Small molecule antagonist of cell surface glycosaminoglycans restricts mouse embryonic stem cells in a pluripotent state.

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Supporting Information

SUPPLEMENTARY MATERIALS AND METHODS

Reagents, tissue culture materials, antibodies, and sources

Product Name	Manufacturer/Distributor	Catalog Number
Cell lines		
E14Tg2a wild-type mESC (E14)	*gift from Cathy Merry (Univ. of Manchester)*	
Oct4-GFP mESCs	PrimCells	PCEMM08
Sox1-GFP mESCs	PrimCells	PCEMM01
Reagents		
Surfen hydrate (cas # 5424-73-3) available at NCI # 12155	Sigma Aldrich	S6951
Sodium chlorate (cas # 7775-09-9)	Acros Organics	223222500
Adhesamine	Calbiochem	362331(discnt)
Protamine sulfate (cas # 9009-65-8)	MP Biomedicals	151971
PD173074 (cas # 219580-11-7)	TSZ Chemicals	RP04
Heparin	Carbosynth	OH03833
Tissue culture reagents		
DPBS without Ca and Mg	Corning	21-031
Non-essential amino acids	Gibco	11140-050
Porcine Gelatin	Sigma	G1890
KO-DMEM	Gibco	10829-018
L-glutamine (200 mM)	Gibco	25030-081
0.05% Trypsin-EDTA	Gibco	25300-054
Heparinase I and III	Sigma Aldrich	H3917-250UN
Heparinase II	Sigma Aldrich	H6512-25UN
Neurobasal medium	Gibco	21103-049
β-mercaptoethanol	Gibco	21985-023
LIF (leukemia inhibitory factor)	Millipore ESGRO	MESG1106
DMEM/F-12 medium	Gibco	11330-032
Knockout Serum Replacement (KSR)	Gibco	10828028
N2 supplement	Gibco	17502048
B27 supplement	Gibco	17504044
Penicillin/Streptomycin (100X)	Sigma	P4333
Western blotting materials		
Cell Lysis Buffer	Cell Signaling Technology	9803
Protease Inhibitor Cocktail	Cell Signaling Technology	5872
PMSF	Cell Signaling Technology	8553
BCA Assay	Thermo Scientific	23225
Luminata HRP substrate	Thermo Scientific	WBLUF0100
Restore Plus Western blot stripping buffer	Thermo scientific	21059
FGF2	Gibco	PHG0264
Bovine serum albumin (BSA)	Spectrum Chemicals	A3611
Tween-20	Fisher Scientific	BP337
ECL Amersham Hyperfilm	GE healthcare	28906839
Protein ladder	Lambda Biotech	G02101
Immobilon FL PVDF membrane	Millipore	IPFL00010

Antibodies		
anti-nestin	Millipore, Clone: 401	MAB353
anti-Oct4	Santa Cruz Biotechnology	sc-25401
chicken anti-Sox1 polyclonal Ab	Millipore	AB5934
anti-β-III-tubulin	Abcam; Clone:EP1569Y	AB52623
anti-HS antibody (F58-10E4)	Amsbio; Clone:F58-10E4	370225
3G10 antibody	Amsbio; Clone: F69-3G10	370260
anti-phosphoErk1/2	Cell Signaling Technology Rbt, Clone: d13.14.4E	4370
anti-Erk	Cell Signaling Technology Rbt, Clone: 137F5	4695
anti-α tubulin	Cell Signaling Technology Mse, Clone: DM1A	3837
anti-SSEA1 (480) Alexa Fluor 647	Santa Cruz Biotechnology	sc-21702
IgM, goat anti-mouse (R-PE)	Molecular Probes	M31504
HRP-goat anti-mouse antibody	Cell Signaling Technology	7076
HRP-goat anti-rabbit antibody	Cell Signaling Technology	7074
AlexaFluor647 goat pAb to chk IgY	Abcam	GR272372-1
Fluoromount-G mounting media	Southern Biotech	0100-01
qPCR materials		
SYBRGreen	Applied Biosystems	4367659
RNAse-Free DNAse	Qiagen	79254
RNeasy Mini Extraction Kit	Qiagen	74104
High capacity cDNA reverse transcription kit	Applied Biosystems	4368814
Heparin ELISA		
TMB substrate	eBioscience	00-4201-56
Heparin coated plates	Bioworld	20140005-3

Stock solutions and storage. Surfen and adhesamine stocks were prepared as 30 mM solutions in DMSO (anhydrous, molecular biology grade), and stored at -20 °C. PD173074 stocks were prepared as 5 mM solutions in DMSO. Protamine Sulfate (source: salmon sperm; 19% sulfate, 24.3% nitrogen; may contain insolubles and histones) stock solutions were prepared as a 20 mg/mL solution, and stored at -20 °C. Protamine has an estimated molecular weight of 10 KDa (used for the analysis in Fig. 2). Heparin was prepared as a 100 μg/mL solution in doubly-distilled water, stored at 4 °C.

***In vitro* culture of mESCs**. Occasionally, Oct4-GFP mESCs were enriched for GFP-positive populations by treatment with 1 μg/mL puromycin in mESC media for two to three days. Gelatin was prepared as a 1.0 % (w/v) autoclaved stock solution in water, and stored at 4 °C. mESCs are cultured in plastic tissue-culture treated plates that were pre-treated for ≥ 10 mins at RT with 0.1% gelatin/DPBS, prepared by a 1:10 dilution of the stock solution in DPBS.

mESC differentiation (N2B27, KSR). Stock solutions of compounds were directly dissolved in N2B27 at the highest concentration. In our hands, we found that DMSO concentrations ≥ 0.025% (v/v) consistently affected pluripotency (data not shown).[1] Thus, we ensured that all compounds were dissolved at a significantly lower DMSO composition. Experiments with the addition of soluble heparin were performed by first adding N2B27 ± additive into the well containing cells, and then heparin to the desired final concentration.

IC_{50} determination. To determine IC_{50} values, $\%GFP^{+ve}$ values for each cell line was plotted against the logarithm of surfen concentrations in nM. The data points were then fitted using GraphPad Prism (v6) using a non-linear curve (equation: log(inhibitor) vs. response – variable slope (four parameters)). Oct4-GFP: R^2 = 0.9914, IC_{50} = 1619 nM, Hill Slope = 6.808. Sox1-GFP: R^2= 0.9978, IC_{50} = 1848 nM, Hill Slope = -7.120.

Differentiation of surfen-treated mESCs. Following a six-day differentiation protocol (in N2B27) as outlined above, cells were washed with DPBS, detached into single cells. Viable cells were counted (via trypan blue exclusion) and re-plated (10,000 cells/cm^2) in N2B27 onto new gelatin-coated twelve-well plates. After an additional six days in N2B27, cells were detached, neutralized, and analyzed by flow cytometry as before. Each experiment was performed with technical duplicates, and repeated successfully with eight biological replicates (see. Fig. S3) to account for variations in differentiation efficiencies.

Immunostaining and fluorescence imaging. Live cells were washed twice with DPBS, fixed with 4% PFA/PBS (RT, 10 min), blocked with immunostaining buffer (1% BSA, 0.1% Triton-X 100, 1% goat serum/DPBS) for 1 hr at RT, then stained with primary antibodies in buffer (4°C, overnight). Oct4 antibody was used at 1:100 dilution, Sox1 at 1:200 dilution, and nestin at 1:300 dilution. Following washes with immunostaining buffer, secondary antibodies were used at a 1:500 dilution in immunostaining buffer, and incubated with the cells for 2-3 hr at RT. Hoescht was used at 10 μg/mL final concentration, and incubated with the cells for 10 mins at RT. Following washes, cells were mounted in Fluoromount-G mounting media. Fluorescence micrographs were obtained either with a Zeiss Axiovert A.1 epifluorescence microscope or a Keyence BZX-700 Fluorescence Microscope.

Viability Assay. Oct4-GFP mESCs were seeded onto gelatin-coated 96-well plates in mESC complete medium overnight. After washing 1x DPBS, cells were incubated in increasing dosages of surfen in mESC media. After 48 hours in culture, cells were washed 2 x DPBS, and re-incubated in 100 μL of KO-DMEM. 20 μL of CellTiter Aqueous reagent (Promega) was then added to each well, and the plate was incubated at 37 °C for 2 hours. Absorbance at 590 nm was then read with a plate reader (Envision Wallac) and viability was quantified. A 100% death consisting of cells-treated for 2 hours with 0.1 % Triton X-100 was used. This experiment was conducted in triplicate conditions, and two biological replicates. A representative experiment is shown in Fig. S9

Staining cell surface HS with antibodies via flow cytometry. Oct4-GFP mESCs cultured on gelatin-coated flasks were re-incubated with complete mESC medium or medium containing 10% (v/v) of Heparinase I, II, and III (1 U/mL). After 18 hr incubation at 37 °C, 5% CO_2, cells were washed 2x DPBS, detached with cell dissociation buffer, and fixed in 1% PFA/PBS on ice for 30-60 min. After washing 2x with DPBS, cells were re-suspended to $1x10^7$ cells/mL 0.1% BSA/PBS, and stored at 4 °C, until ready for staining. To stain for cell surface HS, cells were incubated with either F58-10E4 (1:100) or 3G10 antibody (1:100) for 1 hr on ice, with periodic agitation. After washing twice with 0.1% BSA/PBS, cells were stained with PE-anti-mouse IgM (1:1000) or AF555-α-mouse (1:1000), respectively, for 1 hr on ice, with periodic agitation. After two washes, cells were re-suspended in 0.1% BSA/PBS for flow cytometry analysis.

FGF2 stimulation experiments. Oct4-GFP mESCs (used for all stimulation experiments) were plated ($1x10^5$ cells/cm^2) onto gelatinized six-well plates in mESC growth medium. After 8-12 hours, cells were washed with DPBS, and serum starved overnight (~18 hrs) by replacing with

FBS-free mESC growth medium. Stimulation was performed by adding FGF2 (25 ng/mL) in FBS-free mESC media to cell monolayers for 15 mins at 37 °C, 5% CO_2. The plate was then immediately placed on ice, and total protein was extracted (by scraping) using 1x Cell Lysis Buffer with 1x Protease Inhibitor Cocktail and PMSF (1 mM). For experiments with inhibitor (e.g. surfen or PD173074), cells were pre-incubated for ten minutes at 37 °C, 5% CO_2, prior to the addition of FGF2. For experiments with soluble heparin, heparin (5 μg/mL final concentration) was added after incubation with the inhibitor, and cells were incubated for an additional 10 minutes prior to the addition of FGF2.

Western blot experiments. Cell lysates were quantified for protein content using a standard BCA assay, and 5 μg of total protein was resolved on 10% SDS-PAGE gels and subsequently blotted onto Immobilon-FL 0.45 μm membranes. Membranes were blocked in 5% (w/v) BSA in tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 hour at room temperature. Primary antibody incubations were performed overnight at 4 °C using anti-phospho-ERK1/2 (1:4000), anti-total ERK1/2 (1:5000), or anti-alpha tubulin (1:25,000). Blotted membranes were then washed 3x with TBST and subsequently incubated with HRP-conjugated secondary antibodies, anti-rabbit HRP (1:10,000) or anti-mouse HRP (1:10,000) for 1 hr at RT. After secondary incubations, membranes were washed 3x with TBST and developed using Luminata Forte HRP Detection Reagent and ECL Amersham Hyperfilm. Membranes were stripped and sequentially stained according to the following procedure. Membranes were incubated with Restore PLUS Western blot stripping buffer for 25 mins at RT, and washed 3x with TBST and blocked with 5% BSA/TBST for 1 hr at room temperature before additional antibody incubations were conducted. Densitometry was performed using ImageJ analysis software (National Institute of Health), phospho-ERK1/2 and total-ERK1/2 levels were normalized to α-tubulin levels, then phospho-ERK1/2 was normalized to total-ERK1/2.

FGF2 ELISA. Heparin-coated plates (3 μg heparin per well) were first blocked for 1 hr at RT with 2% BSA/PBS. After washing 3x with 0.05%(v/v) Tween 20/PBS (PBST), the plate was incubated with dilutions of surfen (0.6 to 40 μM) in 1% BSA/PBS, in triplicate wells, for 10-15 mins at RT. Without washing the wells, FGF2 was added to each well to a final concentration of 10 nM, and the plate was left to incubate at RT for 2 hours. After washing 3x with PBST, the wells were incubated with mouse anti-FGF2 antibody (1 μg/mL) in 1% BSA/PBS, for 1 hr at RT. After washing 3x with PBST, the wells were incubated with HRP-conjugated goat anti-mouse antibody (1:1000) in 1% BSA/PBS for another hour at RT. After washing 3x with PBST, 1X TMB substrate (100 μL) was added at RT. After 2-5 mins, the reaction was quenched with 2 N sulfuric acid (100 μL), and the absorbance was read at 450 nm.

Real Time-PCR. Primers were obtained from IDT Technologies and stored as 100 μM solutions in doubly-distilled water. RNA extraction was performed according to manufacturer's instructions, using an RNeasy® Mini Kit. Following treatment of cells with RLT lysis buffer (+ 1% β-mercaptoethanol), cell lysates were homogenized and loaded onto the RNeasy® Mini spin columns. Following column capture of nucleic acids from the lysates, DNase was utilized to remove contaminating genomic DNA. Spin columns were treated with DNase I (27.3 Kunitz units in 80 μL) for 15 minutes as instructed by the kit procedure. Purification of RNA samples was followed by UV-Vis spectroscopy (NanoDrop 2000c, Thermo Scientific) to assess RNA concentration and purity (A_{260}/A_{280}~1.8-2.1). The RNA samples were then converted to cDNA using a High-Capacity cDNA Reverse Transcription Kit. Finally, real-time PCR (Applied Biosystems 7900 HT) was conducted using a 384-well plate, with each well composed of 5 μL cDNA (10 ng/μL; 50 ng), 1 μL primers (10 μM forward and reverse), 10 μL of 2X SYBRGREEN and 4 μL nuclease-free water.

SUPPORTING FIGURES

Figure 1. Surfen maintains pluripotency in Oct4-GFP, Sox1-GFP, and wild-type E14Tg2a mESCs after six days of N2B27 differentiation. Duplicate fluorescence images of mESCs incubated without (left panels) or with surfen (5 μM; right panels) for six days in N2B27 differentiation media, and immunostained for Oct4 (green), Sox1 (red) and DNA (blue). Surfen inhibits Sox1 and maintains Oct4 expression. Non-GFP channels were used for immunostaining to avoid fluorescence bleed-over.

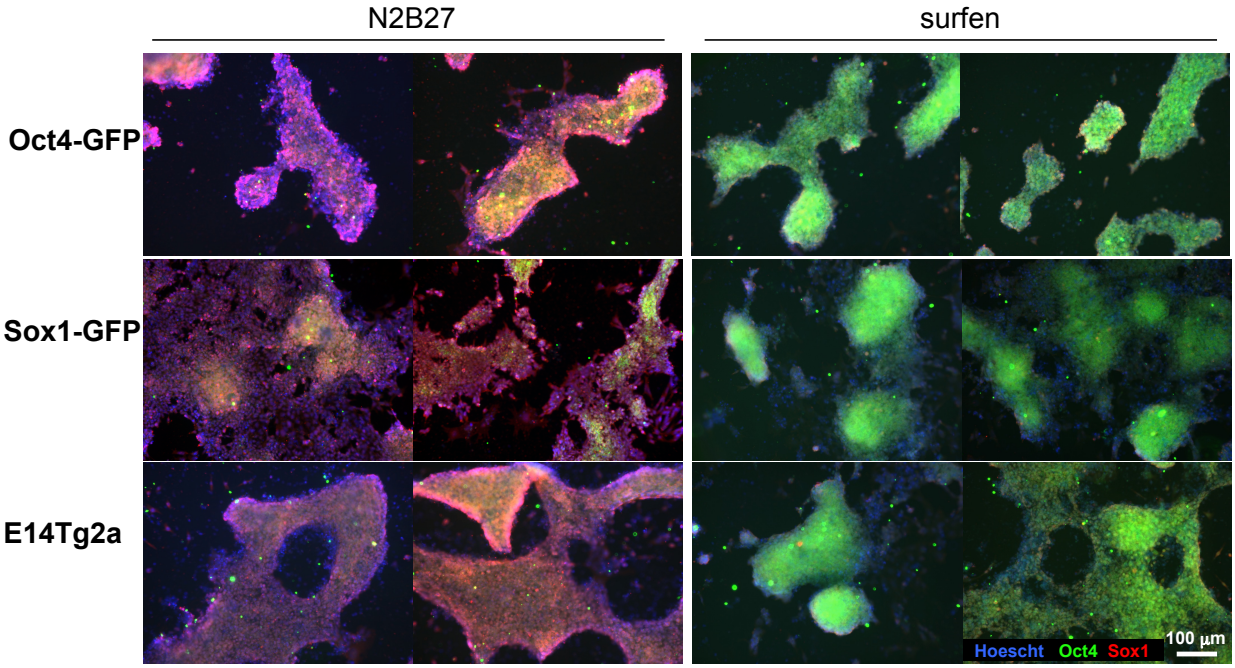


Figure 2. Flow cytometry evaluation of the effects of adhesamine (0.1-10 μM) and protamine (1-100 μg/mL) towards the differentiation of Oct4-GFP and Sox1-GFP mESCs. Protamine MW is 10 kDa. Protamine promotes Oct4-GFP expression but does not inhibit Sox1-GFP expression. Adhesamine does not significantly alter Oct4-GFP nor Sox1-GFP expression levels. Dunnett's multiple comparison test against N2B27 untreated control, ***p<0.0001. Shown are technical duplicates (mean ± SD), repeated with two biological replicates.

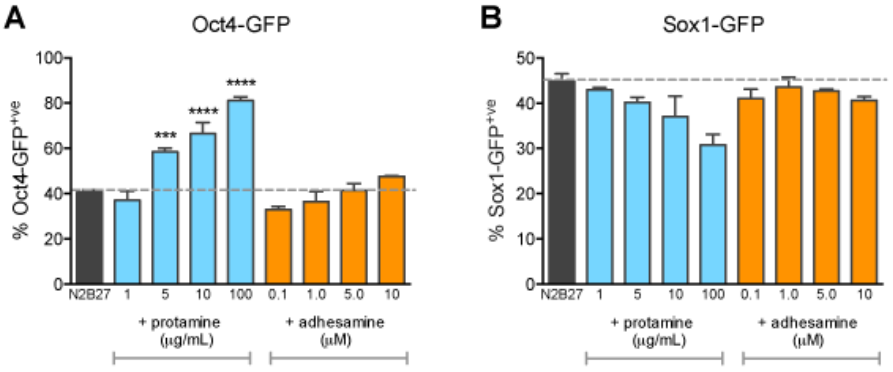


Figure 3. Brightfield microscopy images of mESCs following 24 hr incubation with surfen. At higher concentrations of surfen (10 μM), artifacts on the substrate are visible.

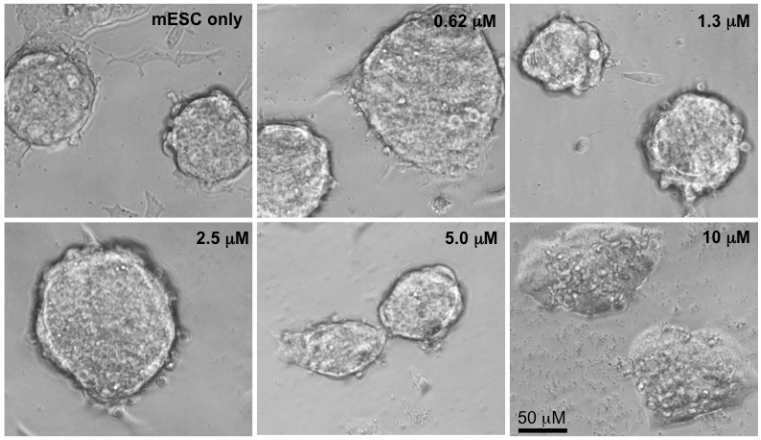


Figure 4. Scatter plot of biological replicates of %GFP-positive cells in Oct4-GFP (eight) and Sox1-GFP (seven) mESCs following six days of N2B27 differentiation with or without surfen. Despite variations in differentiation efficiencies, surfen consistently maintains Oct4 expression and inhibits Sox1 expression after 6 days of differentiation in N2B27. Consistent with reports in literature, Sox1-GFP mESCs can be differentiated into 50 ± 10 % GFP-positive cells. [2,3] Paired t-test against N2B27 untreated control, **** $p < 0.0001$. Shown are eight (Oct4-GFP) or seven (Sox1-GFP) pairs (mean ± SD).

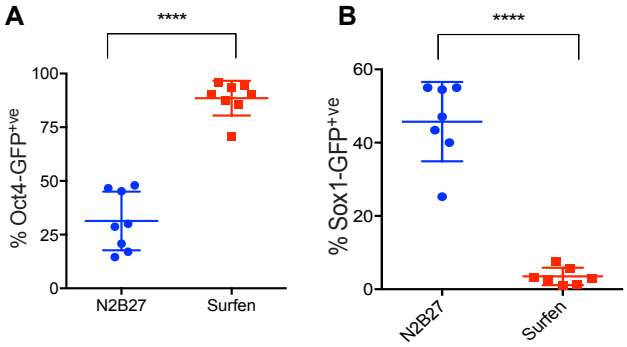


Figure 5. Flow cytometry evaluation of % GFP-positive populations each day of N2B27 differentiation. Surfen maintains pluripotency throughout six days of N2B27 differentiation.

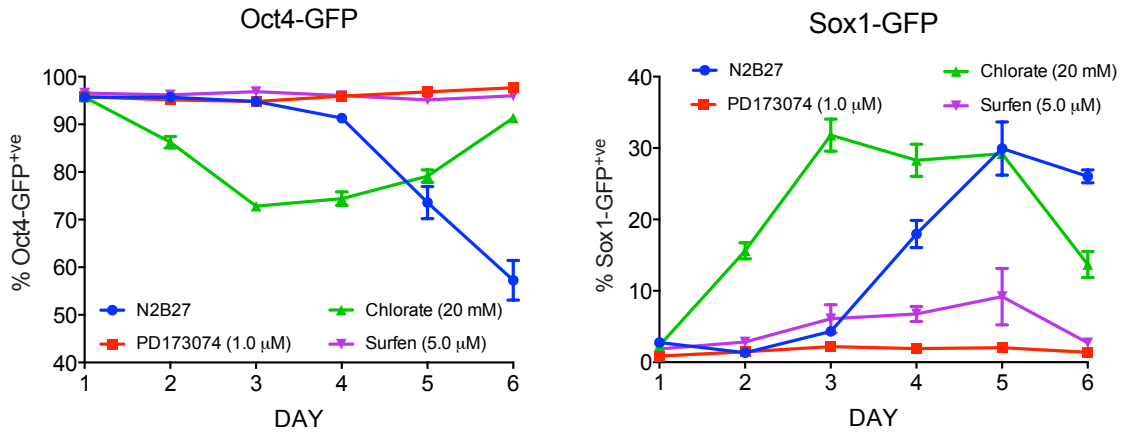


Figure 6. Flow cytometry evaluation of cell-surface HS levels following heparinase treatment, and its effect towards the differentiation of Oct4-GFP mESCs. Heparinase treatment causes reduction of cell surface HS chains but does not maintain pluripotency. Oct4-GFP mESCs cultured in complete medium with heparinase and stained with (A) F58-10E4 antibody or (B) 3G10 antibody. Cells cultured in heparinase show a reduction in geometric mean compared to non-treated cells when probed with F58-10E4, an HS antibody. Cells cultured in heparinase show an increase in 3G10 staining, which detects the enzymatic stub resulting from HS digestion, (C) Heparinase treatment did not significantly affect Oct4 levels compared to untreated control after 6 days in N2B27 differentiation media.

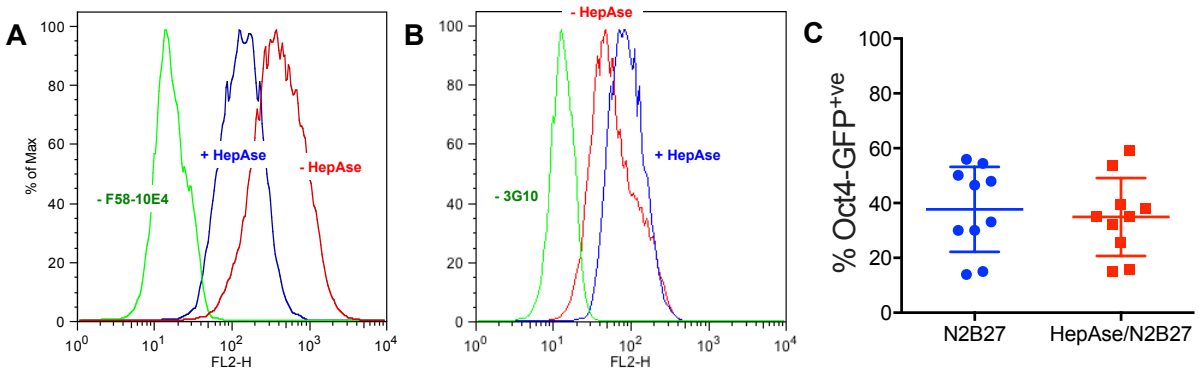


Figure 7. Dose-dependent inhibition of differentiation with surfen treatment. Individual curves for Fig. 2E.

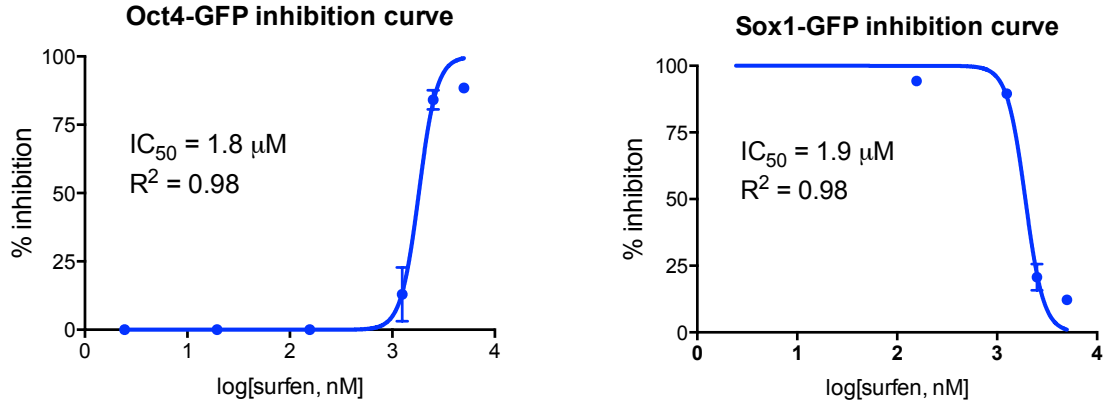


Figure 8. Flow cytometry evaluation of cells after 6 days of N2B27 differentiation, analyzed by SSEA-1 immunostaining and GFP fluorescence. Surfen-treated cells display high SSEA expression in both (A) Oct4-GFP and (B) Sox1-GFP mESCs after 6 days of N2B27 differentiation.

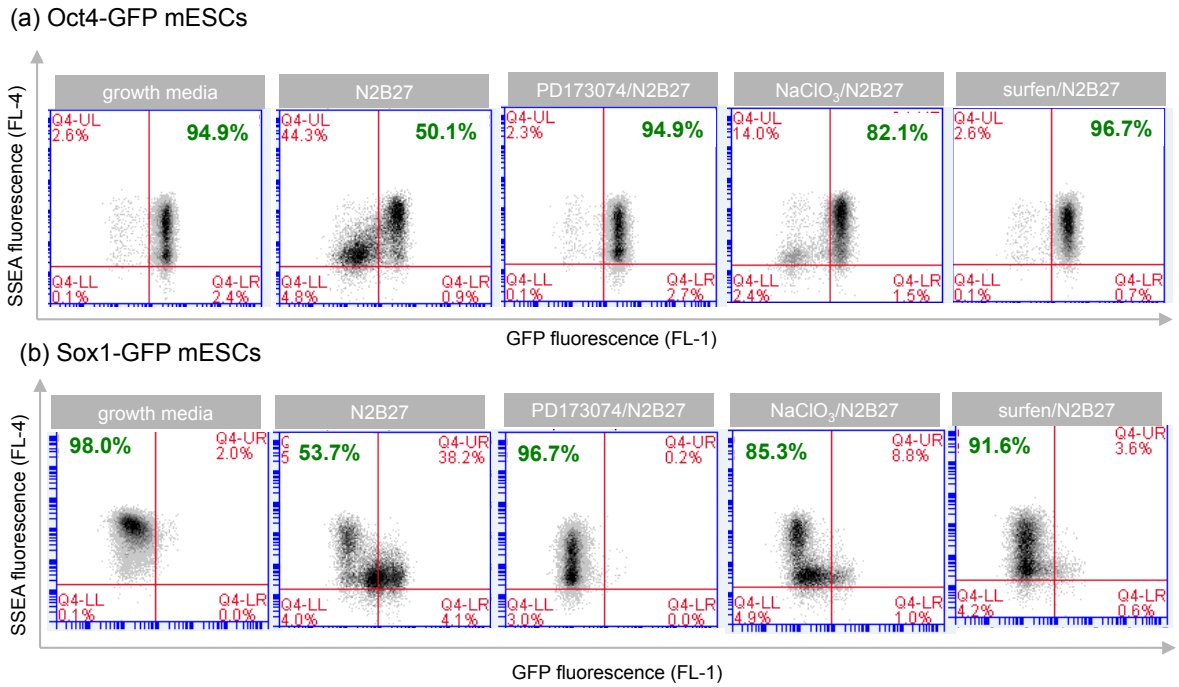


Figure 9. qRT-PCR analysis of surfen-treated cells during N2B27 differentiation. Surfen (5.0 μM) maintains the expression of the pluripotency marker, *Oct4*, and inhibits the expression of neuroectodermal marker, *nestin*.

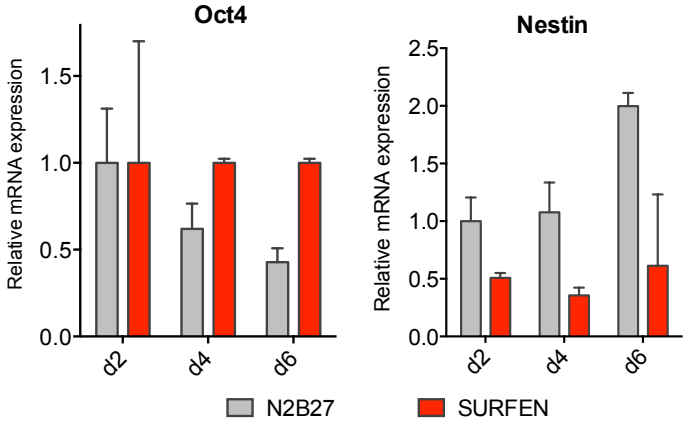


Figure 10. Surfen maintains pluripotency and does not cause Oct4-GFP mESCs to enter an epiblast state. qPCR analysis of cells following six-day treatment with or without surfen in N2B27 media show that surfen maintains pluripotency (high *Rex5* expression), but does not cause an epiblast state, as evidenced by low expression of epiblast markers (*FGF5*, *Nodal*).[4]

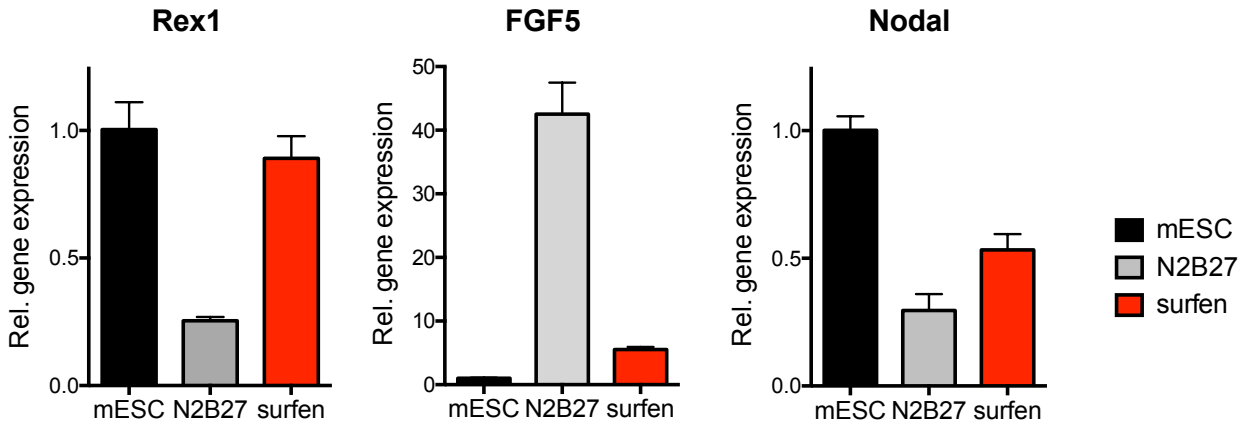


Figure 11. Effects of surfen towards Oct4-GFP mESC viability. CellTiter Aqueous experiment shows that surfen does not exhibit decreased cell viability at 5.0 μM. Dunnett's multiple comparison test against untreated control, ***p<0.0001. Shown are technical triplicates (mean ± SD), repeated with two biological replicates.

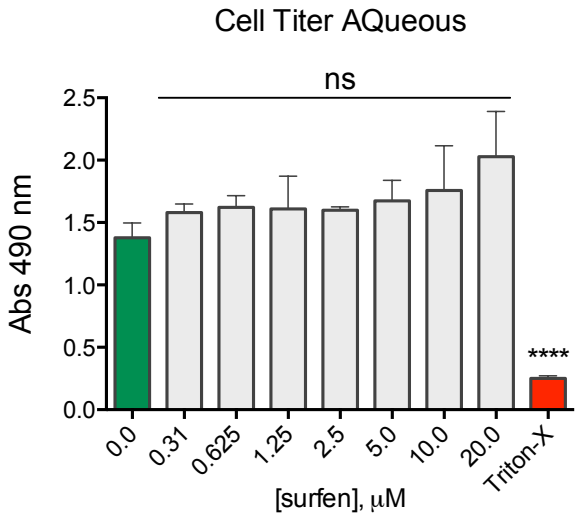


Figure 12. Withdrawal of surfen at D6 allows differentiation to proceed, whereas continuous treatment maintains pluripotency. Fluorescence microscopy images of D13 cells, stained for pluripotency marker Oct4 (green) and the neuroectodermal marker nestin (red), following removal of surfen at D6 (left panels) or continuous treatment with surfen (right panels). High nestin and low Oct4 staining was observed in surfen-withdrawn cells, whereas low nestin and high Oct4 staining was observed in cells continuously treated with surfen. These observations are consistent in Oct4-GFP (used in main text), Sox1-GFP, as well as E14Tg2a wild-type mESCs.

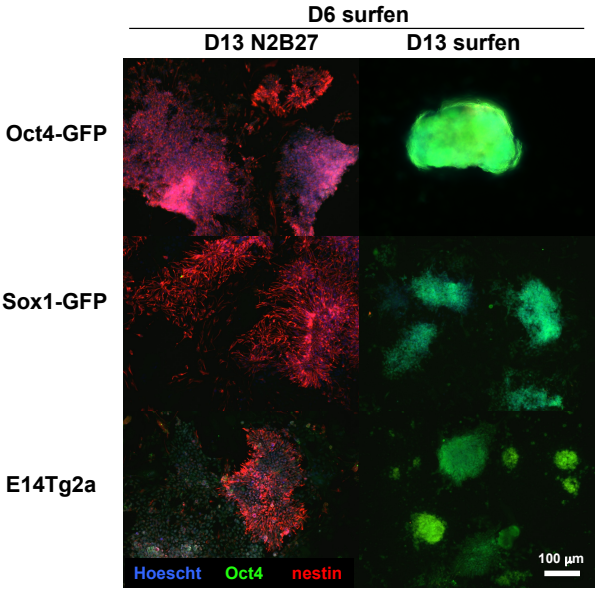


Figure 13. Withdrawal of surfen at D6 allows robust neural differentiation to occur. Triplicate fluorescence microscopy images of Oct4-GFP cells, stained for neural differentiation markers nestin (green) and β-III-tubulin (red), six days after differentiation without (top panels) or with surfen (middle panels). Surfen inhibited expression of nestin and β-III tubulin. Following these six days of surfen treatment, surfen was removed and the cells were allowed to differentiate for an additional seven days in N2B27 (bottom panels). Neural differentiation, as evidenced by high nestin and β-III tubulin cells, occurred robustly in these latter cells.

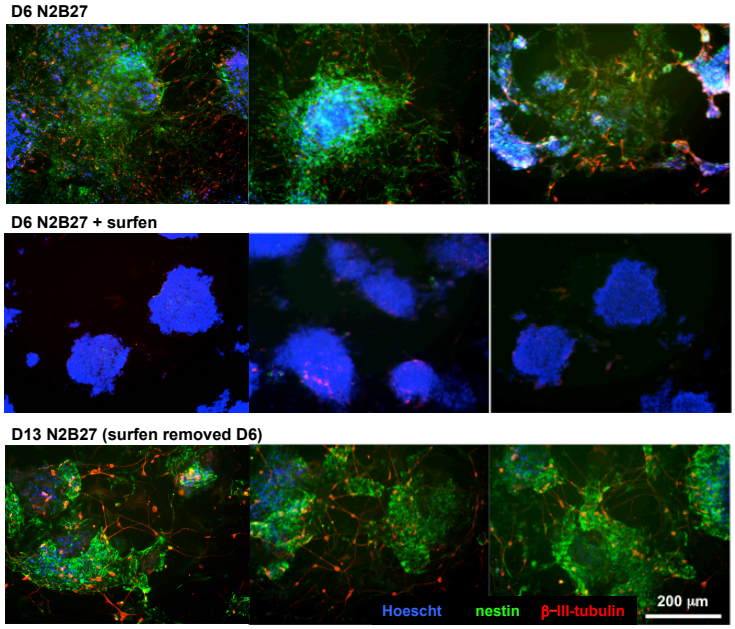


Figure 14. Surfen inhibits Erk phosphorylation in a dose-dependent manner. Original Western blot images (top) and corresponding densitometry bar graph (bottom) of Oct4-GFP mESC lysates treated with increasing dosages of surfen. (Fig. 4A).

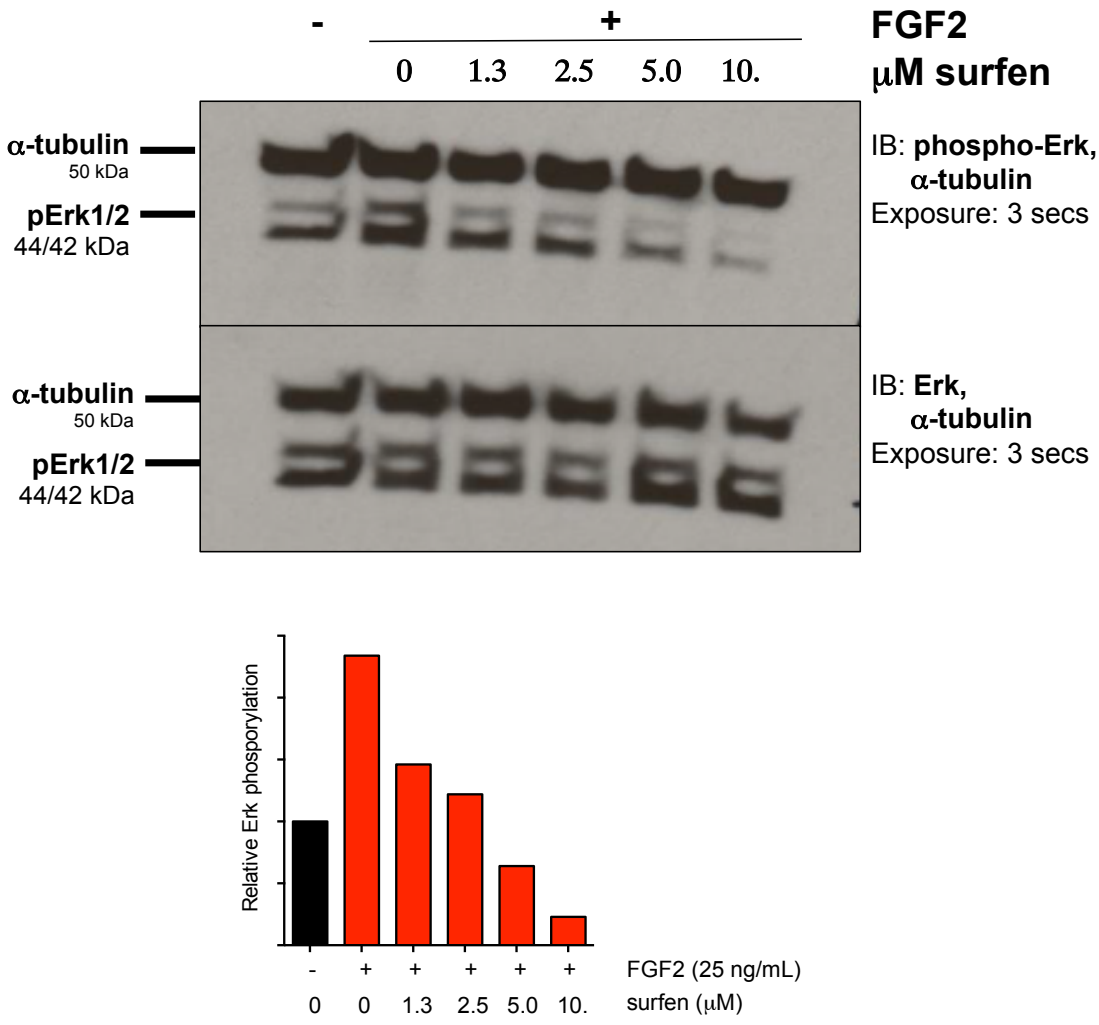


Figure 15. Surfen inhibits FGF2 binding to heparin. Heparin-coated ELISA plates were treated with FGF2 and increasing amounts of surfen.

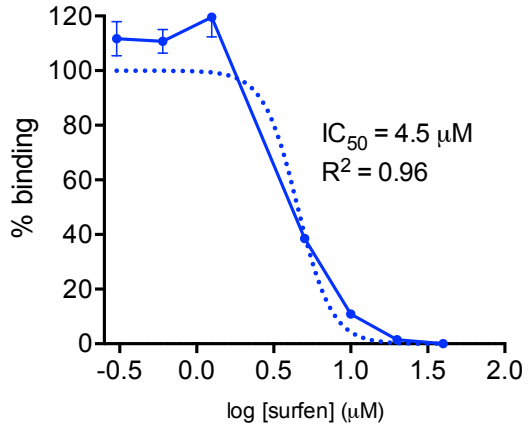


Figure 16. Soluble heparin (5 μg/mL) rescues Erk phosphorylation of surfen-treated (5 μM) Oct4-GFP mESCs. Original Western blot images (top) and corresponding densitometry bar graph (bottom) of lysates treated with surfen and rescued with soluble heparin (Fig. 4B).

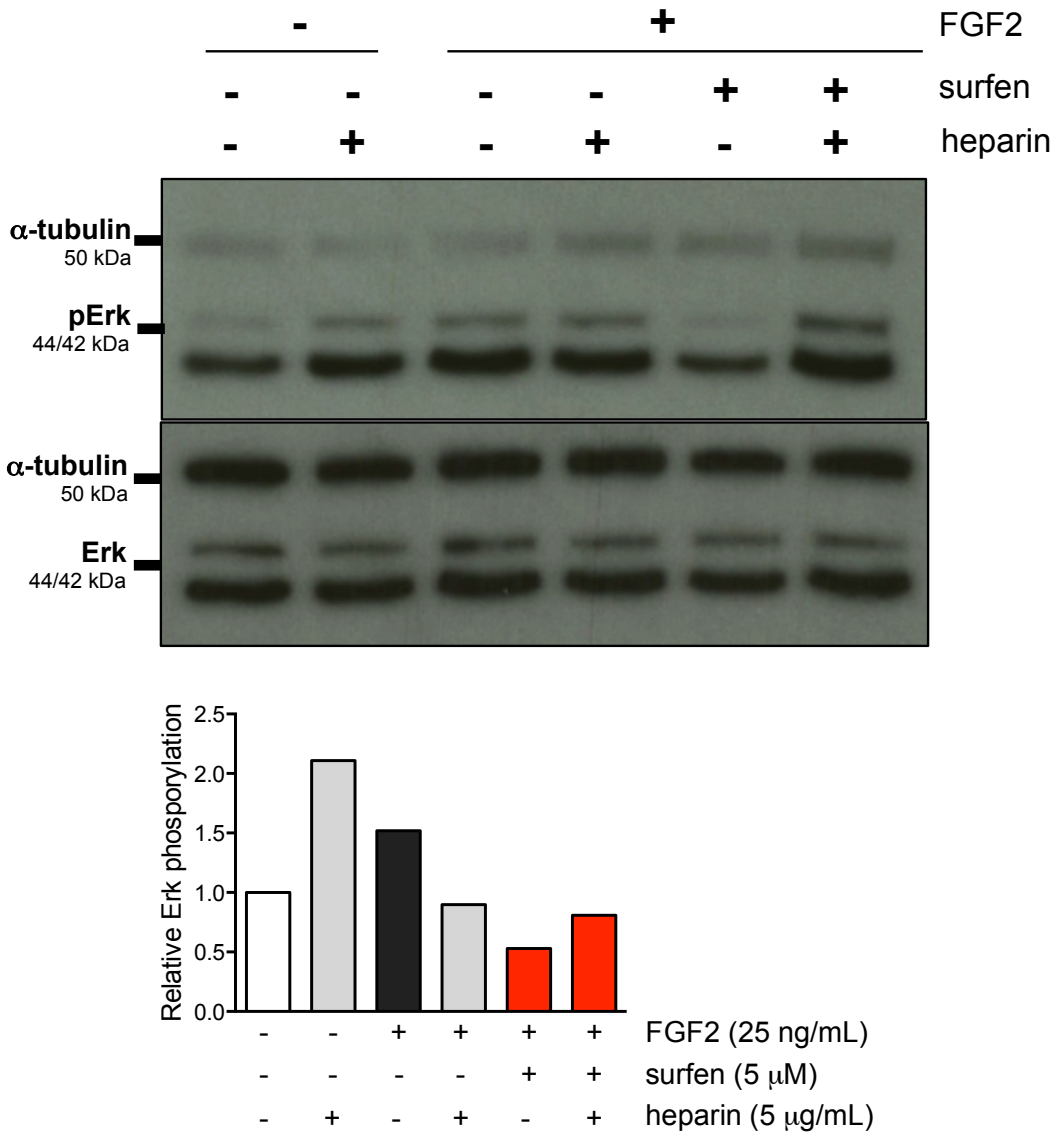
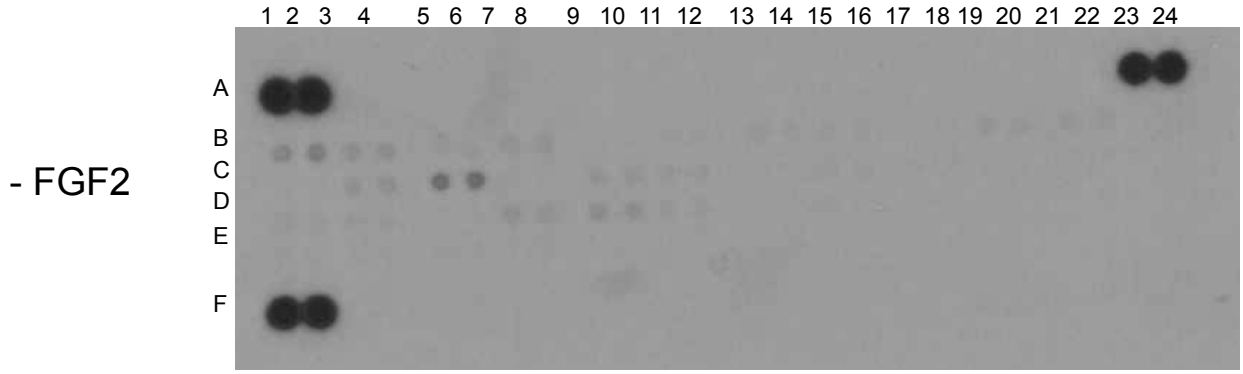
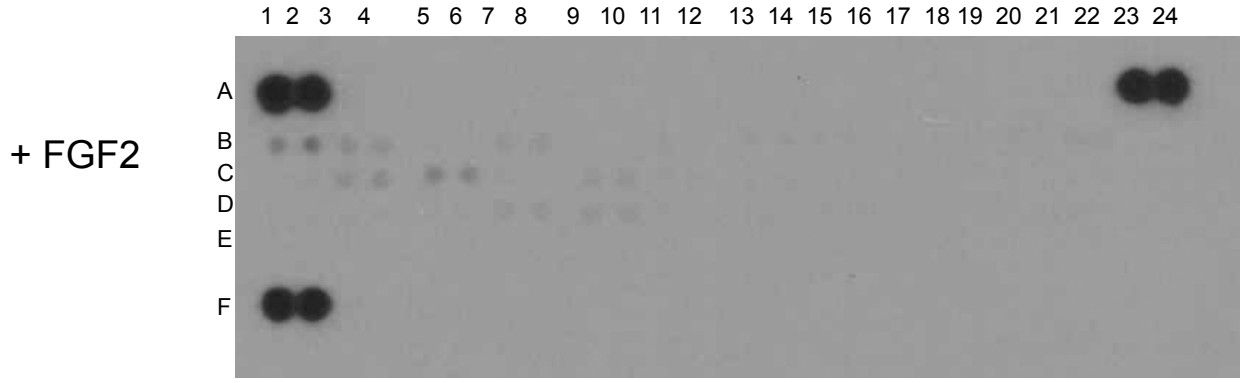
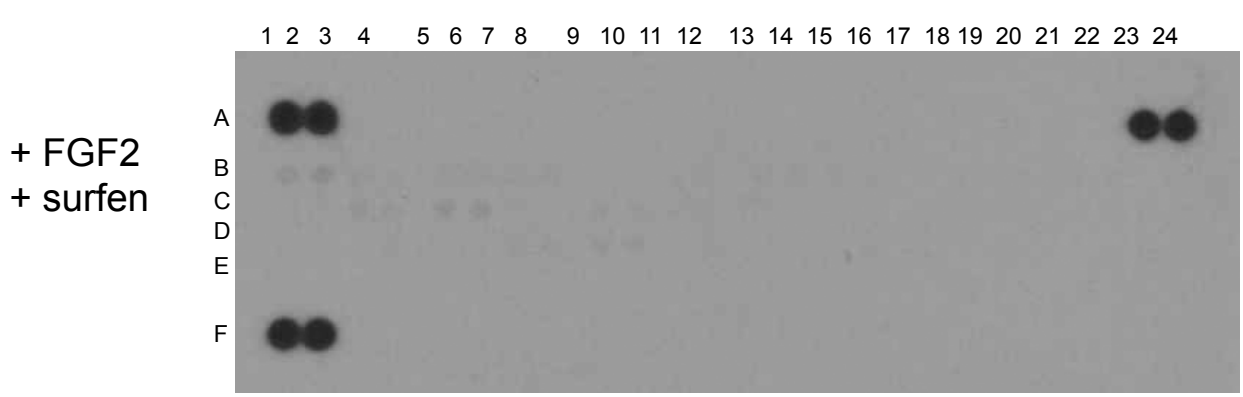


Figure 17. RTK (Receptor Tyrosine Kinase) array analysis of surfen-treated embryonic Oct4-GFP mESCs following FGF2 activation. Surfen is a broad-spectrum phosphorylation inhibitor of many RTKs (see Figure 5 in main text), similar to results observed for Ext1$^{-/-}$ mESCs.[5] Duplicate capture antibodies (labeled in the table below) are spotted in these membranes (R&D Systems Cat. # ARY014). PBS control spots are located in E7-8. Increased PDGFRα expression is observed in these mESCs, although expression of this RTK is known to fluctuate within a population of mESCs.[6]







	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	
A	reference																						reference		A
B	EGFR		ErbB2		ErbB3		ErbB4		FGFR2 (IIIc)		FGFR3		FGFR4		Insulin R		IGF-1 R		Axl		Dtk		Mer		B
C	HGFR		MSPR		PDGFRα		β		SCFR		Flt-3		MCSFR		c-Ret		Tie-1		Tie-2		TrkA		TrkB		C
D	TrkC		VEGFR1		VEGFR2		VEGFR3		MuSK		EphA1		EphA2		EphA3		EphA6		EphA7		EphA8		EphB1		D
E	EphB2		EphB4		EphB6		PBS																		E
F	reference																								F
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	

Table 1. Primers used for RT-PCR.

AMPLICON NAME	Forward primer (5'-3'')	Reverse primer (5'-3')	Product size (bp)
GAPDH (housekeeping)	TGC CTG CTT CAC CAC CTT CT	CCA ATG TGT CCG TCG TGG AT	83
β-actin (housekeeping	GGG GTG TTG AAG GTC TCA AA	TGT TAC CAA CTG GGA CGA CA	168
Oct4 (embryonic)	TTG CCT TGG CTC ACA GCA TC	TGT TCC CGT CAC TGC TCT GG	82
Rex1 (embryonic)	GGC TGC GAG AAG AGC TTT ATT CA	AGC ATT TCT TCC CGG CCT TT	79
Sox1 (ectoderm)	GGC CGA GTG GAA GGT CAT GT	TCC GGG TGT TCC TTC ATG TG	93
β -III-tubulin (Tubb3) (ectoderm)	TGA TGA CGA GGA ATC GG AAG C	GGA CAG ATG CTG CTT GTC TTG G	101
nestin (ectoderm)	CTA CCA GGA GCG CGT GGC	TCC ACA GCC AGC TGG AAC TT	219
Brachyury "T" (mesoderm)	TTG AAC TTT CCT CCA TGT GCT GA	TCC CAA GAG CCT GCC ACT TT	82
Foxa2 (mesoderm)	ACT GGA GCA GCT ACT ACG	CCC ACA TAG GAT GAC ATG	152
Sox17 (endoderm)	AGC CAT TTC CTC CGT GGT GT	AAC ACT GCT TCT GGC CCT CAG	104
FGF5 (epiblast)	CCT TGC GAC CCA GGA GCT TA	CCG TCT GTG GTT TCT GTT GAG G	98
Nodal (epiblast)	ACT GAG GGC CCA CTC ACC AT	CGG TGA ACG TCT CCA TCC AA	103

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