THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

doi: 10.1387/ijdb.113374sm

SUPPLEMENTARY MATERIAL

corresponding to:

JNK activity supports multiple phases of 3D-mammary epithelial acinus formation

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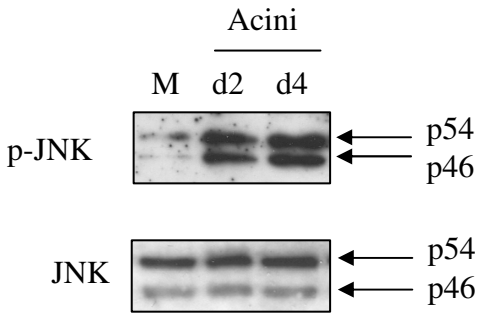
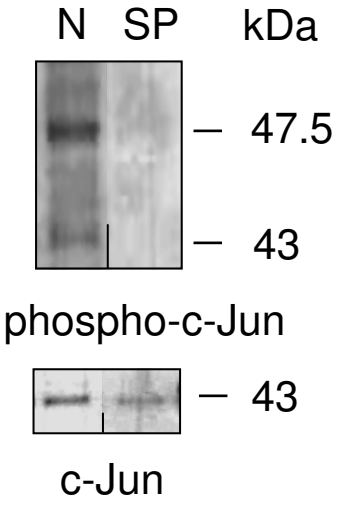
Detection of JNK activity during acinus formation by primary MECs

Supplementary data - 1

i



ii



Failure of acinus formation by primary MECs when JNK is inhibited by SP600125 is accompanied by loss of JNK dependent c-Jun phosphorylation. Western analysis

Figure S1

Markers of EMT expressed in 3-D structures exposed to SP600125

Suppressed occludin expression and localisation

Induced occupation of E-boxes in occludin promoter by Snail1; and loss of AP-1 occupation;

Elevated fibronectin expression

Depressed E-cadherin and increased N-cadherin expression

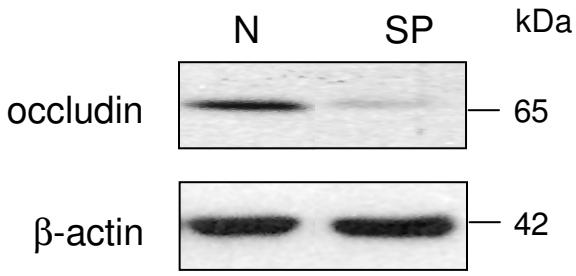
Increased MMP-2 and -9 expression; and Snail1 occupation of MMP promoters; and

In monolayer culture, increased nucleus-to-nucleus distance, transition to mesenchymal phenotype, and induction of focal adhesions at wound borders.

That inhibition of JNK precipitates changes reminiscent of EMT was confirmed by detecting the appearance of a comprehensive range of markers of EMT. Figs S2a - e.

Supplementary data - 2

i



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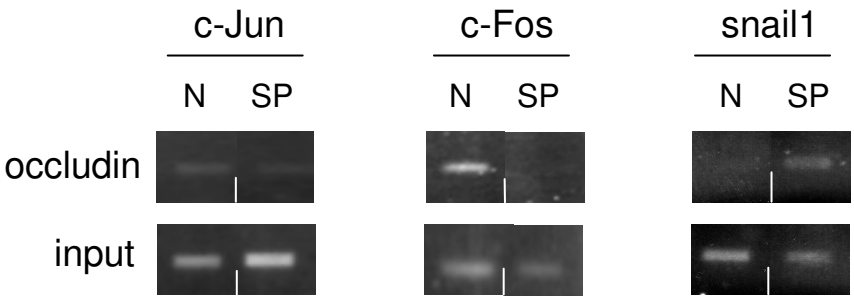
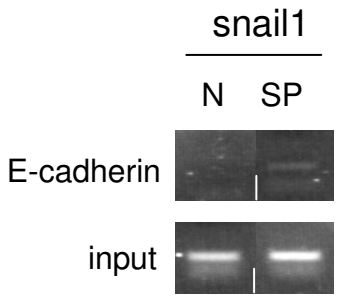


Failure of acinus formation by primary MECs when JNK is inhibited by SP600125 is accompanied by loss of occludin expression [western analysis, [i]]; and, loss of c-Jun and c-Fos occupation of the AP-1 binding element in the proximal promoter of the occludin promoter with reciprocal increase in snail biding to E-boxes in this promoter [ChIP analysis, [ii]].

Figure S2a

i



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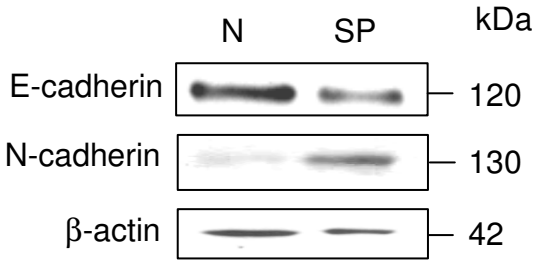
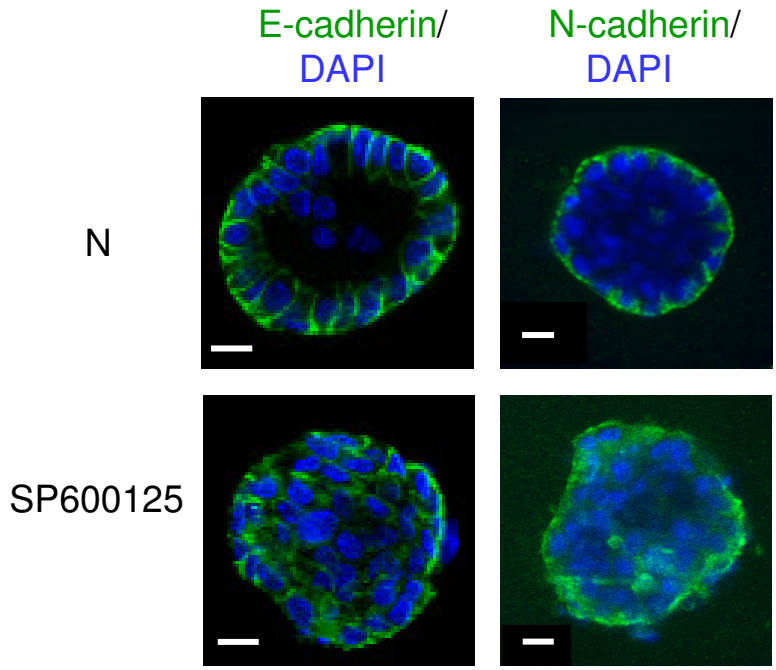
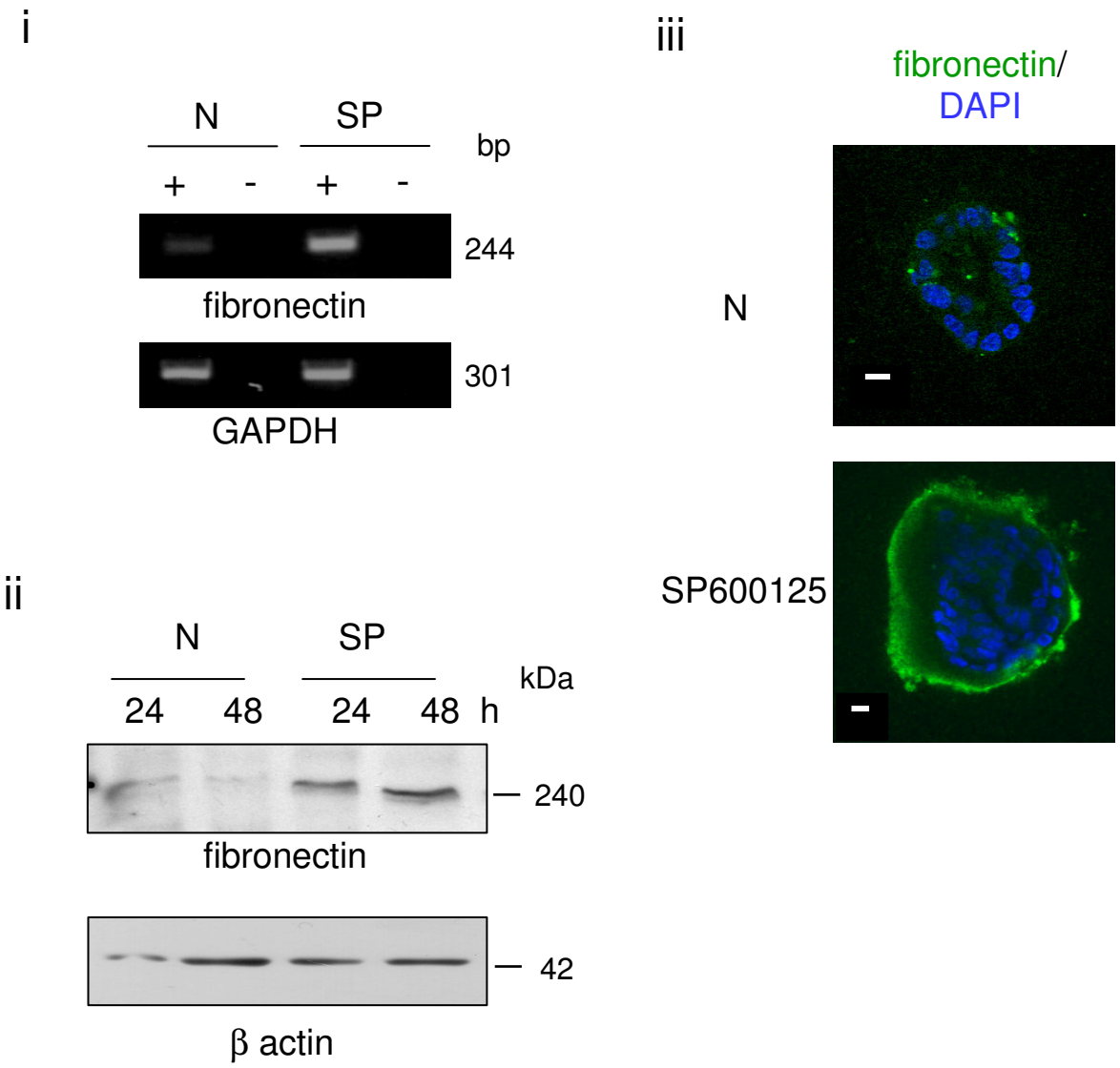


iii



Failure of acinus formation by primary MECs, when JNK is inhibited by SP600125, is accompanied by an increase in snail biding to E-boxes in the E-cadherin promoter [ChIP analysis, [i]], by loss of E-cadherin and gain of N-cadherin expression [western analysis, [ii]]; and, disruption of polarised E-cadherin distribution [immunefluorescence confocal microscopy].

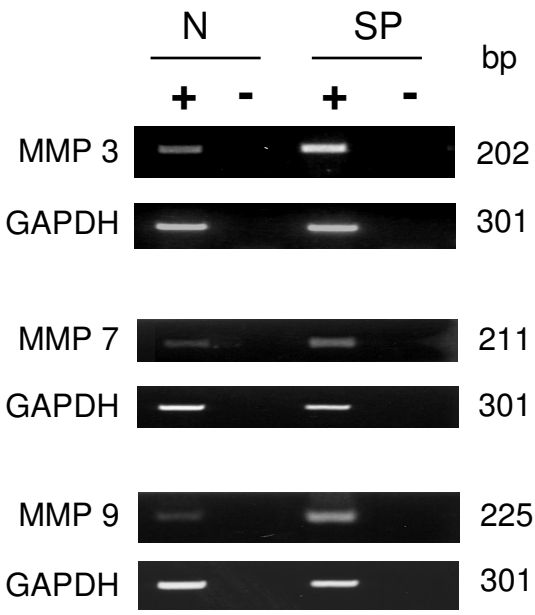
Figure S2b



Failure of acinus formation by primary MECs, when JNK is inhibited by SP600125, is accompanied by an increase in levels of expression of fibronectin mRNA [RT-PCR analysis, [i]] and fibronectin protein [western analysis, [ii] and immunefluorescence confocal microscopy, [iii]].

Figure S2c

i



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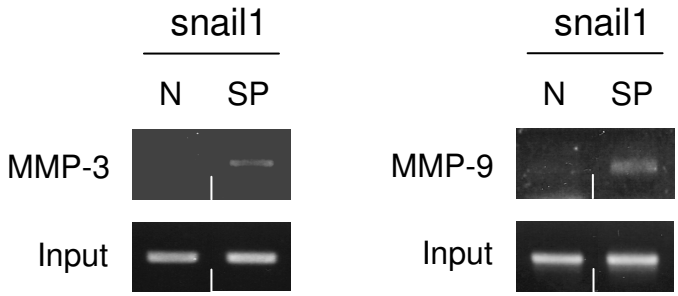
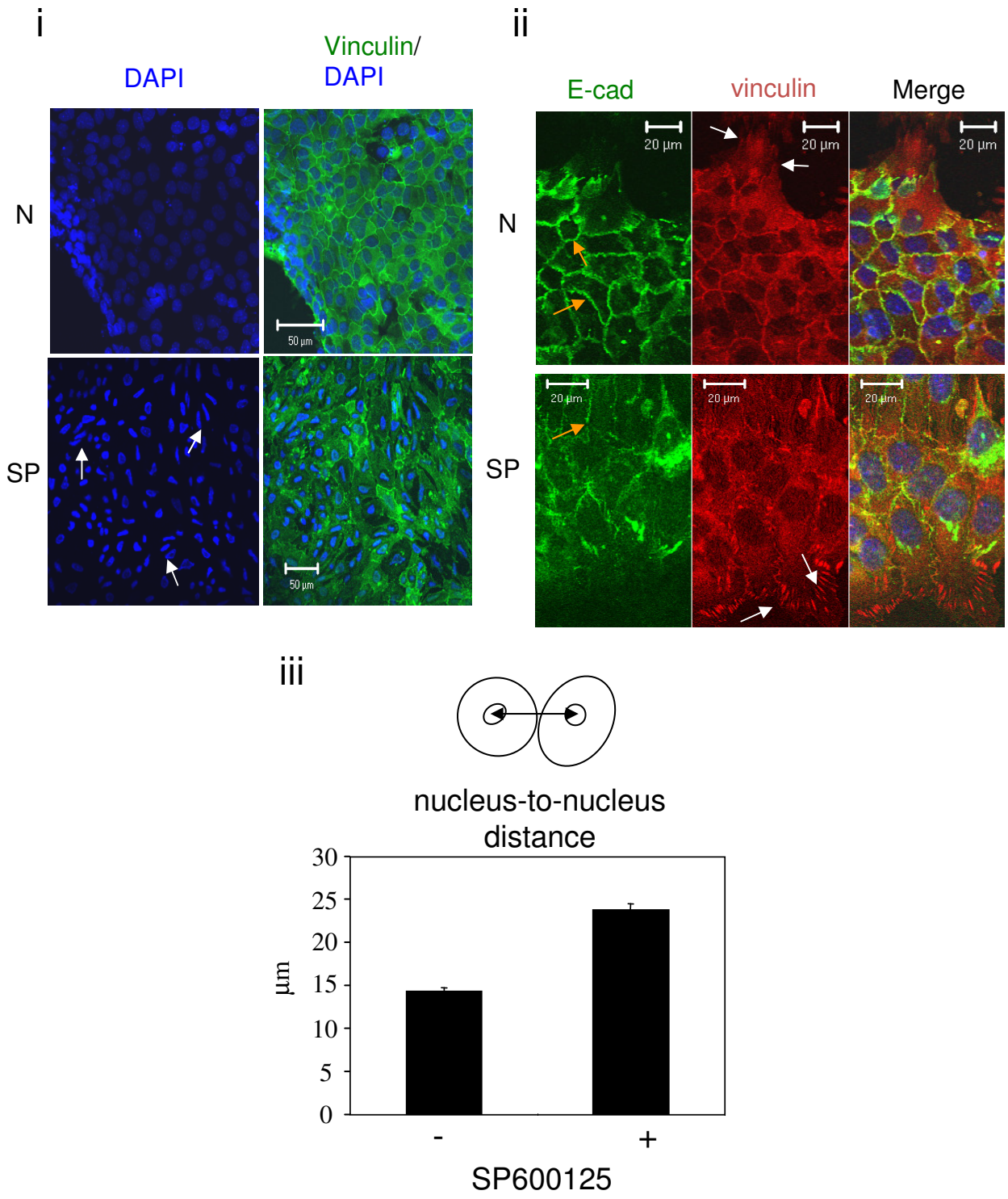


Failure of acinus formation by primary MECs, when JNK is inhibited by SP600125, is accompanied by increased MMP-3, -7 and -9 mRNA expression [RT-PCR analysis, [i]], and increase in snail biding to E-boxes MMP-3 and -9 proximal promoters [ChIP analysis, [ii]].

Figure S2d

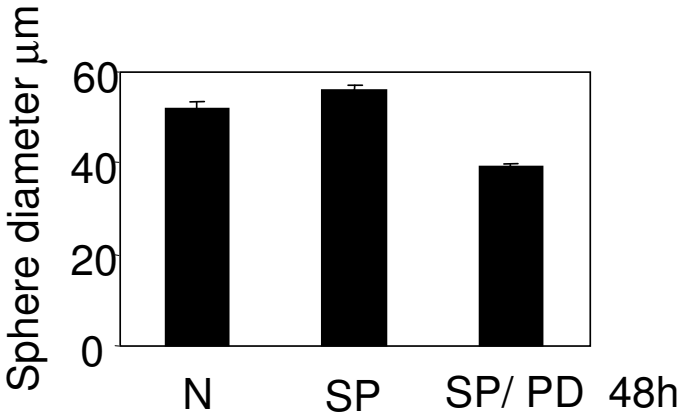


MCEs cultured as monolayers on dilute EHS-ECM, serum starved and scratch-wounded, in the presence of the JNK inhibitor, SP600125 display a 'metastable' cell phenotype with elongated nuclei [i], lose E-cadherin expression and show abundant focal adhesions at their wounded edge [ii], and show significantly increased 'nucleus-to-nucleus' distance, relative to controls [iii].

Figure S2e

Inhibition of ERK signalling during acinus formation protects from the effects of JNK inhibition

Supplementary data - 3

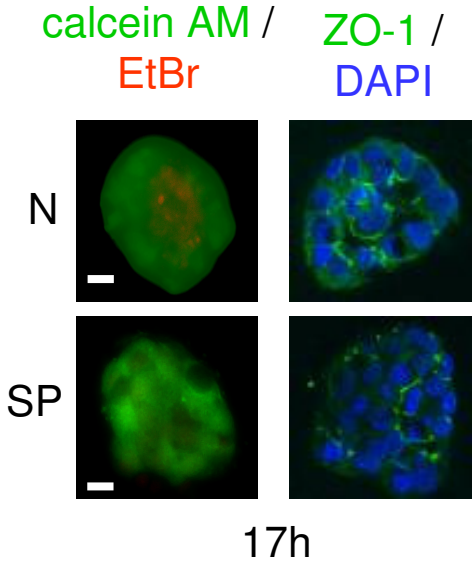


Inhibition of ERK signalling during acinus formation protects from the effects of JNK inhibition [See Fig 2a-e] but reduces acinus size. Mean sphere diameter in multiple fields, in independent experiments, was measured using the con-focal microscope; results are expressed as mean $\pm$ sem, n = 4.

Figure S3

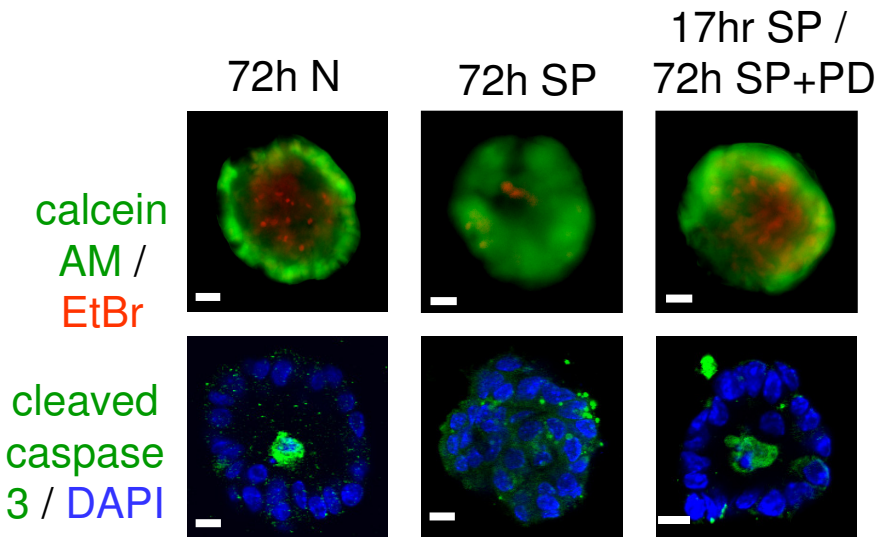
Inhibition of ERK signalling during acinus formation can reverse the effects of JNK inhibition

Supplementary data - 4



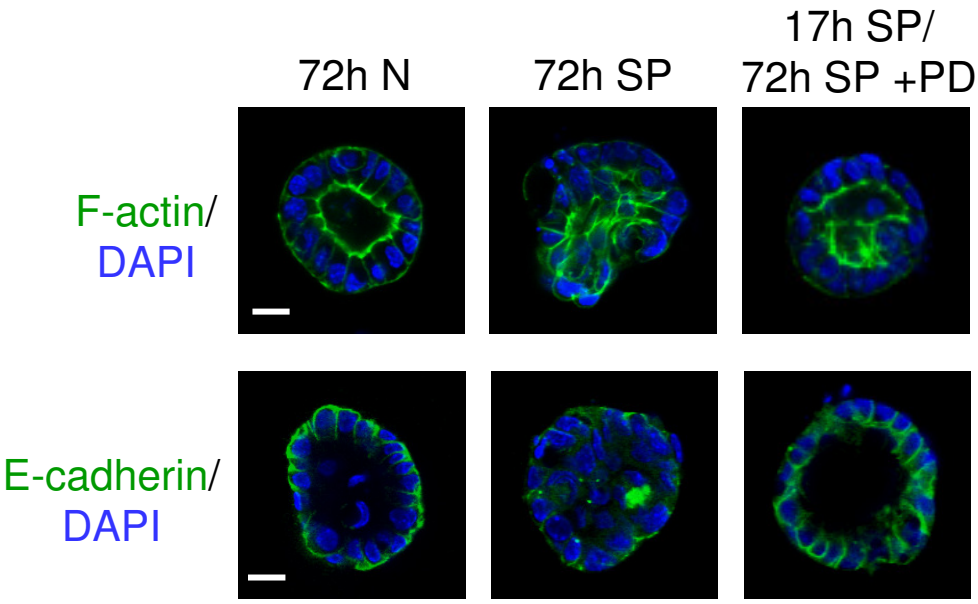
The effects of JNK inhibition, using SP600125, on acinus formation by primary MECs are detactable 17h after cell seeding on EHS ECM: *left-hand panels,* luminal cell death [vital dye staining with EtBr, orange] is suppressed; and, *right-hand panels*, polarised baso-lateral E-cadherin [green] distribution is impaired [confocal immunefluorescence microscopy]. In the following series of analyses the ERK pathway inhibitor, PD98059, was added 17h after cell seeding in order to demonstrate its ability to reverse the effects of JNK inhibition [by Sp600125 added at time of cell seeding.

Figure S4a



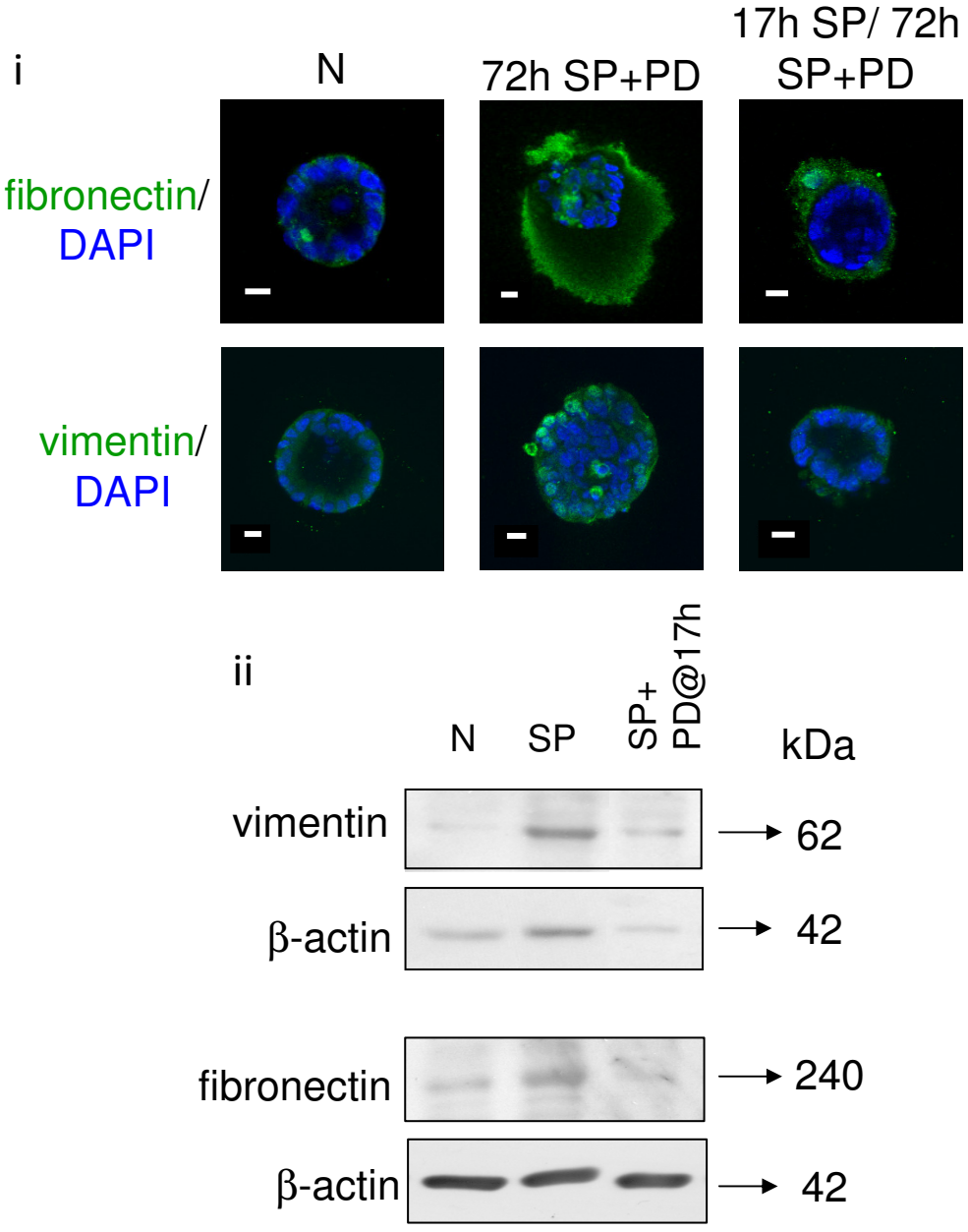
The effects of JNK inhibition, using SP600125, on acinus formation by primary MECs are reversed by addition of the ERK pathway inhibitor, PD98059, 17h after cell seeding on EHS ECM: *top panels,* luminal cell death [vital dye staining with EtBr, orange] is reinitiated; and, *bottom panels*, luminal cell apoptosis, reflected by cleaved caspase 3 staining [green] is again detectable [at 72h] [confocal immunefluorescence microscopy].

Figure S4b



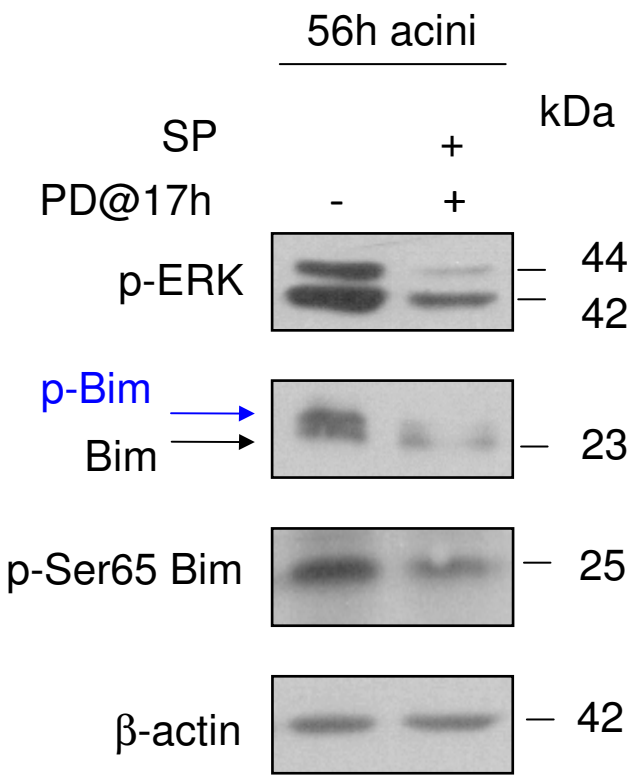
The effects of JNK inhibition, using SP600125, on acinus formation by primary MECs are reversed by addition of the ERK pathway inhibitor, PD98059, 17h after cell seeding on EHS ECM: polarised apical distribution of F-actin [*top panels,* green] and, baso-lateral E-cadherin staining [*bottom panels*, green] is re-established [at 72h] [confocal immunefluorescence microscopy].

Figure S4c



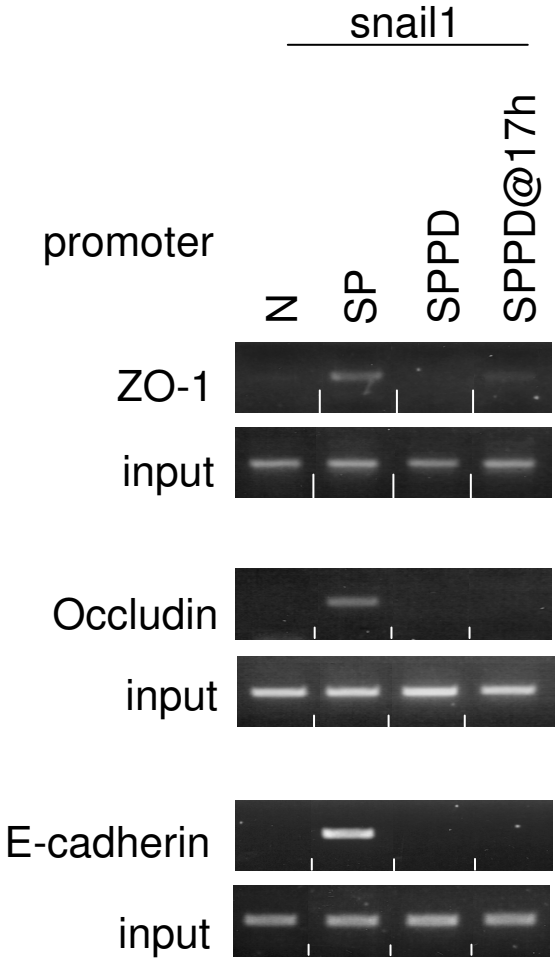
The effects of JNK inhibition, using SP600125, on acinus formation by primary MECs are reversed by addition of the ERK pathway inhibitor, PD98059, 17h after cell seeding on EHS ECM: expression of EMT markers, fibronectin and vimentin is suppressed [at 72h] [*top panels,* confocal immunefluorescence microscopy] and, [*bottom panels*, western analysis].

Figure S4d



The effects of JNK inhibition, using SP600125, on acinus formation by primary MECs are reversed by addition of the ERK pathway inhibitor, PD98059, 17h after cell seeding on EHS ECM: ERK phosphorylation / activation and ERK-dependent Bim phosphorylation [as reflected both by 'band shift' [*2nd panel*] and anti-phospho-Bim antibody [*3rd panel*] is suppressed [at 72h] [western analysis].

Figure S4e



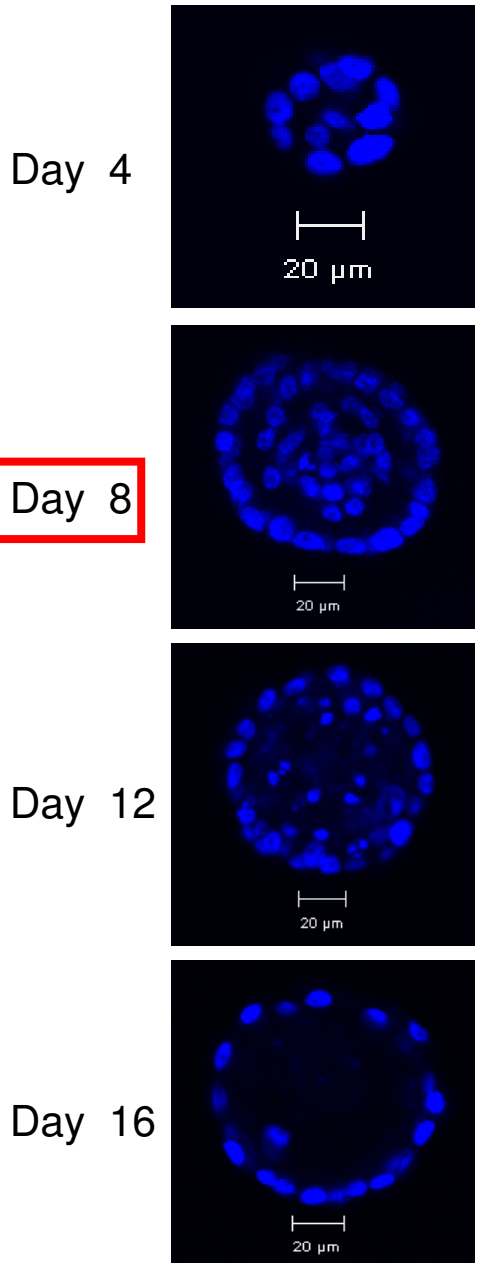
The effects of JNK inhibition, using SP600125, on acinus formation by primary MECs are reversed by addition of the ERK pathway inhibitor, PD98059, 17h after cell seeding on EHS ECM: Snail occupation of E-boxes in the proximal promoters of the ZO-1, occludin and E-cadherin genes is suppressed [at 72h] [ChIP analysis]. Here the effect of PD98059 added at time of seeding and 17h thereafter was studied.

Figure S4f

Effect of TGFβ on MCF10A acinus formation

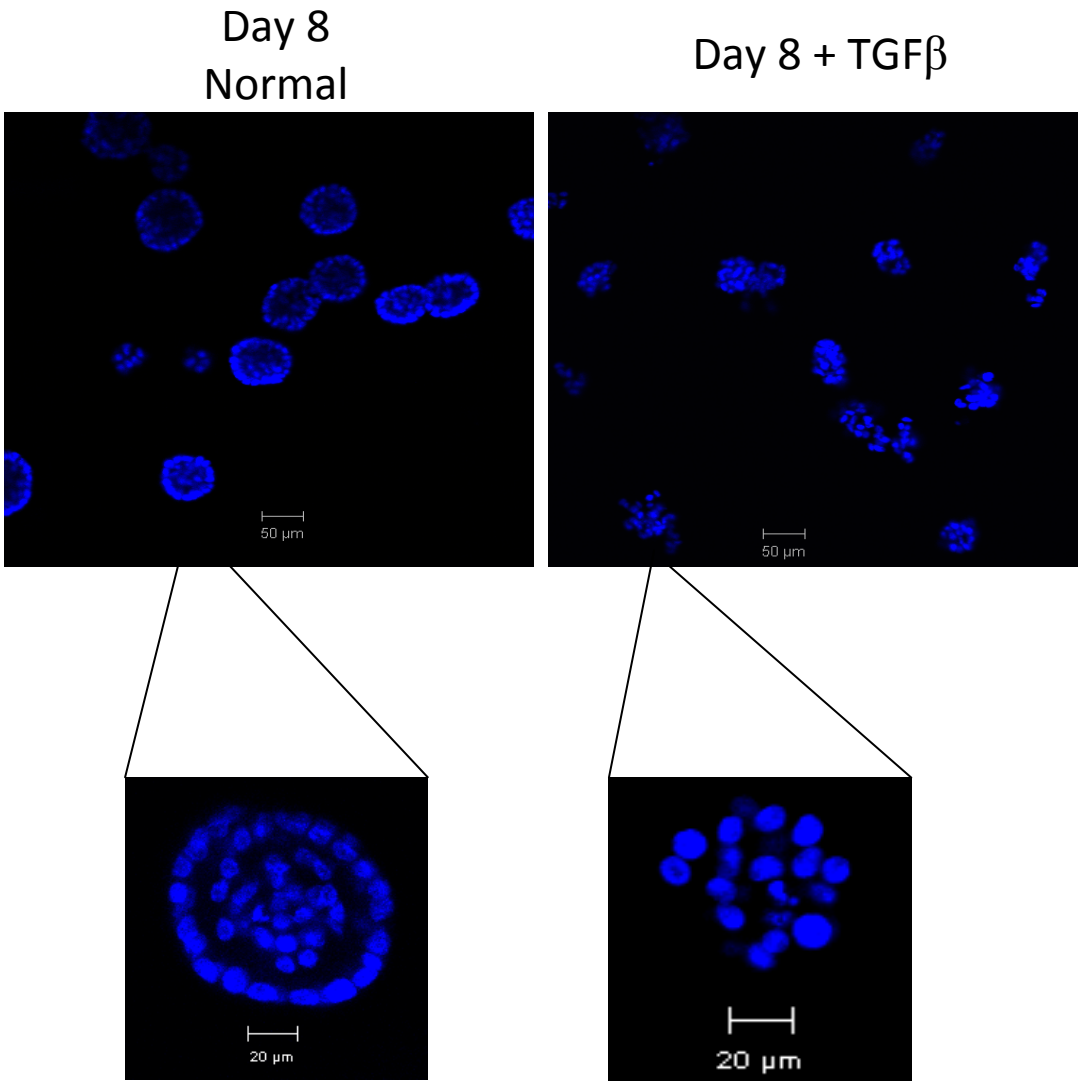
Supplementary data - 5

MCF10A



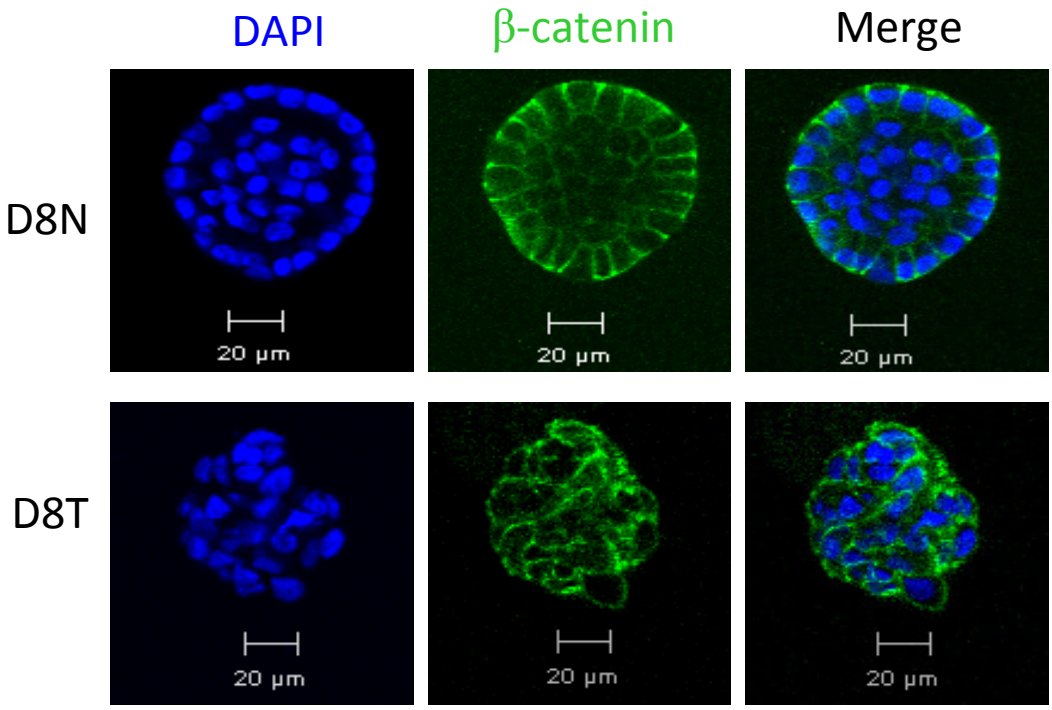
Growth pattern of MCF10A acini: Note that on day 8 of culture two sub-populations of cells are clearly seen: 1. Well ordered cells in intimate contact with the surrounding matrix; and, 2. Disordered luminal cells that will die, and be removed to generate the empty lumen. We have chosen to concentrate our analyses on MCF10A acini at day 8

Figure S5a



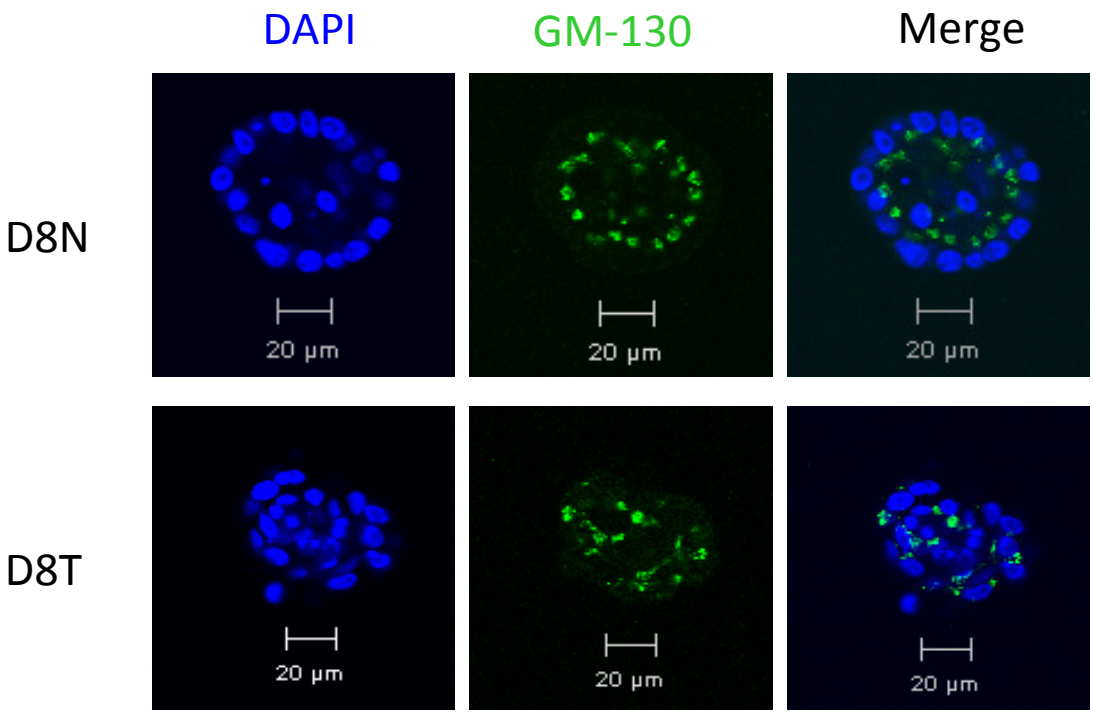
Effect of TGFβ on MCF10A acinus formation. MCF-10A acini cultured to day 8 in the absence or presence of TGFβ (5ng/ml). DAPI (blue) tracks acinus organisation. [confocal fluorescence microscopy]

Figure S5b



β-catenin fails to localise to the basolateral membrane of cells in MCF-10A acini cultured in the presence of TGFβ. Confocal fluorescence microscopy sections of MCF-10A acini cultured to day 8 in the absence (N) or presence (T) of TGFβ (5ng/ml). Acini were stained with anti- β-catenin antibody (green) and counterstained with DAPI (blue).

Figure S5c

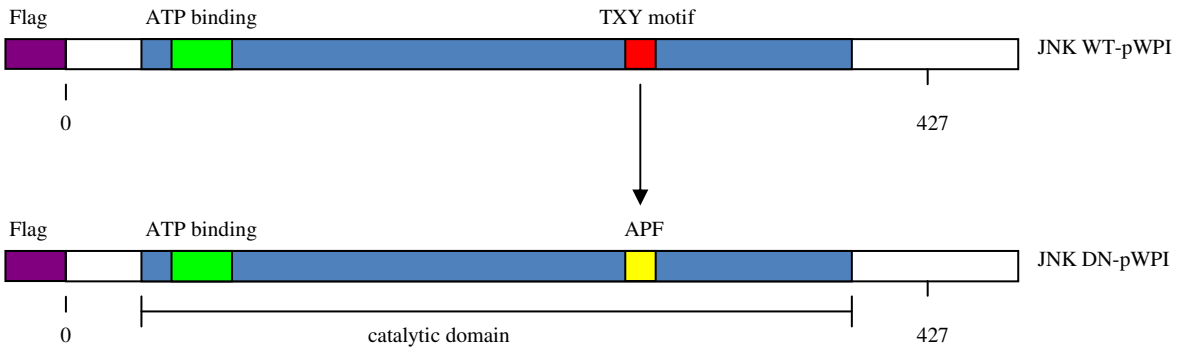


The GM-130, golgi marker, fails to localise to the apical surface of cells in structures treated with TGFβ (T). Confocal fluorescence microscopy sections of MCF-10A acini cultured to day 8 in the absence (N) or presence (T) of TGFβ (5ng/ml). Acini were stained with anti-GM-130 antibody (green) and counterstained with DAPI (blue).

Figure S5d

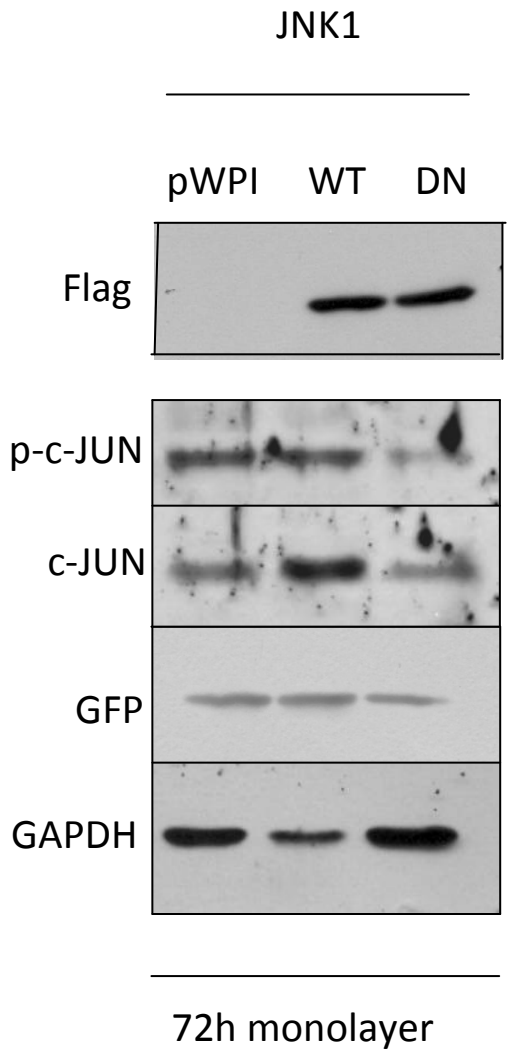
Validation of action of JNK1 dominant negative mutant protein in MCF10A cells cultured as a monolayer

Supplementary data - 6



Schematic of JNK constructs used for lentiviral infection of MCF10A cells showing the Protein kinase domain 26-321 (Ser/Thr protein kinases catalytic domain); ATP binding region 32-40, and TXY motif 183-185. MAP kinases are activated by dual phosphorylation within protein the kinase domain and JNK is activated by dual phosphorylation on sites Thr-183 and Tyr-185. The JNK DN construct used in this study has replaced the dual phosphorylation motif THr(183)-Pro-Tyr(185) with Ala-Pro-Phe which is incapable of being activated.

Figure S6a



Validation of action of over-expressed dominant negative JNK1 in MCF10A cells cultured as a monolayer. Over-expression of the JNK1 DN suppresses JNK-dependent c-Jun phosphorylation.

Figure S6b

Supplementary Materials and Methods

Supplementary data - 7

(I) Antibodies used in Western blot analysis

Antibodies used in western blot analysis were against JNK, ERK, phospho-ERK(thr-202/tyr-204), Src, phospho-Src(tyr-416), paxillin, phospho-paxillin(tyr-118) and lamin A-C, Tubulin, phospho-cjun (ser63) (Cell Signalling Technology), ZO-1, phospho-Bim(ser-65) and GAPDH (Chemicon), occludin, E-cadherin, N-cadherin and fibronectin (BD Transduction Laboratories), β-actin, vimentin, vinculin and BimEL (Sigma-Aldrich), phospho-EGFR(tyr-1173) and cjun (Abcam), EGFR, FAK, MKP-1, MKP-2 and MKP-3 (Santa Cruz Biotechnology Inc.) and phospho-FAK(tyr-397) (Biosource), were used according to the manufacturer's recommendations.

(II) The primary staining antibodies used were against: cyclin D1 and ki67 (Santa Cruz Biotechnology Inc.), GM130, β-catenin, ZO-1 (Chemicon), E-cadherin, N-cadherin and fibronectin (BD Transduction Laboratories), vimentin and vinculin (Sigma-Aldrich), cleaved caspase 3, phospho-Src(Y416) and phospho-Pax (Y118) (Cell Signaling Technology), active β1-integrin (BD Pharmingen), pFak (Y407 and Y397) (upstate-Millipore) and EGFR (Novus Biologicals).

(III) measure of nucleus to nucleus distance

Nucleus-to-nucleus distance was measured using the Zeiss LSM 510 Meta laser scanning confocal microscope; 40X high magnification images were analysed using Zeiss LSM software to find the distance from the geometrical centre of nuclei in adjacent cells. More than 50 distances were measured for each treatment in three independent cell preparations. Only cells in contact with each other were selected for analysis and adjacent cell pairs were chosen randomly. Once all nucleus-to-nucleus distances were compiled, average distance (per experiment) and standard error of the mean (SEM) for each experimental condition was calculated using GraphPad software. An unpaired student's t-test was used to analyse the statistical significance of differences between treatments.

Figure S7a

Figure S7b: ***Organisation of gene proximal promoters in terms of transcription factor binding sites.***

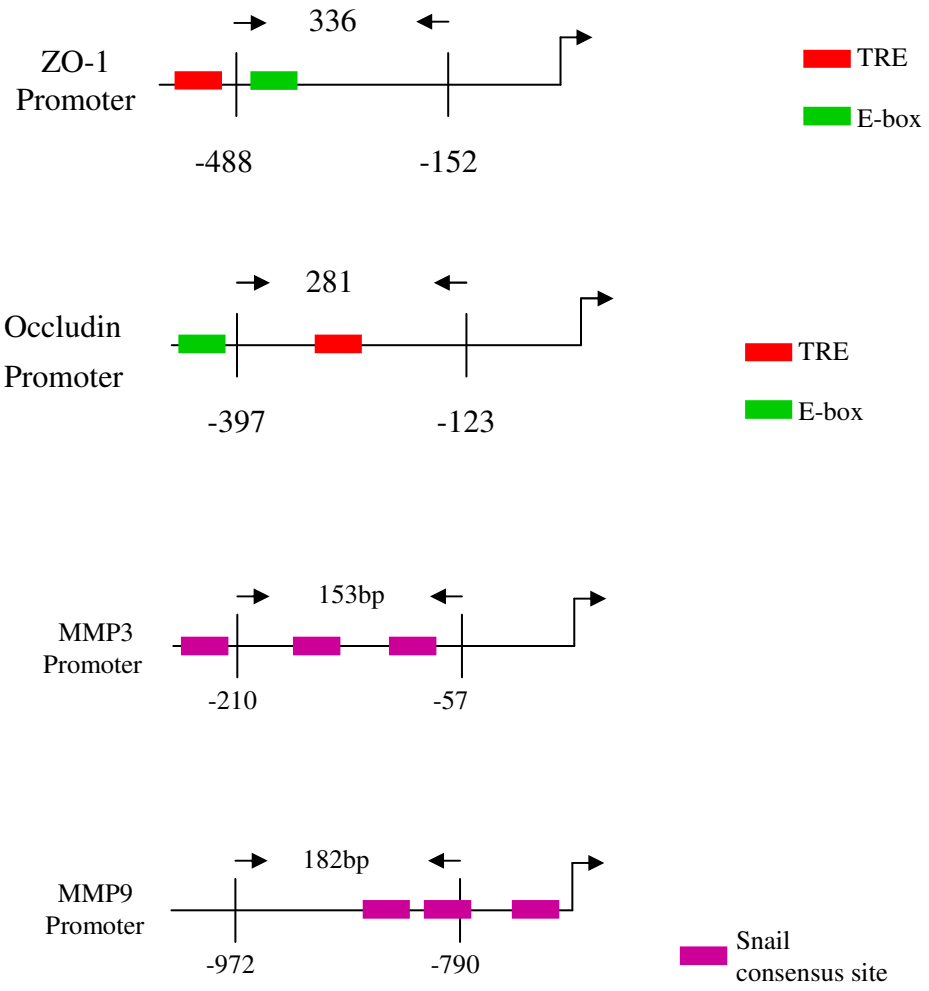
Investigation of ZO-1, Occludin, β4-Integrin, E-cadherin and Cyclin D1gene promoters from up to 5000bp upstream of the transcriptional start site. Table includes, gene promoter under investigation, family/matrix of promoter binding factor, further +/-strand binding, the core similarity of the sequence, the matrix similarity to the sequence and finally the nucleotide binding sequence itself, where red denotes the suggested binding sequence and capital letters indicating the core consensus binding site of the particular binding factor. Data was obtained from Matinspector.

Gene Promoter	Family/Matrix	Further Information	Position from - to	S	Core sim.	Matrix sim.	Sequence (red: ci-value >60 capitals: core seq.)
ZO-1	AP1F/AP1	Activator Protein 1	2047-2057	-	1.000	0.874	gggGAGTcaggt
	AP1F/AP1	Activator Protein 1	2574-2584	+	1.000	0.884	aatGAGTGagt
	AP1F/AP1	Activator Protein 1	3405-3415	-	1.000	0.918	tttGAGTaacc
	EBOX/ATF6	Member of b-zip family	3206 - 3218	-	1.000	0.945	ccaCCACgcccgg
	EBOX/NMYC	N-Myc	4333 - 4345	+	1.000	0.930	attccgCGTGtcc
	EBOX/USF	Upstream stimulating factor 1/2	4599 - 4611	+	0.914	0.942	gagaCACAtggtg
	EBOX/MYCMAX	MYC-MAX binding sites	4843 - 4855	-	1.000	0.911	ctgccgCGCGatc
Occludin	AP1F/AP1	Activator protein 1	263 - 273	+	1.000	0.987	tgtgACTCaca
	AP1F/AP1	Activator protein 1	263 - 273	-	1.000	0.987	tgtgAGTCaca

Continued……..

Figure S7b: ***Organisation of gene proximal promoters in terms of transcription factor binding sites. [continued]***

Gene promoter	Family/Matrix	Further Information	Position from - to	S	Core sim.	Matrix sim.	Sequence (red: ci-value >60 capitals: core seq.)
E-cadherin	EBOX/MYCMAX	c-Myc/Max heterodimer	945-957	+	0.860	0.930	caaccaCATTGgta
	EBOX/MYCMAX	c-Myc/Max heterodimer	946-958	-	0.750	0.861	ctacCATGtggtt
	EBOX/NMYC	N-Myc	1596-1608	+	1.000	0.965	gtggcaCGTTGcgt
	EBOX/NMYC	N-Myc	1597-1609	-	1.000	0.981	aacgcaCGTGcca
MMP3	EBOX/MYCMAX	MYC-MAX binding sites	1888-1900	+	0.877	0.923	atgccaTGTGaca
	EBOX/USF	Upstream stimulating factor 1/2	1889-1901	-	0.914	0.955	atgtCACAtggca
	EBOX/USF	Upstream stimulating factor	2282-2294	+	1.000	0.915	tactctCGTGact
MMP9	EBOX/NMYC	N-Myc	630-642	+	1.000	0.978	ttcacaCGTGagc
	EBOX/NMYC	N-Myc	631-643	-	1.000	0.977	tgctcaCGTGtga
	EBOX/USF	Upstream stimulating factor 1/2	1014-1026	+	0.829	0.901	ctgaCACCtgagg
	EBOX/MYCMAX	c-Myc/Max heterodimer	3890-3902	+	0.860	0.920	gaagcaCATGaag
	EBOX/ATF6	Member of b-zip family	3965-3977	-	1.000	0.930	ctgCCACcatggt



PCR strategies for ChIP analyses

Figure S7c

Gene	Sequence	Product size
Occludin Promoter	Rev: CTGCATCCAAGGGTCCCT	281bp
	Fwd: CGAGCACACCCAAAATGG	
ZO-1 Promoter	Rev: CTGTCGCCTAAGGAAAGA	336bp
	Fwd: TTAAGGCAGTCGGTTTGTCC	
E-cadherin Promoter	Rev: AAACAAACAGGATGGATCGC	154bp
	Fwd: CATGCTGGGCTACATAGCAA	

PCR primers used in ChIP analyses

Figure S7d

Gene	Sequence	Product size
Vimentin	Fwd: ATGCTTCTCTGGCACGTCTT	206bp
	Rev: AGCCACGCTTTCATACTGCT	
GAPDH	Fwd: ACCACAGTCCATGCCATCAC	301bp
	Rev: TCCACCACCCTGTTGCTG TA	
	Rev: TGGATGTGCATCTTCAGAG	
Fibronectin	Fwd: AATGGAAAAGGGGAATGGAC	244bp
	Rev: CTCGGTTGTCCTTCTTGCTC	
MMP1	Fwd: ATCCTGGCCACCTTCTTCTT	202bp
	Rev: TTTCTCGGAGCCTGTCAACT	
	Rev: CAGGTCAGTTCCCTGGTTGT	
	Rev: GCATTGGGTATCCATCCATC	
MMP3	Fwd: CAGACTTGTCCCGTTTCCAT	173bp
	Rev: GGTGCTGACTGCATCAAAGA	
MMP7	Fwd: TAGGCGGAGATGCTCACTTT	211bp
	Rev: TTCTGAATGCCTGCAATGTC	
MMP9	Fwd: CGTCGTGATCCCCACTTACT	225bp
	Rev: AACACACAGGGTTTGCCTTC	

PCR primers used in RT-PCR analyses

Figure S7e