensitive PCR), U = unmethylated, M = methylated. *DAB2* re-expression was measured by qRT-PCR following treatment with 5-azacytidine (5-AZA), trichostatin A (TSA) or combination. Arrows indicate the direction and degree of change of expression. Nd = not determined throughout.