Journal Club: iPSCs

Jesse Levine

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Gholson Lyon

Pluripotent Stem Cells Induced from Mouse Somatic Cells by Small-Molecule Compounds

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Goals

- Develop a combination of small molecule compounds capable of reprogramming mouse somatic cells into pluripotent stem cells in the absence of exogenous “master genes”

VC6T

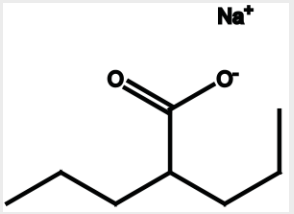
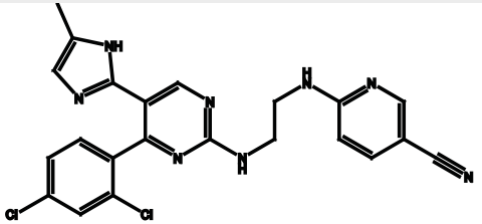
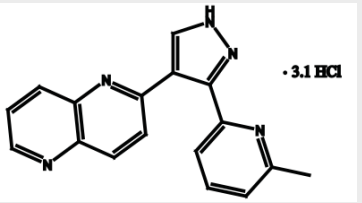
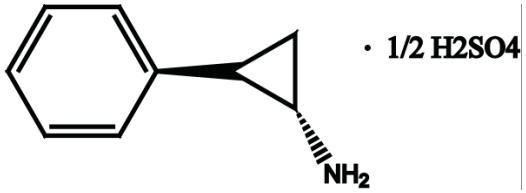
Name	Abbreviation	Function	Structure
Valproic acid sodium salt	VPA, **V**	Histone deacetylase inhibitor	
CHIR99021	CHIR, **C**	GSK3-β inhibitor	
616452	**6**	TGF-β inhibitor	
Tranylcypromine	Tranyl, **T**	H3K4 demethylation inhibitor	

Table S1 (B)

- **Part 1: Find Oct4 substitute**
- Part 2: Test small molecule cocktail
- Part 3: Screen for late reprogramming molecule
- Part 4: Resolve incomplete reprogramming
- Part 5: Optimize cocktail
- Part 6: Screen for reprogramming booster
- Part 7: Additional cells of origin
- Part 8: Characterize CiPSC lines
- Part 9: Determine essential small molecules
- Part 10: Investigate role of small molecules

Part 1: Find Oct4 Substitute

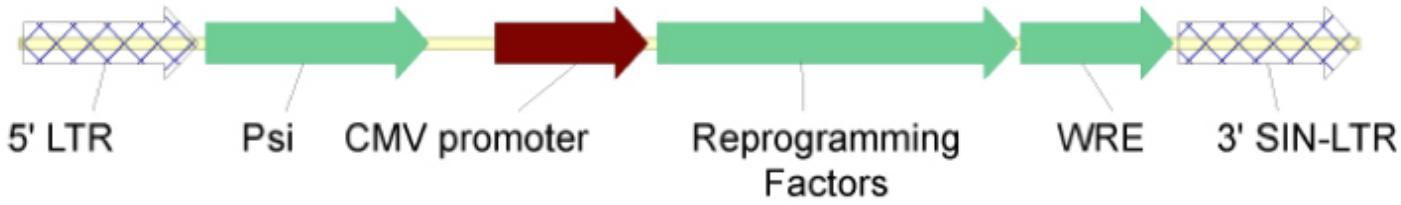
Mice:

- *OG* - Oct4/EGFP transgenic C57BL/6J mice carrying EGFP (enhanced green fluorescence protein) under control of an Oct4 18-kb genomic fragment containing the minimal promoter and proximal and distal enhancers; can come close to mimicking the endogenous embryonic expression pattern of Oct-4 in transgenic mice

Part 1: Find Oct4 Substitute

Methods:

- Lentiviral infection: Sox2, Klf4 and c-Myc (SKM)



Two Supporting Factors Greatly Improve the Efficiency of Human iPSC Generation, Cell Stem Cell, Volume 3, Issue 5, 6 November 2008, Pages 475-479, ISSN 1934-5909, http://dx.doi.org/10.1016/j.stem.2008.10.002.

- Small molecule screen
  - 20k OG MEFs/well; 12 well plate
  - Infect with lentivirus encoding SKM
  - Replace with LIF-free ESC culture medium
  - Add individual chemicals from small-molecule libraries to each well
  - Change medium and chemicals every 4 days
  - 14-20 days or until GFP+ colonies
  - Primary hits confirmed and optimized

Part 1: Find Oct4 Substitute

Small molecule libraries

Library	Source	Number of small-molecule compounds
BBP-2080NPs library	BioBioPha	2,080
The Spectrum Collection	MicroSource Discovery Systems	2,000
Sigma LOPAC®,1280	Sigma	1,280
Prestwick Chemical Library®	Prestwick Chemical	1,200
Tocriscreen™ Total	Tocris	1,120
US Drug Collection	MicroSource Discovery Systems	1,040
ICCB Known Bioactives Library	Enzo	480
Protein Kinase Inhibitor Library I, II, III	Millipore	324
StemSelect Small Molecule Regulators	Calbiochem	303
Nuclear Receptor Ligand Library	Enzo	76
Selected Small Molecules*	Our lab	88

*This library was generated in-house, including 88 selected small molecules related to pluripotency, reprogramming or epigenetic modification.

Table S1 (A)

Part 1: Find Oct4 Substitute

SKM/SK: Primary hits

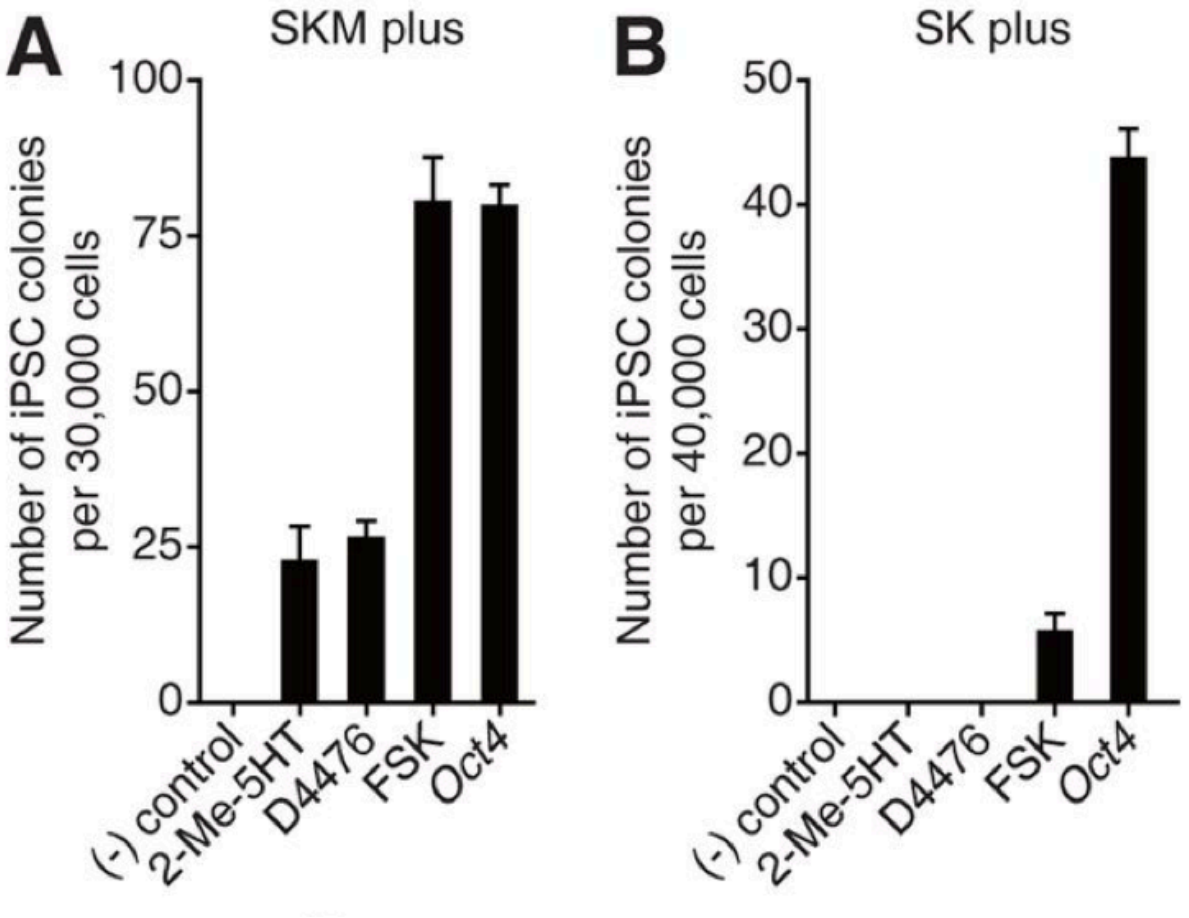


Fig. 1

Part 1: Find Oct4 Substitute

SKM/SK: Primary hits

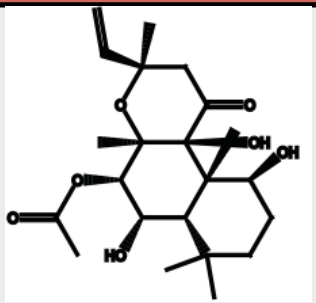
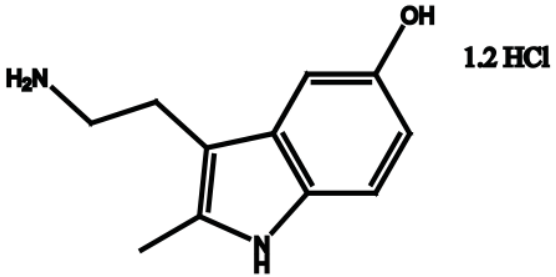
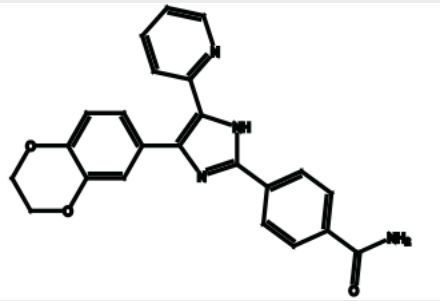
Name	Abbreviation	Function	Structure
Forskolin	FSK, **F**	Activates adenylate cyclase	
2-Methyl-5-hydroxytryptamine	2-Me-5HT	5-HT3 agonist	
D4476		CK1 inhibitor	

Table S1 (B)

Part 1: Find Oct4 Substitute

Characterization of iPSC colonies induced from SKM or SK-infected MEFs with FSK treatment

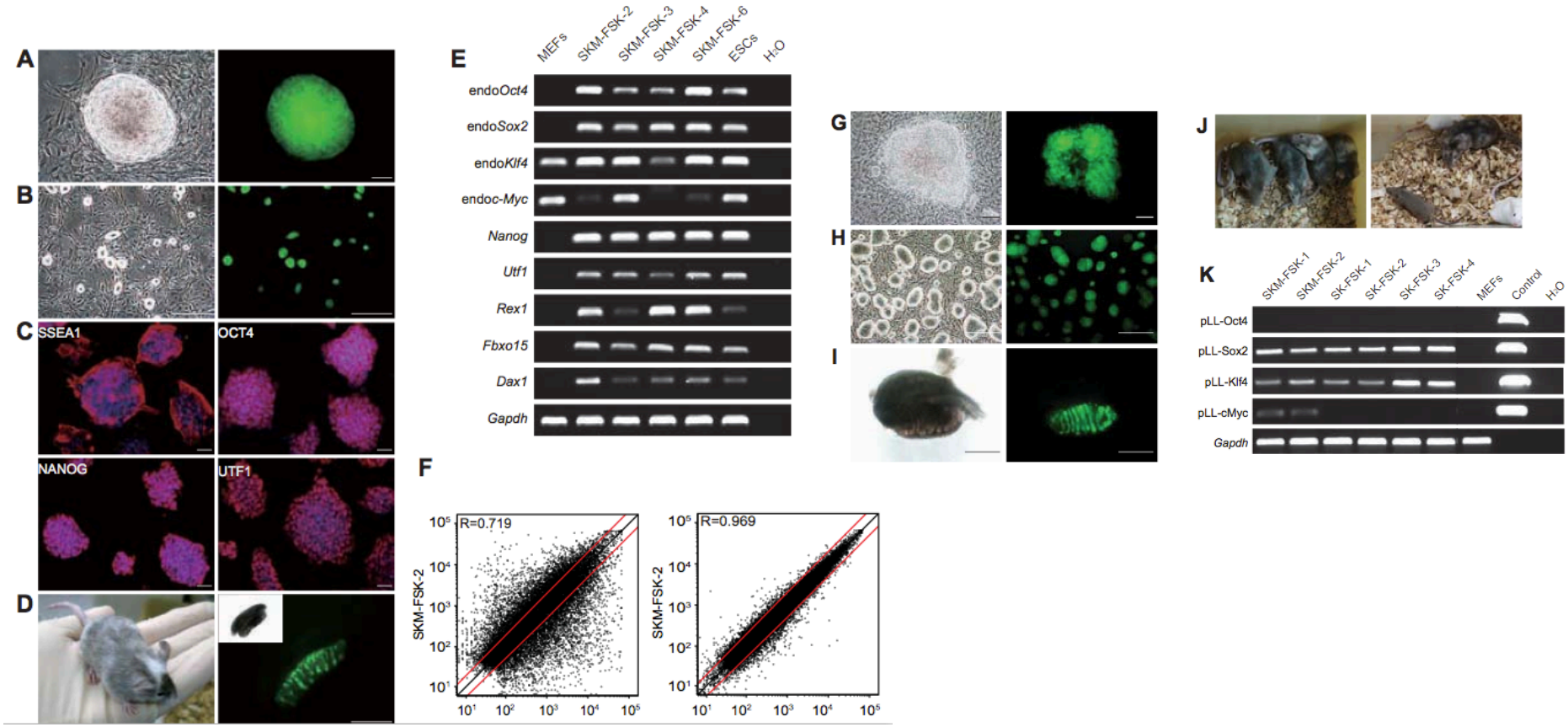


Fig. S1

Part 1: Find Oct4 Substitute

Characterization of iPSC colonies induced from SKM or SK-infected MEFs with 2-Me-5HT or D4476 treatment

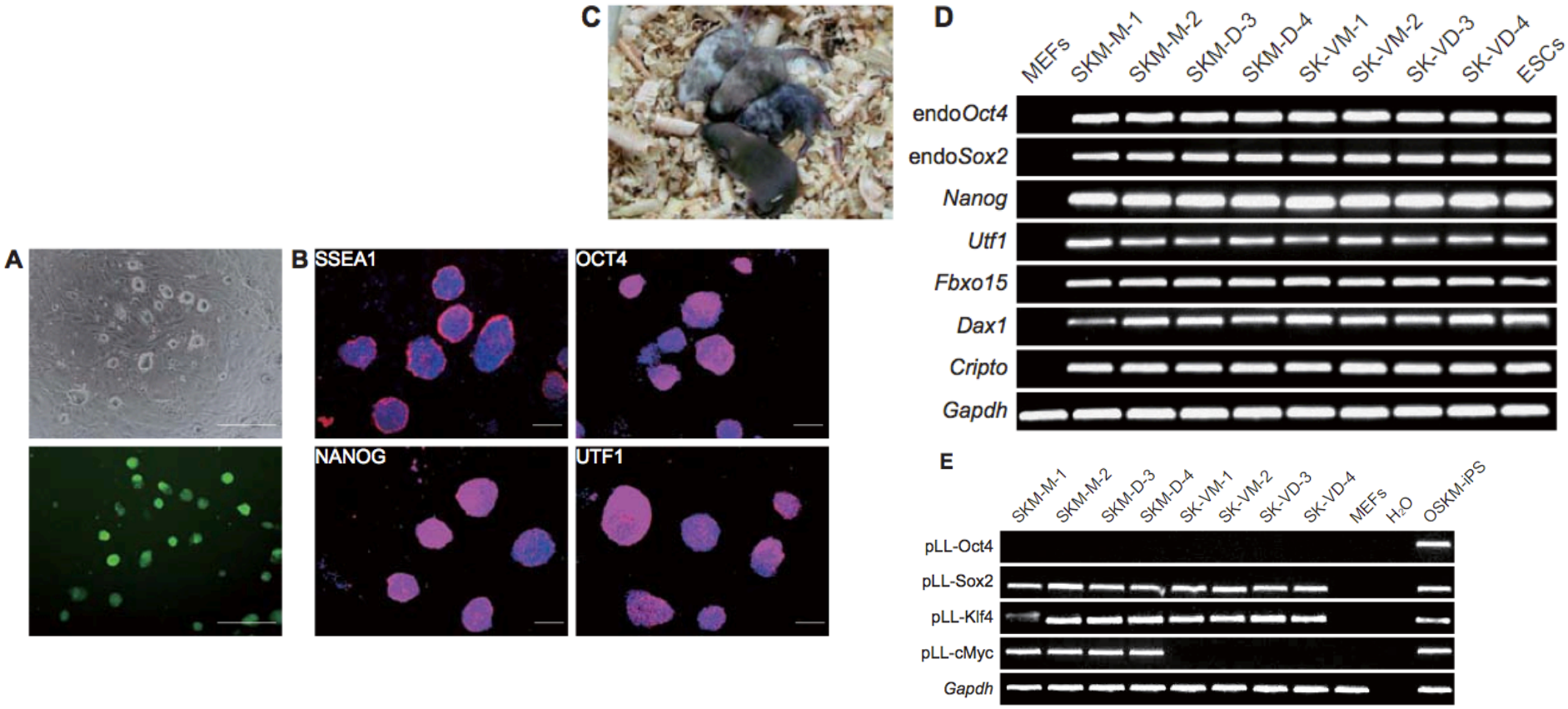


Fig. S2

- Part 1: Find Oct4 substitute
- **Part 2: Test small molecule cocktail**
- Part 3: Screen for late reprogramming molecule
- Part 4: Resolve incomplete reprogramming
- Part 5: Optimize cocktail
- Part 6: Screen for reprogramming booster
- Part 7: Additional cells of origin
- Part 8: Characterize CiPSC lines
- Part 9: Determine essential small molecules
- Part 10: Investigate role of small molecules

Part 2: Test small molecule cocktail

VC6TF

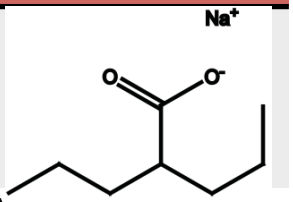
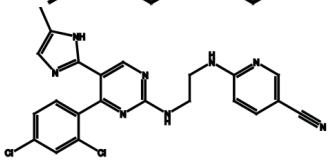
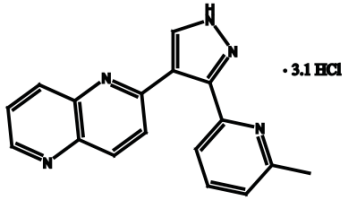
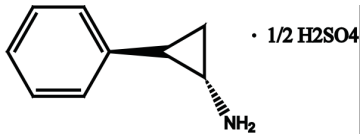
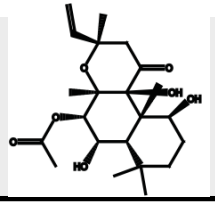
Name	Abbreviation	Function	Structure
Valproic acid sodium salt	VPA, **V**	Histone deacetylase inhibitor	
CHIR99021	CHIR, **C**	GSK3-β inhibitor	
616452	**6**	TGF-β inhibitor	
Tranylcypromine	Tranyl, **T**	H3K4 demethylation inhibitor	
Forskolin	FSK, **F**	Activates adenylate cyclase	

Table S1 (B)

Part 2: Test small molecule cocktail

VC6TF

Mice: OG-MEFs

Methods:

- Plate cells: 50k/well; 6 well plate
- Replace medium with chemical reprogramming medium containing small molecule cocktail
- Change medium every 4 days

Part 2: Test small molecule cocktail

VC6TF: Characterization of GFP+ clusters; day 24

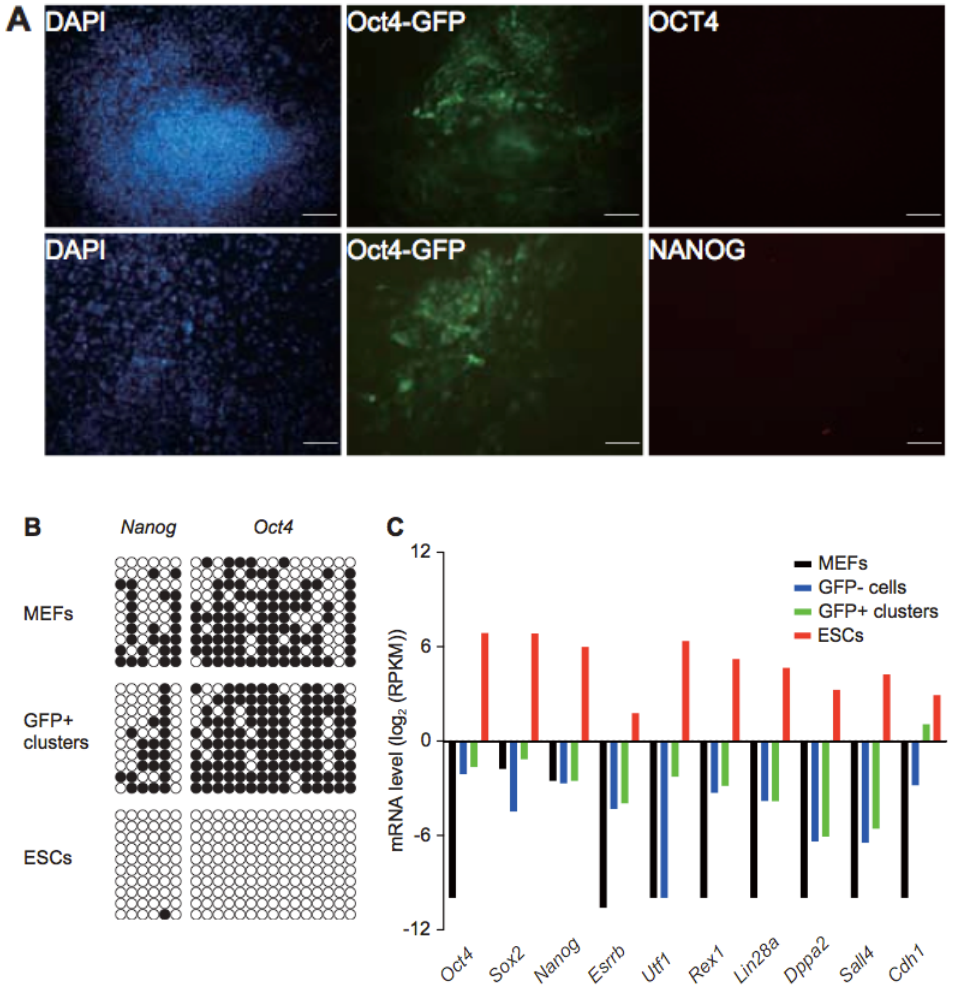


Fig. S3

- Part 1: Find Oct4 substitute
- Part 2: Test small molecule cocktail
- **Part 3: Screen for late reprogramming molecule**
- Part 4: Resolve incomplete reprogramming
- Part 5: Optimize cocktail
- Part 6: Screen for reprogramming booster
- Part 7: Additional cells of origin
- Part 8: Characterize CiPSC lines
- Part 9: Determine essential small molecules
- Part 10: Investigate role of small molecules

Part 3: Screen for late reprogramming molecule

Mice:

- Infected OG MEFs
- MEFs with DOX-inducible Oct4 from Tet-On POU5F1 strain B6;129-*Gt(ROSA)26Sortm1(rtTA*M2)Jae Col1a1tm2(tetO-Pou5f1)Jae/J*

Methods:

- Infect OG MEFs with Fu-tet-hOct4 and FUdeltaGW-reTA lentiviruses
- Culture medium containing VC6T and DOX (DOX first 4-8 days)
- Individual chemicals from small-molecule library in each well
- Change medium and chemicals every 4 days
- Continue 16-24 days or until GFP+ colonies appear
- Confirm and optimize primary hits

Part 3: Screen for late reprogramming molecule

VC6T + DOX: Primary hits

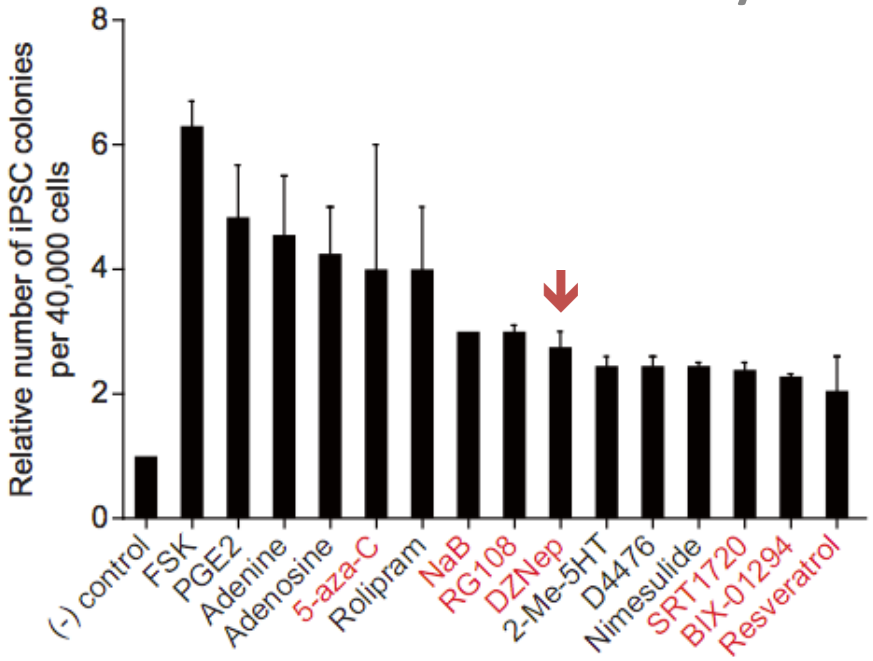


Fig. S4

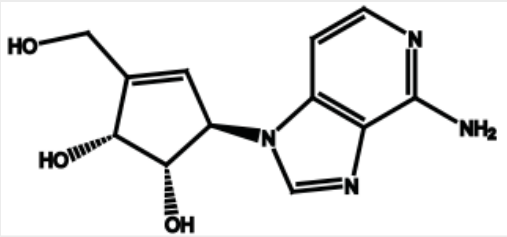
Name	Abbreviation	Function	Structure
3-deazaneplanocin	DZNep, **Z**	S-Adenosylhomocysteine Hydrolase inhibitor and histone methyltransferase EZH2 inhibitor	

Table S1 (B)

Part 3: Screen for late reprogramming molecule

VC6TFZ

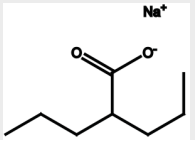
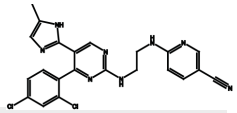
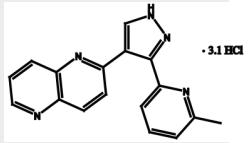
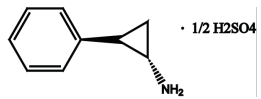
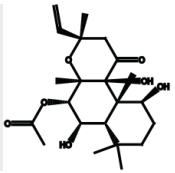
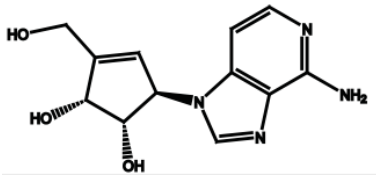
Name	Abbreviation	Function	Structure
Valproic acid sodium salt	VPA, **V**	Histone deacetylase inhibitor	
CHIR99021	CHIR, **C**	GSK3-β inhibitor	
616452	**6**	TGF-β inhibitor	
Tranylcypromine	Tranyl, **T**	H3K4 demethylation inhibitor	
Forskolin	FSK, **F**	Activates adenylate cyclase	
3-deazaneplanocin	DZNep, **Z**	S-Adenosylhomocysteine Hydrolase inhibitor and histone methyltransferase EZH2 inhibitor	

Table S1 (B)

Part 3: Screen for late reprogramming molecule

VC6TFZ: GFP positive cells induced

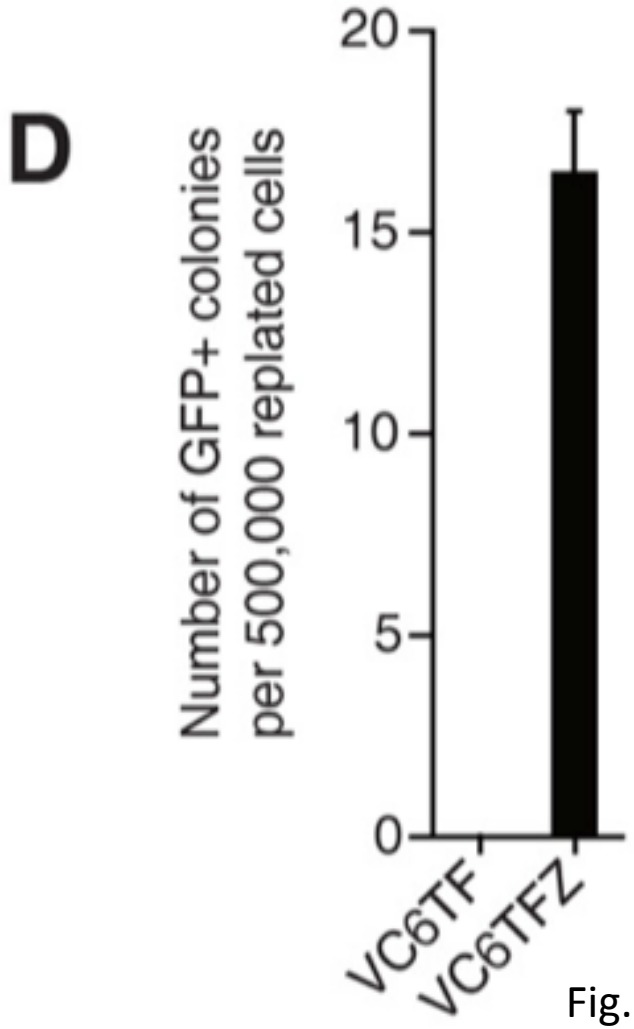


Fig. 1

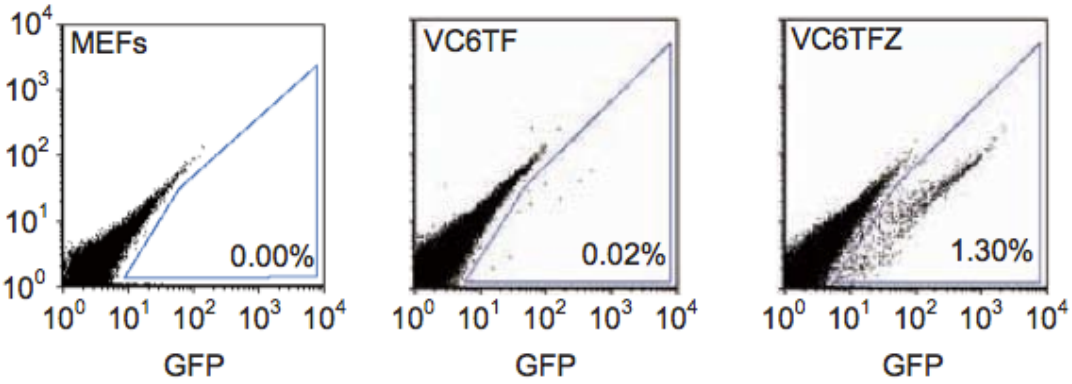


Fig. S5

- Part 1: Find Oct4 substitute
- Part 2: Test small molecule cocktail
- Part 3: Screen for late reprogramming molecule
- **Part 4: Resolve incomplete reprogramming**
- Part 5: Optimize cocktail
- Part 6: Screen for reprogramming booster
- Part 7: Additional cells of origin
- Part 8: Characterize CiPSC lines
- Part 9: Determine essential small molecules
- Part 10: Investigate role of small molecules

Part 4: Resolve incomplete reprogramming

VC6TFZ: timeline of CiPSC generation

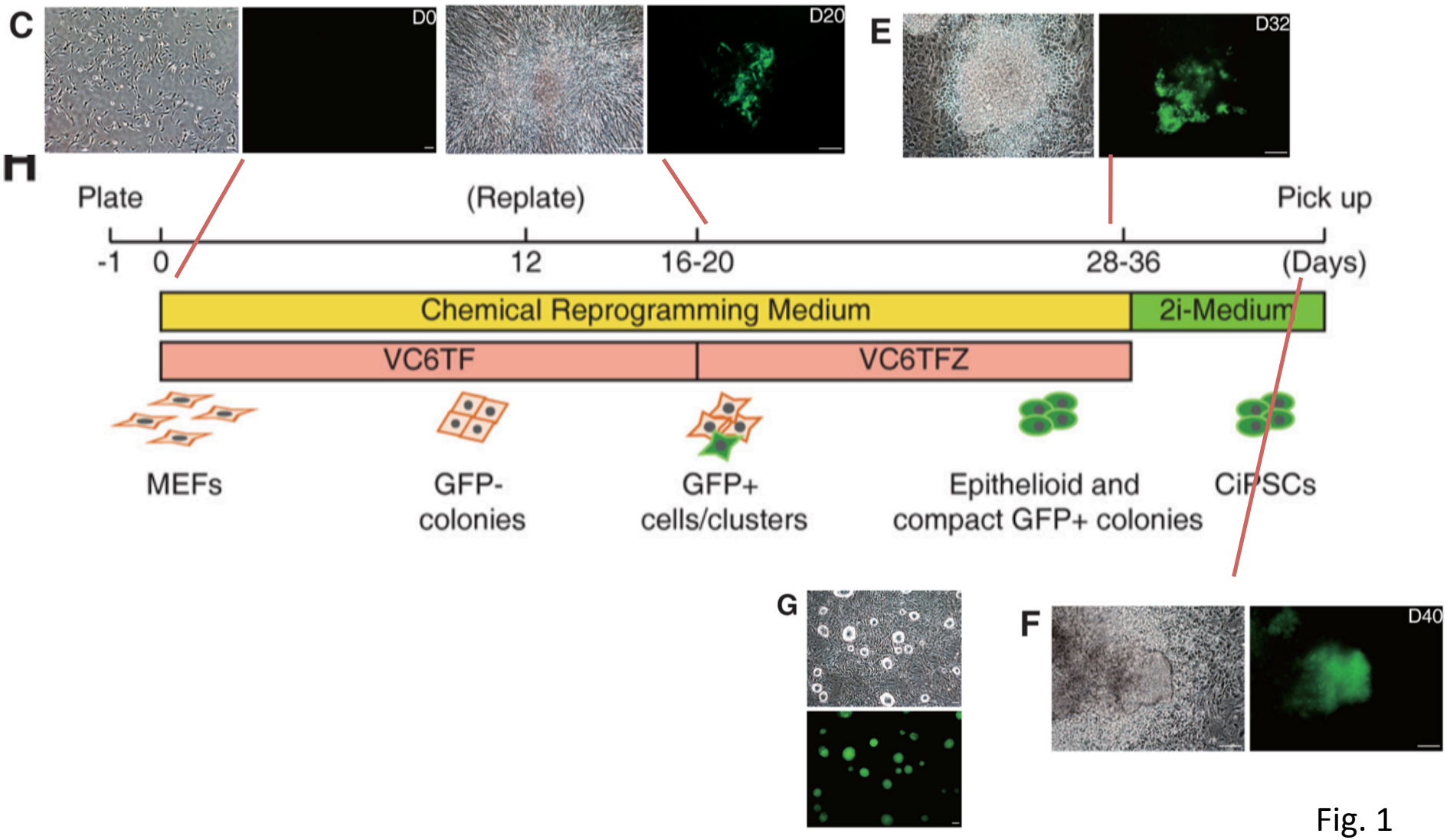
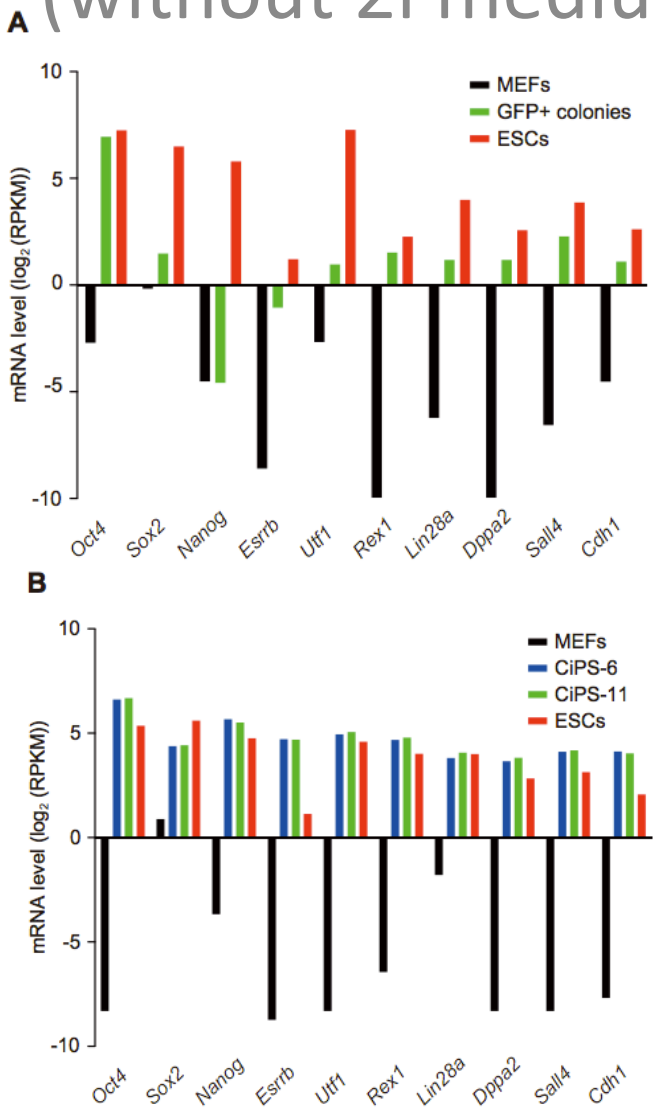
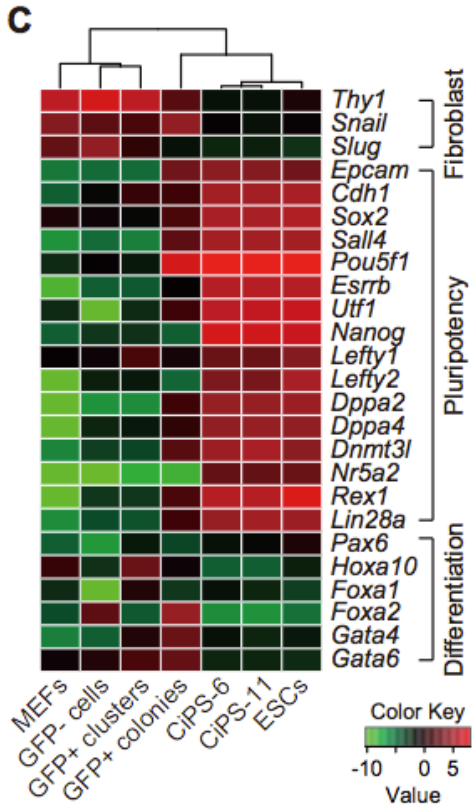


Fig. 1

Part 4: Resolve incomplete reprogramming

VC6TFZ: RNA-seq analysis of GFP positive colonies (without 2i medium/incomplete) and CiPSCs





Heat map:

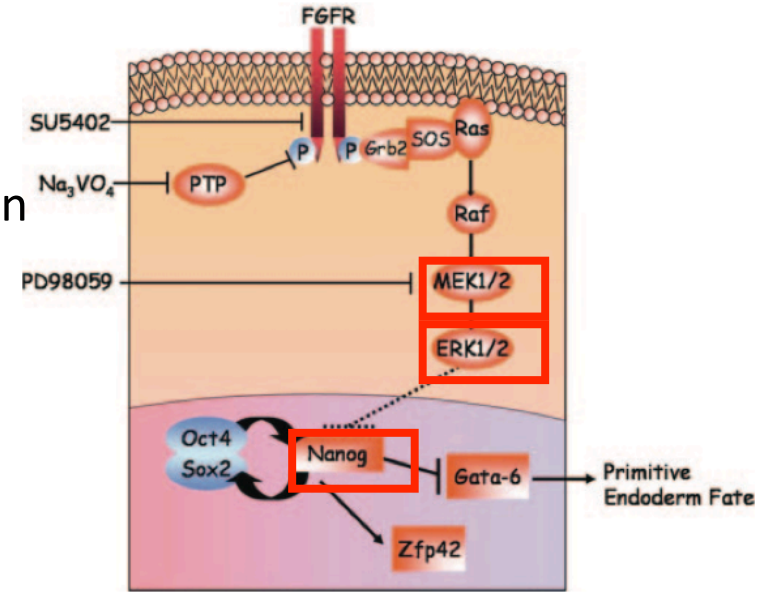
- Value in the color key indicates $\log_2$ changes
- Generated using R

Fig. S6

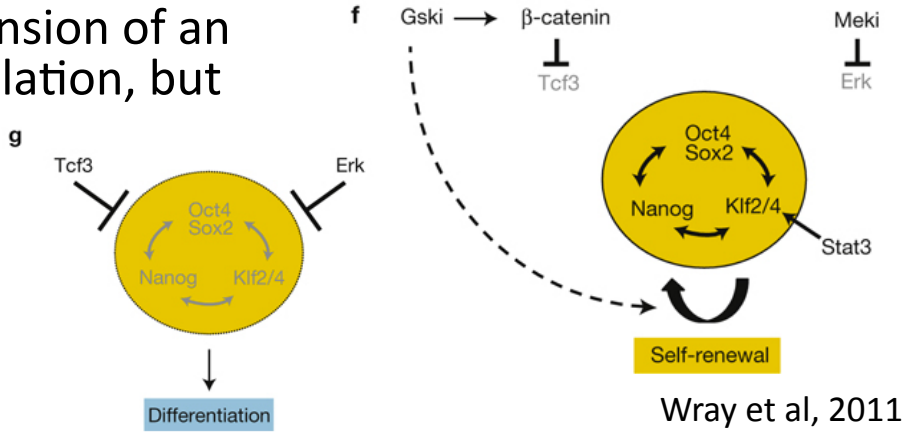
Part 4: Resolve incomplete reprogramming

2i medium:

- Small molecule inhibition of MAP kinase (MEK) and glycogen synthase kinase 3 (GSK3)
- MEK inhibition is the main reprogramming cue in 2i and also exerts selection against pre-iPS cells
  - Phospho-Erk (p-Erk) signal extinguished
  - Upregulation of Nanog expression
- GSK inhibition generates intracellular β-catenin, which interacts with Tcf3 and abolishes its repressor effect on multiple genes in the pluripotent network
- GSK inhibition also supports embryonic stem cell propagation through stimulatory effects on metabolic and biosynthetic processes
- 2i treatment does not select for expansion of an already resident pluripotent subpopulation, but actively induces conversion to pluripotency in pre-iPS cell



Hamazaki et al, 2006



Wray et al, 2011

- Part 1: Find Oct4 substitute
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- **Part 5: Optimize cocktail**
- Part 6: Screen for reprogramming booster
- Part 7: Additional cells of origin
- Part 8: Characterize CiPSC lines
- Part 9: Determine essential small molecules
- Part 10: Investigate role of small molecules

Part 5: Optimize cocktail

VC6TFZ: concentrations and treatment durations of individual chemicals

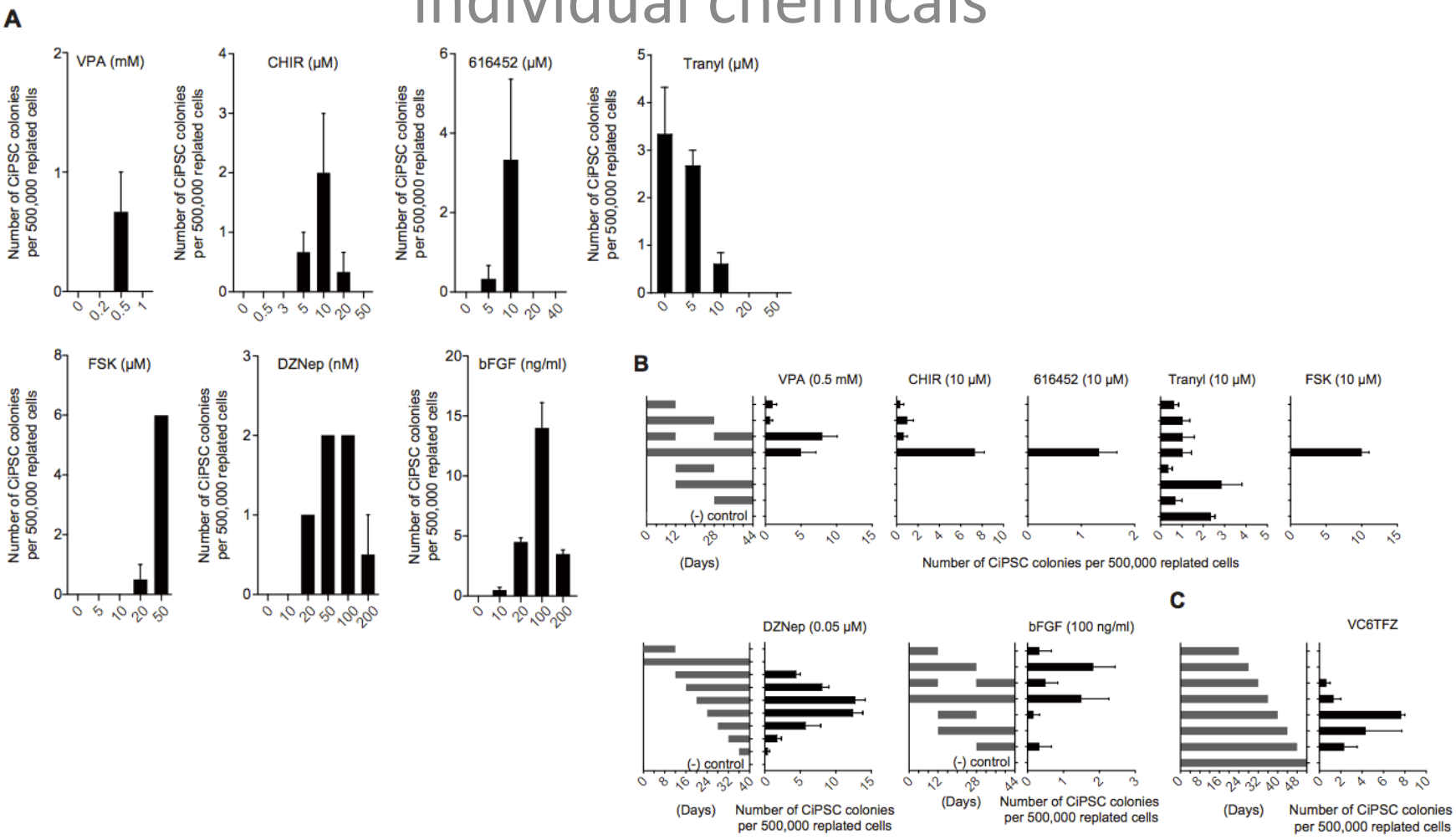


Fig. S7

- Part 1: Find Oct4 substitute
- Part 2: Test small molecule cocktail
- Part 3: Screen for late reprogramming molecule
- Part 4: Resolve incomplete reprogramming
- Part 5: Optimize cocktail
- **Part 6: Screen for reprogramming booster**
- Part 7: Additional cells of origin
- Part 8: Characterize CiPSC lines
- Part 9: Determine essential small molecules
- Part 10: Investigate role of small molecules

Part 6: Screen for reprogramming booster

VC6TFZ + TTNPB: effect of TTNPB and characterization of generated CiPSCs

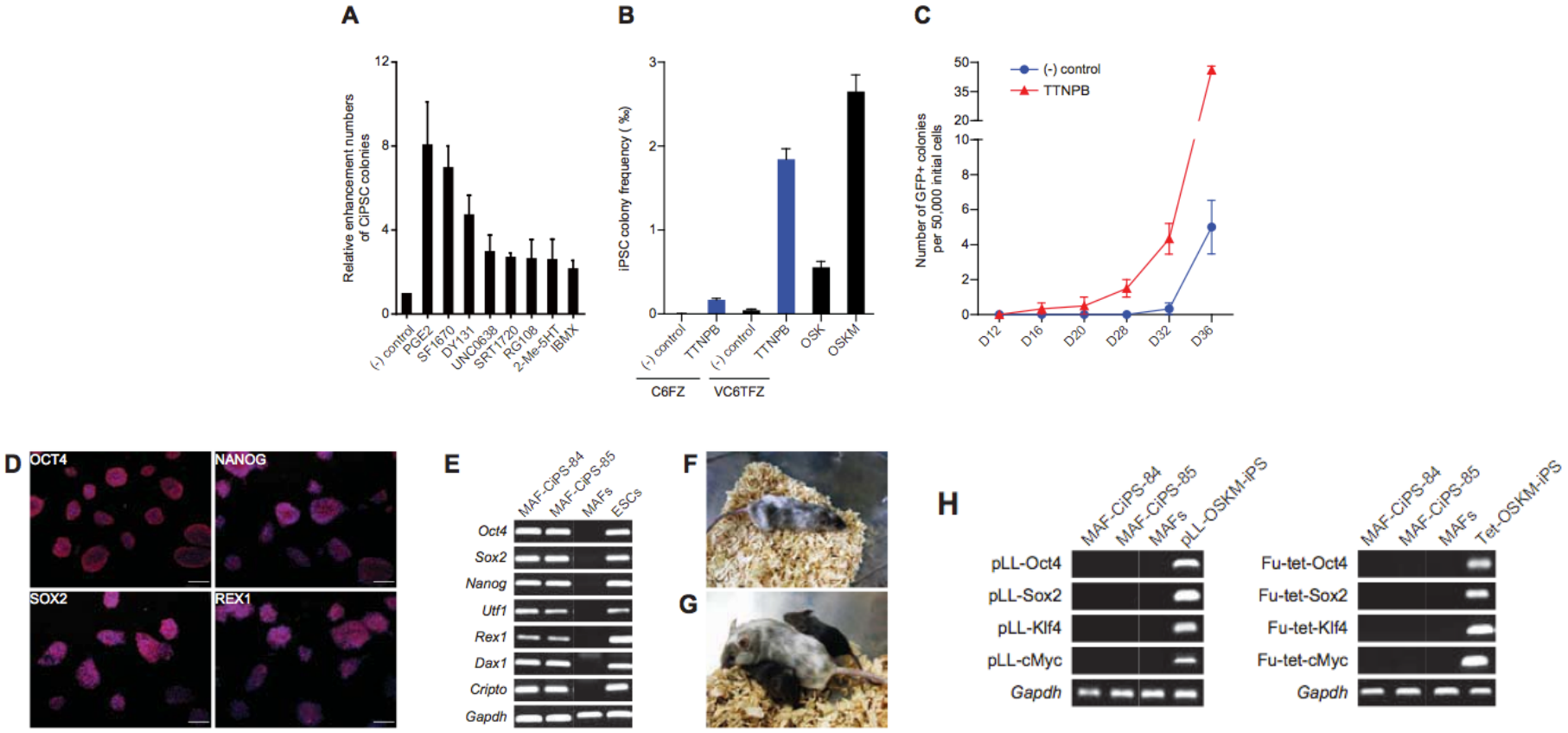


Fig. S8

Part 6: Screen for reprogramming booster

VC6TFZ + TTNPB: effect of TTNPB and characterization of generated CiPSCs

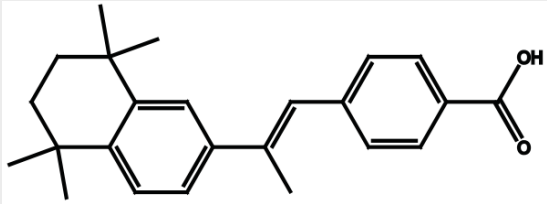
Name	Abbreviation	Function	Structure
TTNPB	N	Selective and highly potent retinoic acid analog with affinity for retinoic acid receptors (RAR) α, β, and γ, which are nuclear transcription factors. Produces ligand-activated transcription of genes that possess retinoic acid responsive elements.	

Table S1 (B)

- Part 1: Find Oct4 substitute
- Part 2: Test small molecule cocktail
- Part 3: Screen for late reprogramming molecule
- Part 4: Resolve incomplete reprogramming
- Part 5: Optimize cocktail
- Part 6: Screen for reprogramming booster
- **Part 7: Additional cells of origin**
- Part 8: Characterize CiPSC lines
- Part 9: Determine essential small molecules
- Part 10: Investigate role of small molecules

Part 7: Additional cells of origin

Methods:

- Plate cells: 50k/well; 6 well plate
- Replace medium with chemical reprogramming medium containing small molecule cocktail
- Change medium every 4 days
- DZNep added day 16 or day 20
- Small molecule cocktail (including DZNep) removed day 20; replace with 2i medium

H

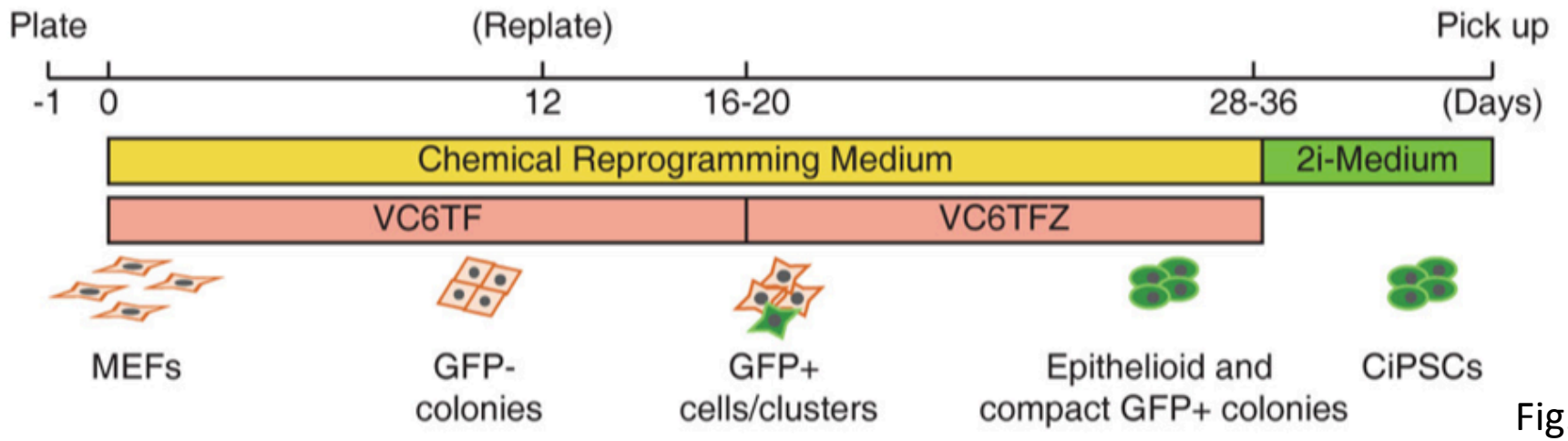


Fig. 1

Part 7: Additional cells of origin

VC6TFZ: morphology of CiPSC colonies generated from MNFs, MAFs, ADSCs and WT MEFs; genomic PCR analyzing pOct4-GFP cassettes in the CiPSCs derived from WT MEFs

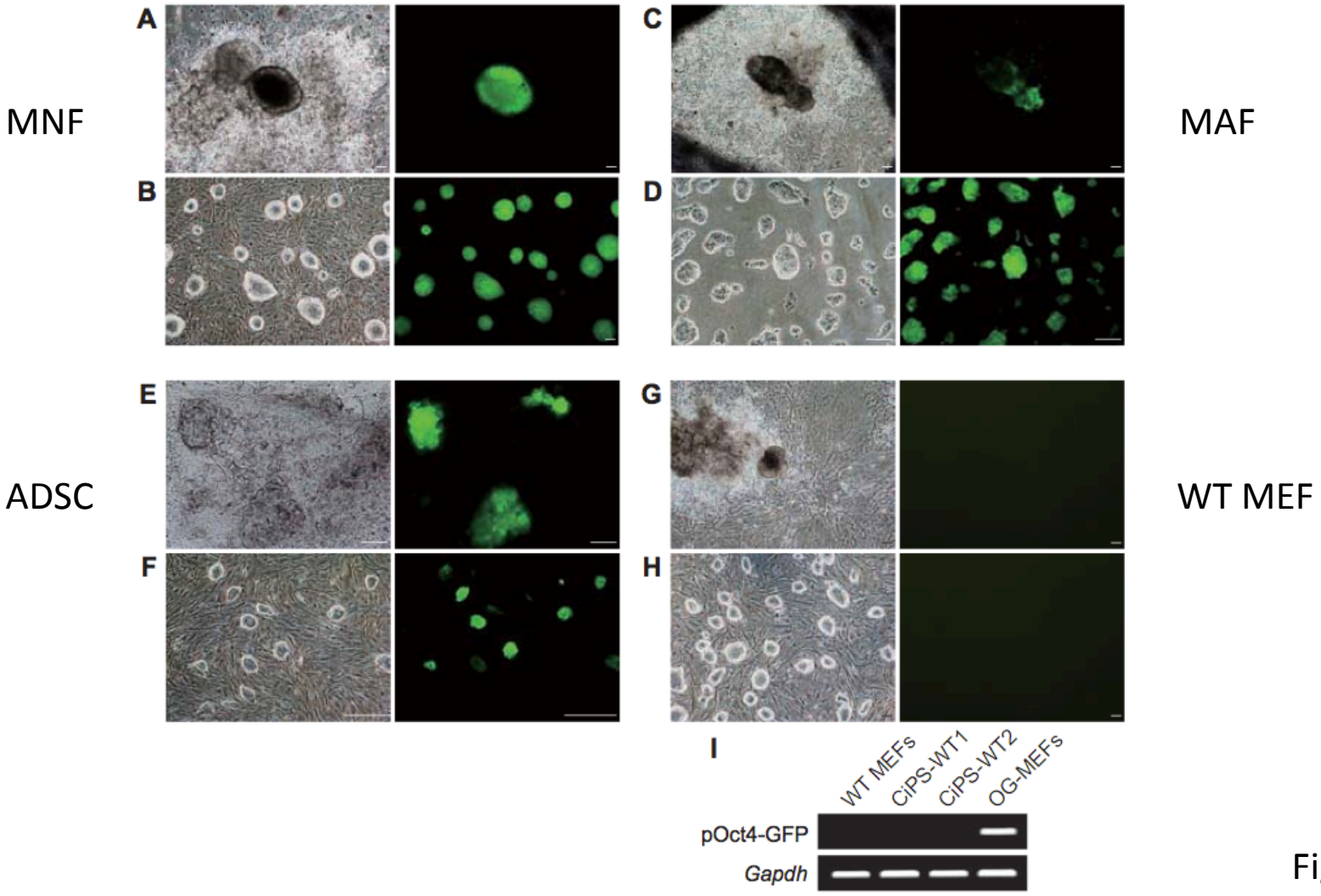
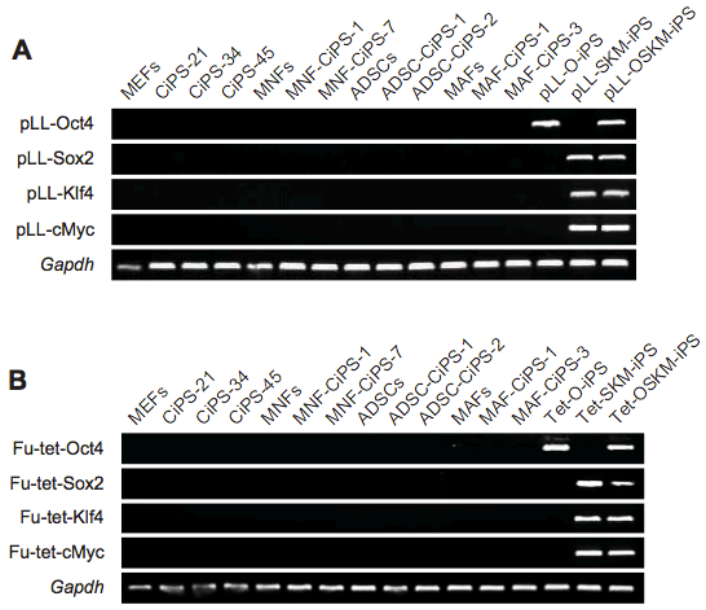


Fig. S9

- Part 1: Find Oct4 substitute
- Part 2: Test small molecule cocktail
- Part 3: Screen for late reprogramming molecule
- Part 4: Resolve incomplete reprogramming
- Part 5: Optimize cocktail
- Part 6: Screen for reprogramming booster
- Part 7: Additional cells of origin
- **Part 8: Characterize CiPSC lines**
- Part 9: Determine essential small molecules
- Part 10: Investigate role of small molecules

Part 8: Characterize CiPSC lines

VC6TFZ: CiPSCs free of transgene contamination



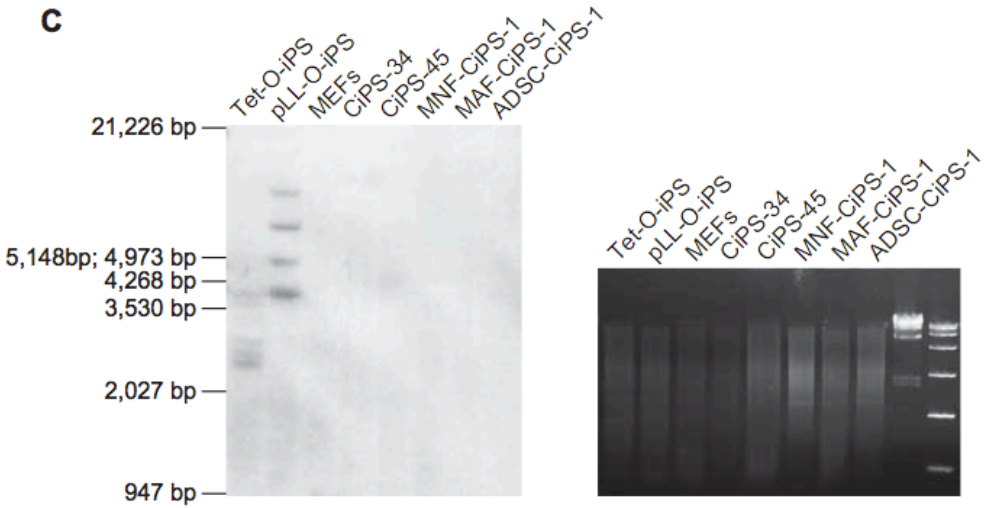


Fig. S10

Part 8: Characterize CiPSC lines

VC6TFZ: MEF-derived

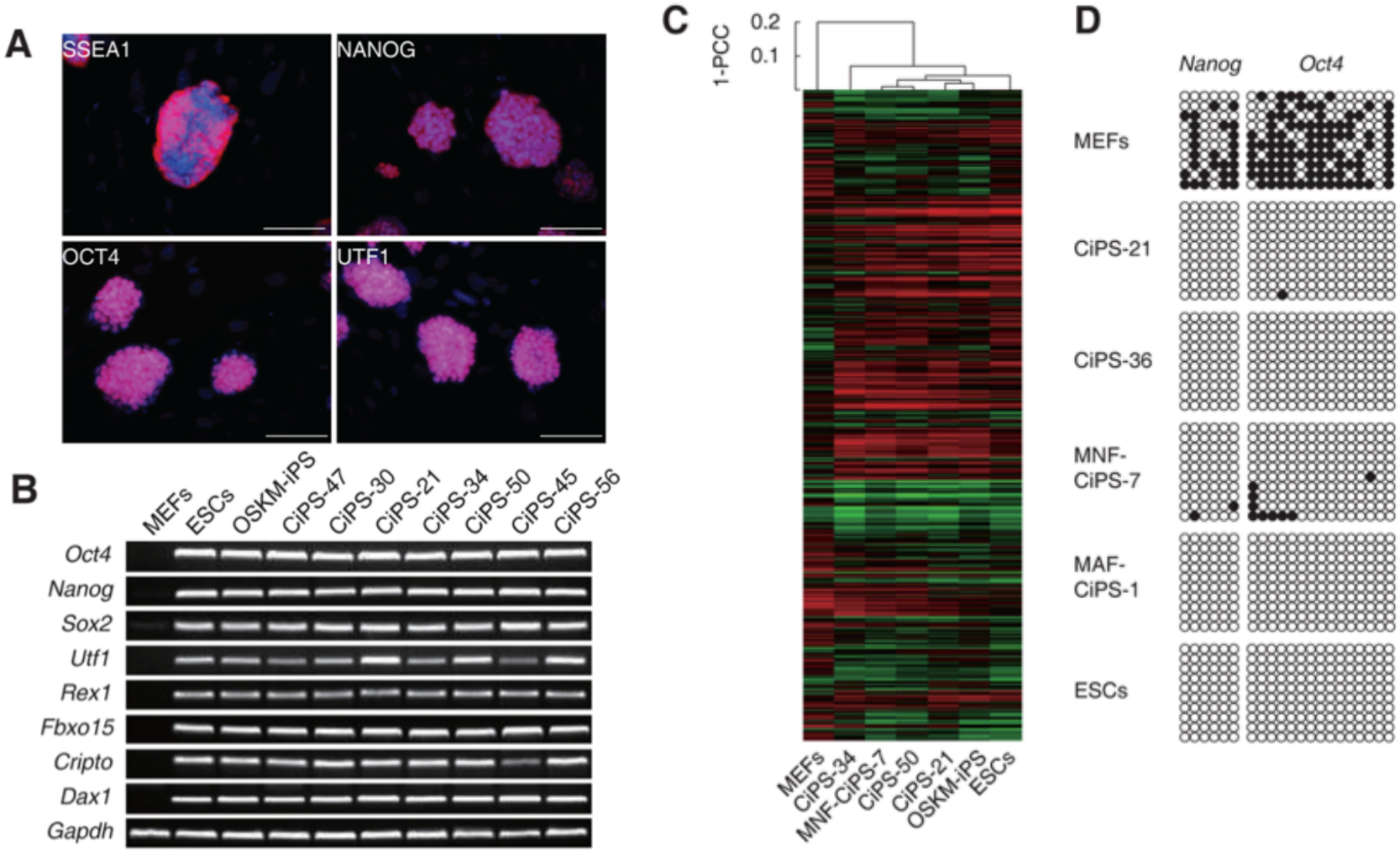


Fig. 2

Part 8: Characterize CiPSC lines

VC6TFZ

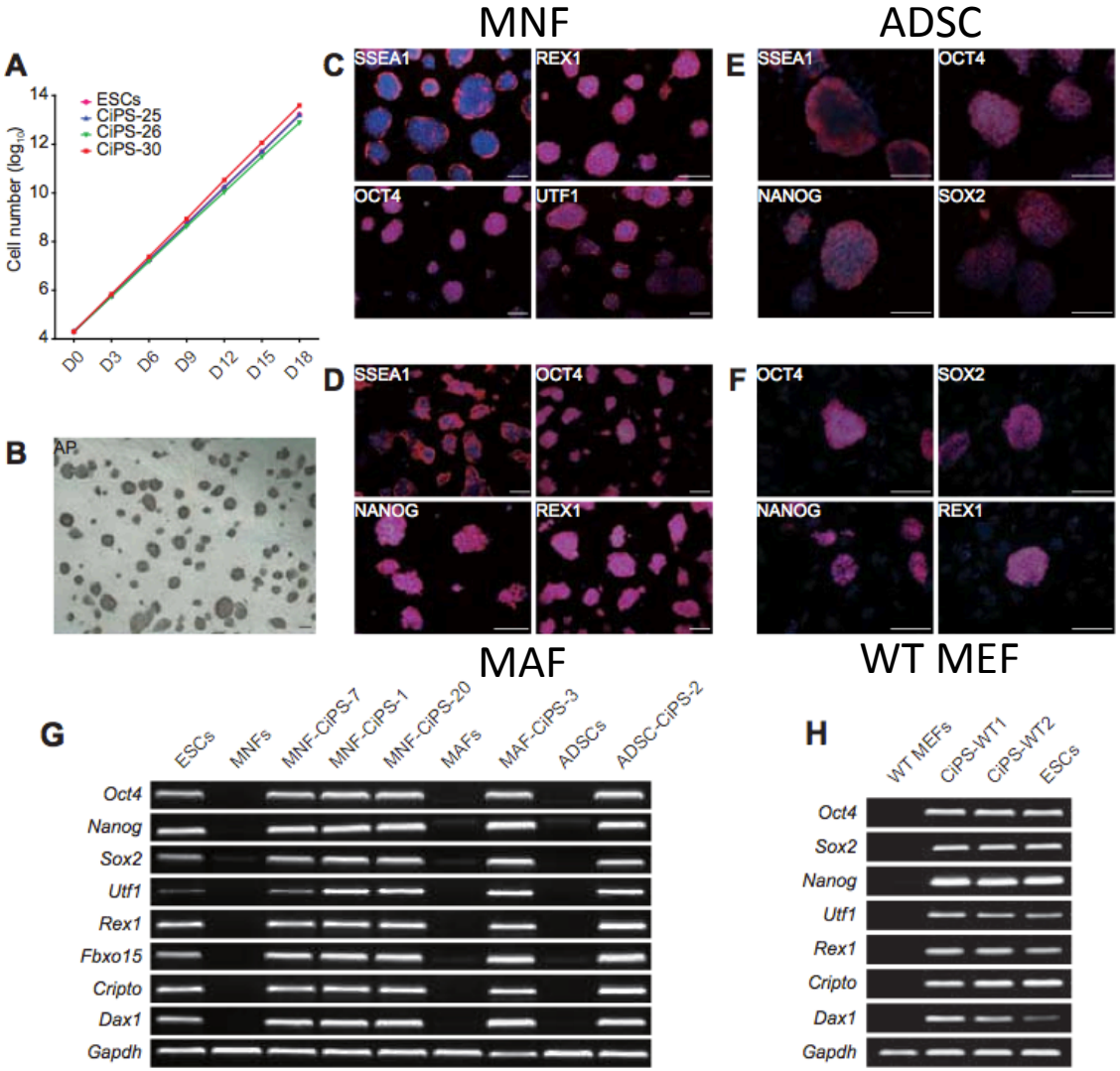
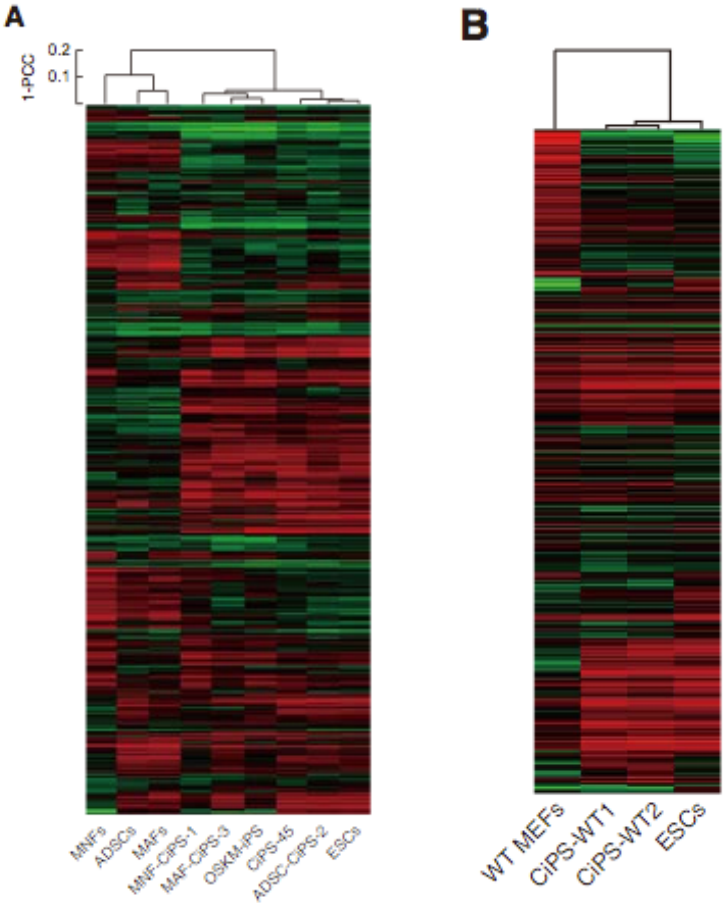
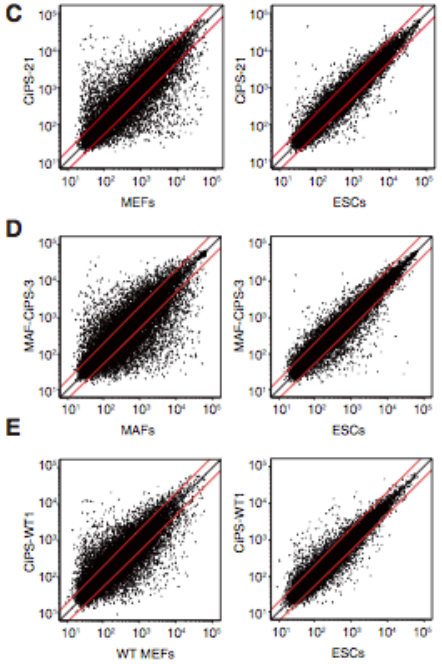


Fig. S11

Part 8: Characterize CiPSC lines

VC6TFZ





F

	MEFs	CiPS-34	CiPS-21	MNF-CiPS-7	CiPS-50	ESCs
MEFs	1	0.806	0.803	0.803	0.798	0.771
CiPS-34		1	0.959	0.963	0.956	0.922
CiPS-21			1	0.966	0.962	0.948
MNF-CiPS-7				1	0.961	0.941
CiPS-50					1	0.949
ESCs						1

G

	MAFs	MAF-CiPS-3	ESCs
MAFs	1	0.766	0.746
MAF-CiPS-3		1	0.946
ESCs			1

H

	WT MEFs	CiPS-WT1	CiPS-WT2	ESCs
WT MEFs	1	0.808	0.809	0.742
CiPS-WT1		1	0.981	0.948
CiPS-WT2			1	0.944
ESCs				1

Fig. S12

Part 8: Characterize CiPSC lines

VC6TFZ: Histone H3 modifications at Oct4, Sox2 and Nanog promoter regions

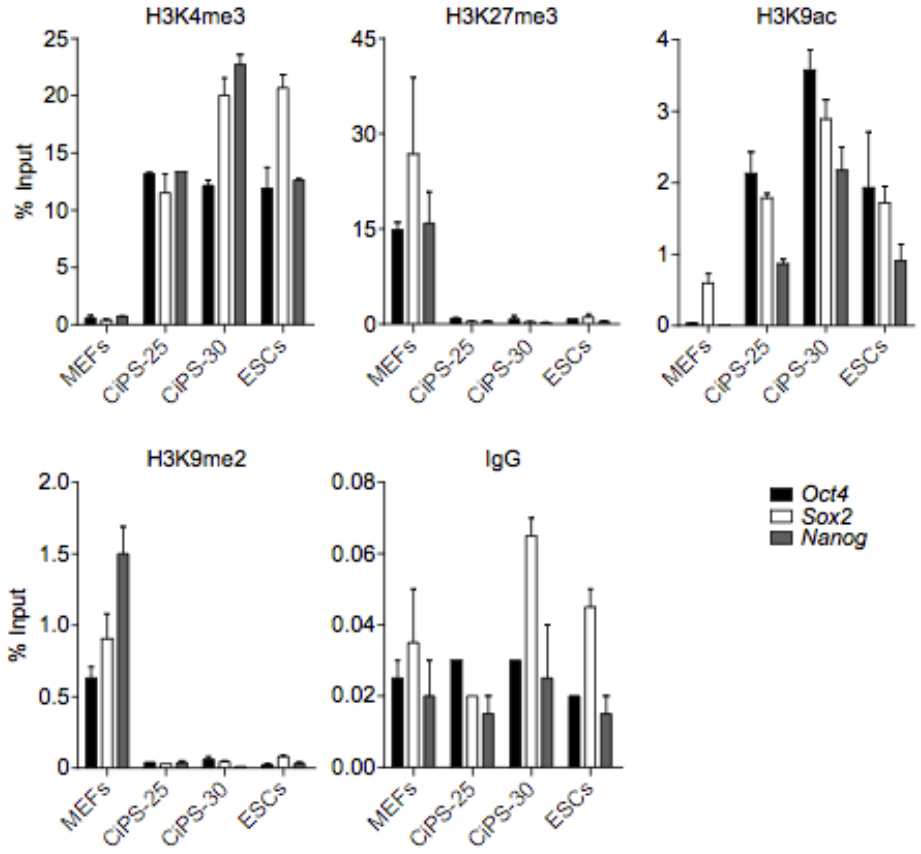
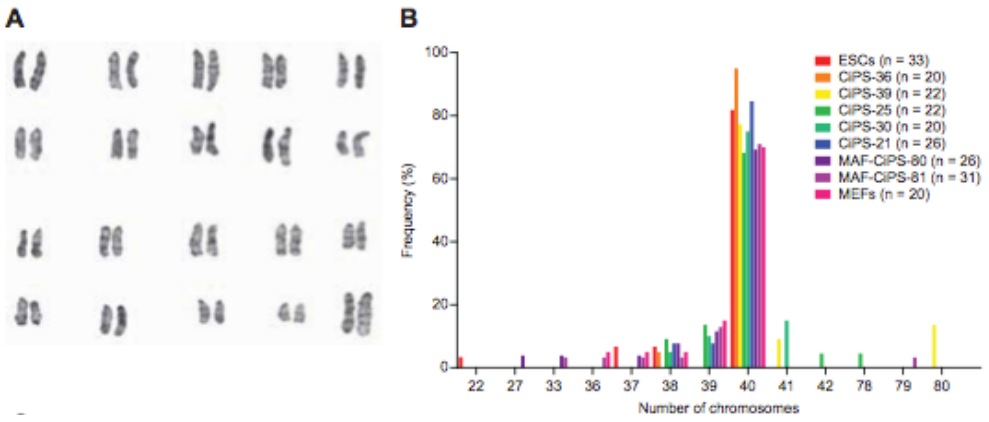
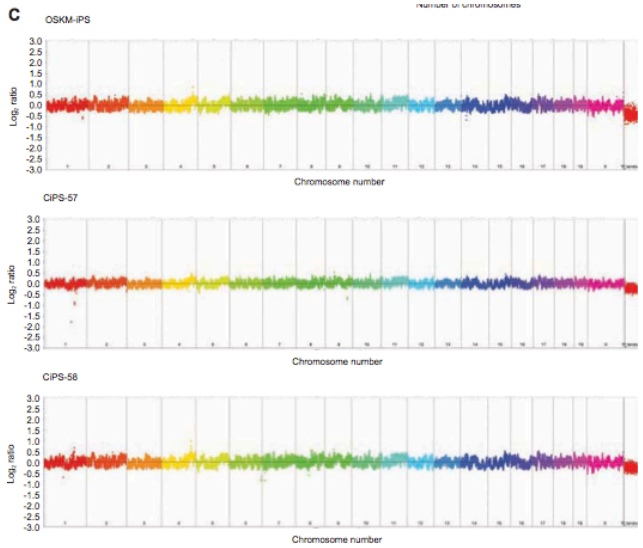


Fig. S13

Part 8: Characterize CiPSC lines

VC6TFZ: genetic integrity of CiPSCs





[Fig. S14]

Part 8: Characterize CiPSC lines

VC6TFZ: pluripotency of CiPSCs

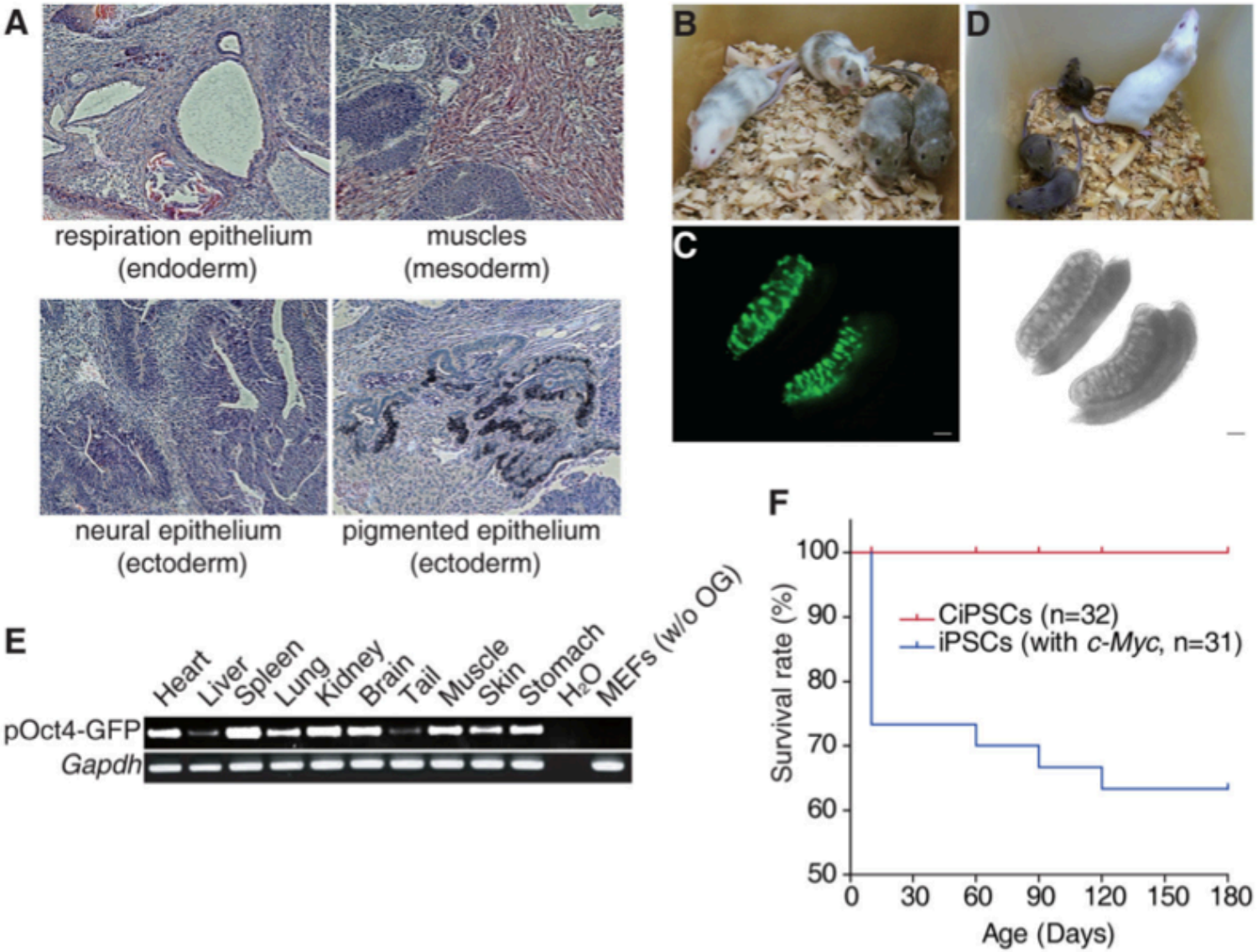


Fig. 3

Part 8: Characterize CiPSC lines

VC6TFZ: pluripotency of CiPSCs

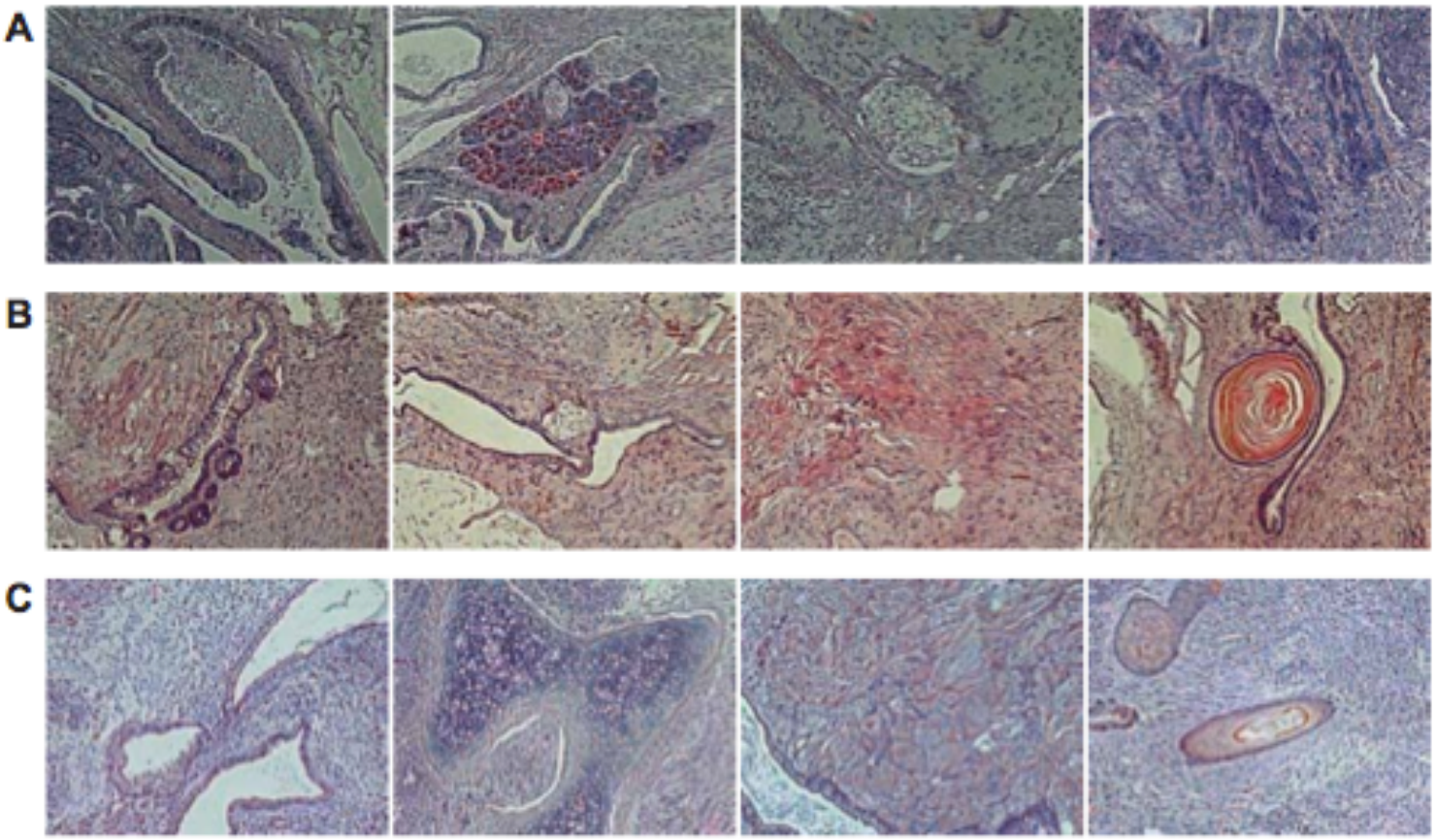
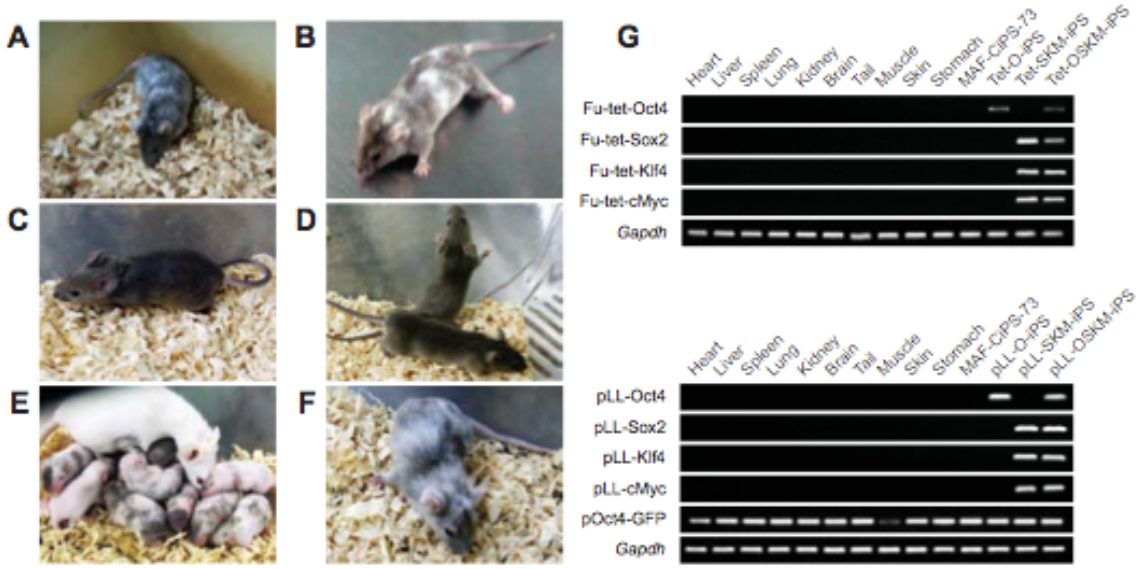


Fig. S15

Part 8: Characterize CiPSC lines

VC6TFZ: in vivo developmental potential of CiPSCs



H

Clone number	Injected 8-cell embryos	Number of born mice	Number of live chimeras	Chimerism (%)	Integration into gonads (E13.5d)	Transmission into next generation
CiPS-21	24	9	2♂	100%, 95%	–	Yes
CiPS-34	12	7	2♂, 2♀	100%, 90%, 60%, 50%	Yes	Yes
CiPS-36	37	16	5♂	95%, 60%, 30%, 20%, 10%	Yes	No
CiPS-45	14	10	2♂, 1♀	70%, 50%, 40%	Yes	–
MAF-CiPS-3	12	4	1♂,1♀	95%, 80%	Yes	No
MAF-CiPS-62	10	5	1♀	90%	–	No
MAF-CiPS-63	14	4	2♂	40%, 20%	–	–
MAF-CiPS-73	34	6	1♂,1♀	60%, 40%	Yes	–

I

Clone number	Injected blastocysts	Number of born mice	Number of live chimeras	Chimerism (%)	Integration into gonads (E13.5d)	Transmission into next generation
CiPS-34	38	19	12	95%, 95%, 95%, 80%, et al.	–	–
CiPS-47	37	17	13	90%, 90%, 80%, 80%,et al.	–	–
MAF-CiPS-62	56	13	7	90%, 75%, 75%, 50%, et al.	Yes	Yes
MAF-CiPS-63	25	15	7	90%, 80%, 70%, 60%, et al.	–	Yes
MAF-CiPS-73	26	11	5	80%, 80%, 70%, 70%, et al.	Yes	–
MAF-CiPS-80	14	4	4	95%,80%,20%,10%	Yes	–
MAF-CiPS-83	14	6	5	80%,70%, 50%, 50%, 40%	–	–

Fig. S16

- Part 1: Find Oct4 substitute
- Part 2: Test small molecule cocktail
- Part 3: Screen for late reprogramming molecule
- Part 4: Resolve incomplete reprogramming
- Part 5: Optimize cocktail
- Part 6: Screen for reprogramming booster
- Part 7: Additional cells of origin
- Part 8: Characterize CiPSC lines
- **Part 9: Determine essential small molecules**
- Part 10: Investigate role of small molecules

Part 9: Determine essential small molecules

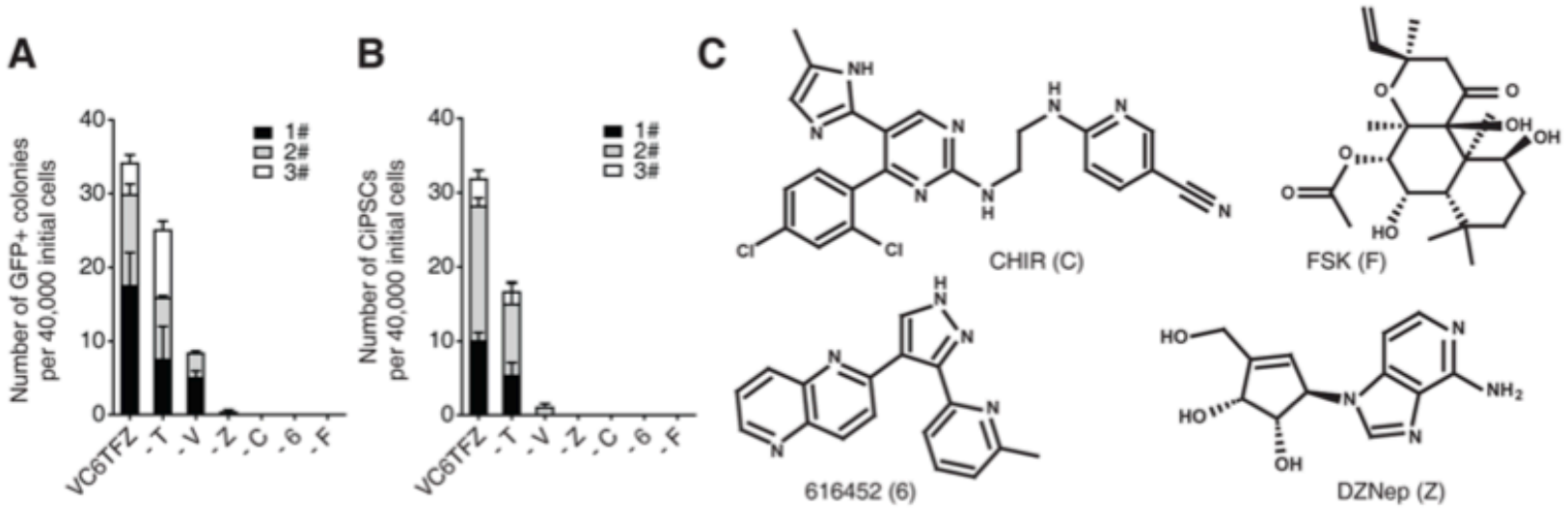


Fig. 4

Part 9: Determine essential small molecules

Characterization of CiPSCs induced by C6FZ

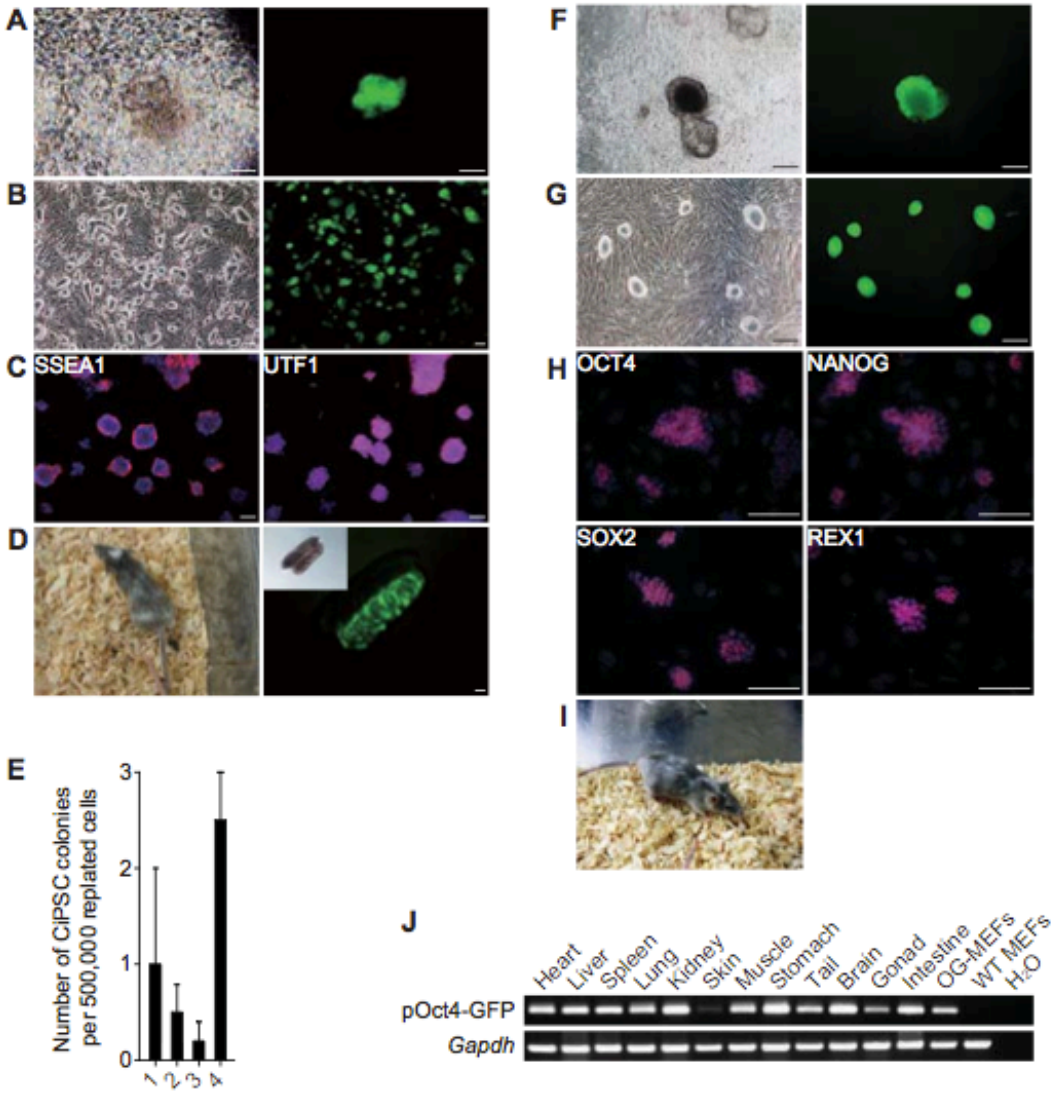


Fig. S20

- Part 1: Find Oct4 substitute
- Part 2: Test small molecule cocktail
- Part 3: Screen for late reprogramming molecule
- Part 4: Resolve incomplete reprogramming
- Part 5: Optimize cocktail
- Part 6: Screen for reprogramming booster
- Part 7: Additional cells of origin
- Part 8: Characterize CiPSC lines
- Part 9: Determine essential small molecules
- **Part 10: Investigate role of small molecules**

Part 10: Investigate role of small molecules

Biological activity of FSK during chemical reprogramming

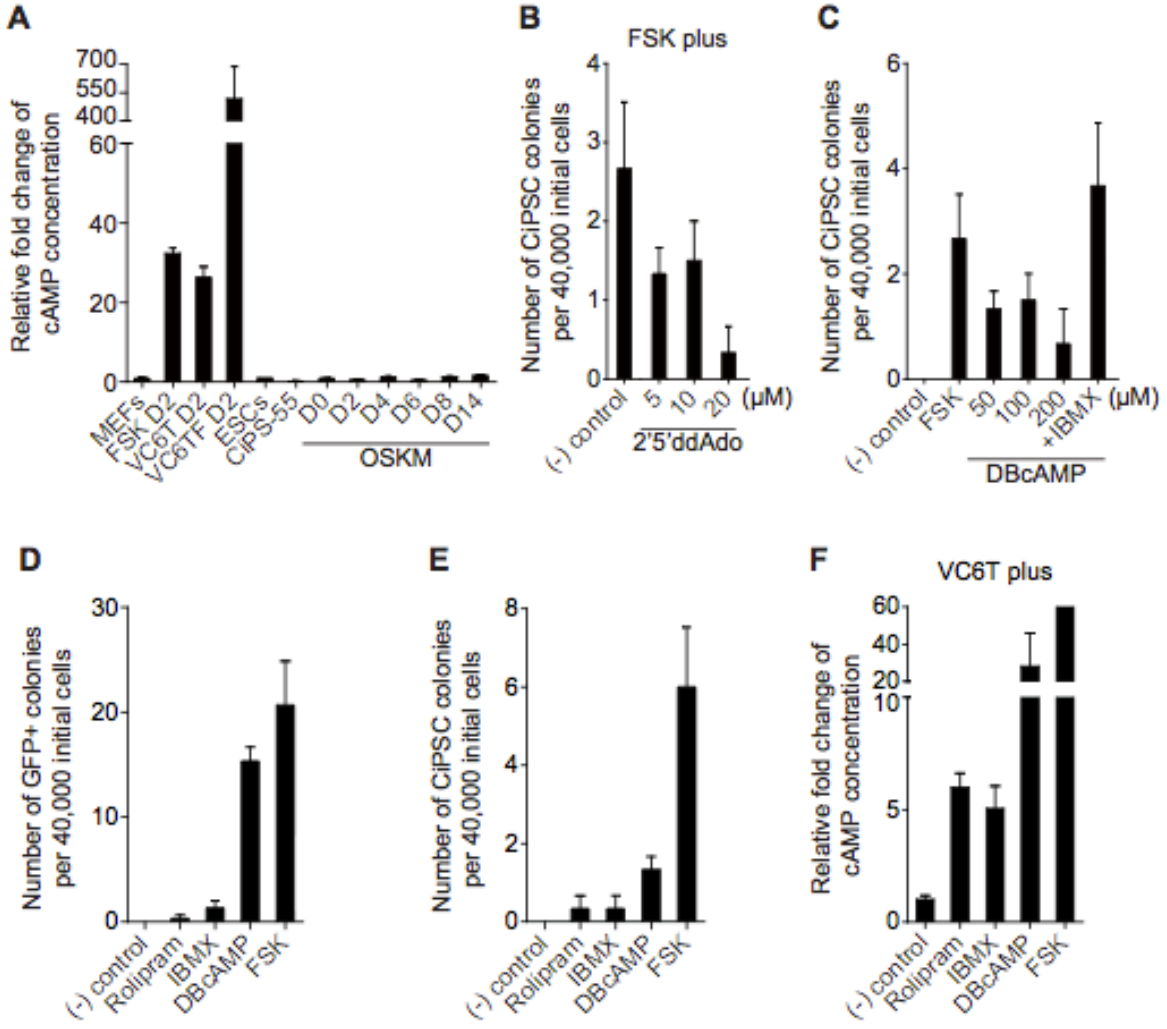


Fig. S17

Part 10: Investigate role of small molecules

Function of DZNep in chemical reprogramming

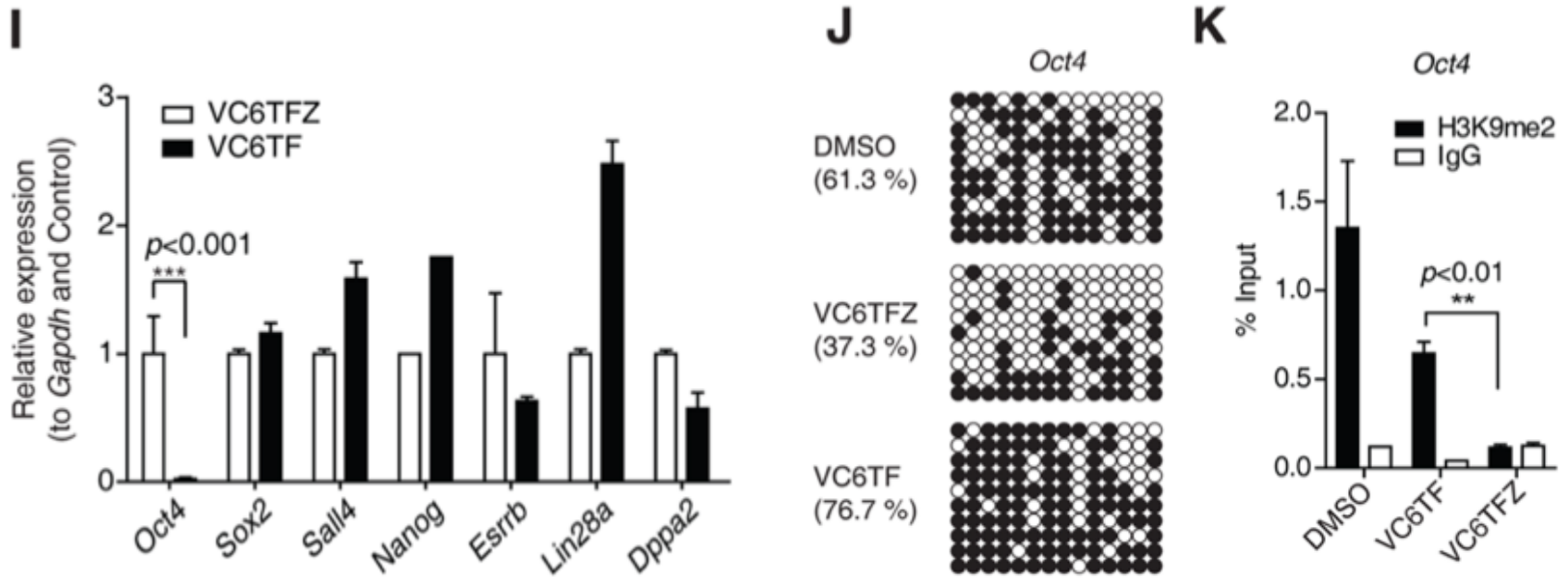


Fig. 4

Part 10: Investigate role of small molecules

Function of DZNep in chemical reprogramming

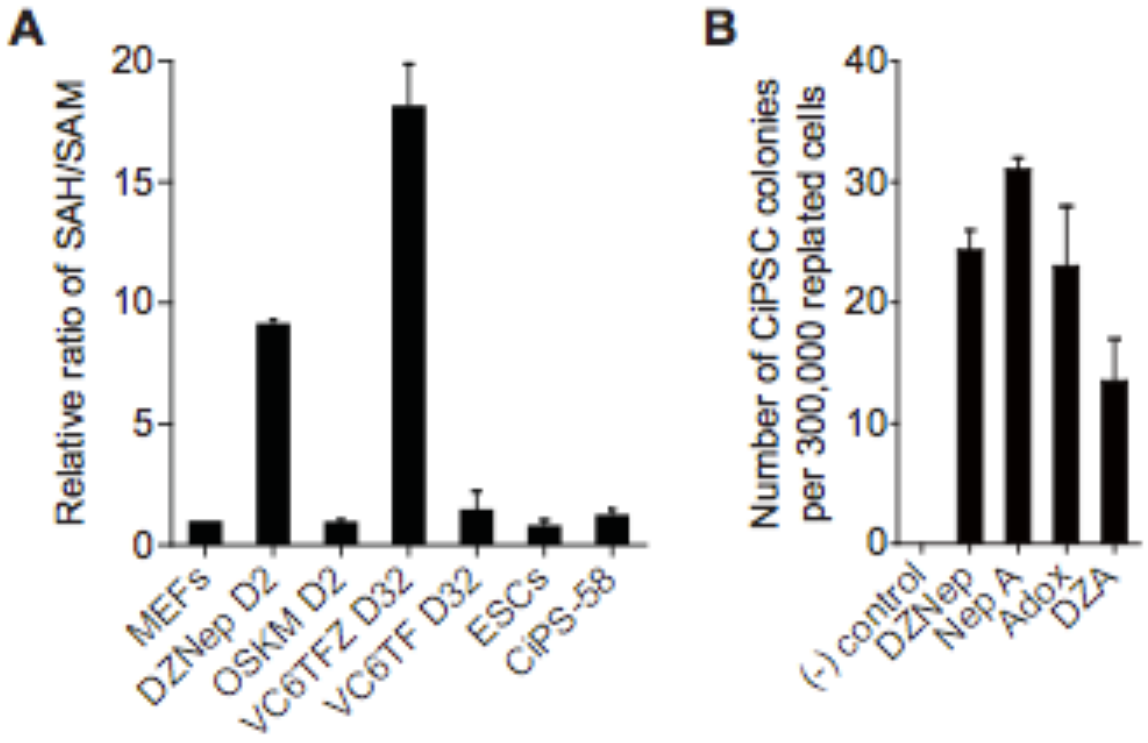


Fig. S18

Part 10: Investigate role of small molecules

Function of DZNep in chemical reprogramming

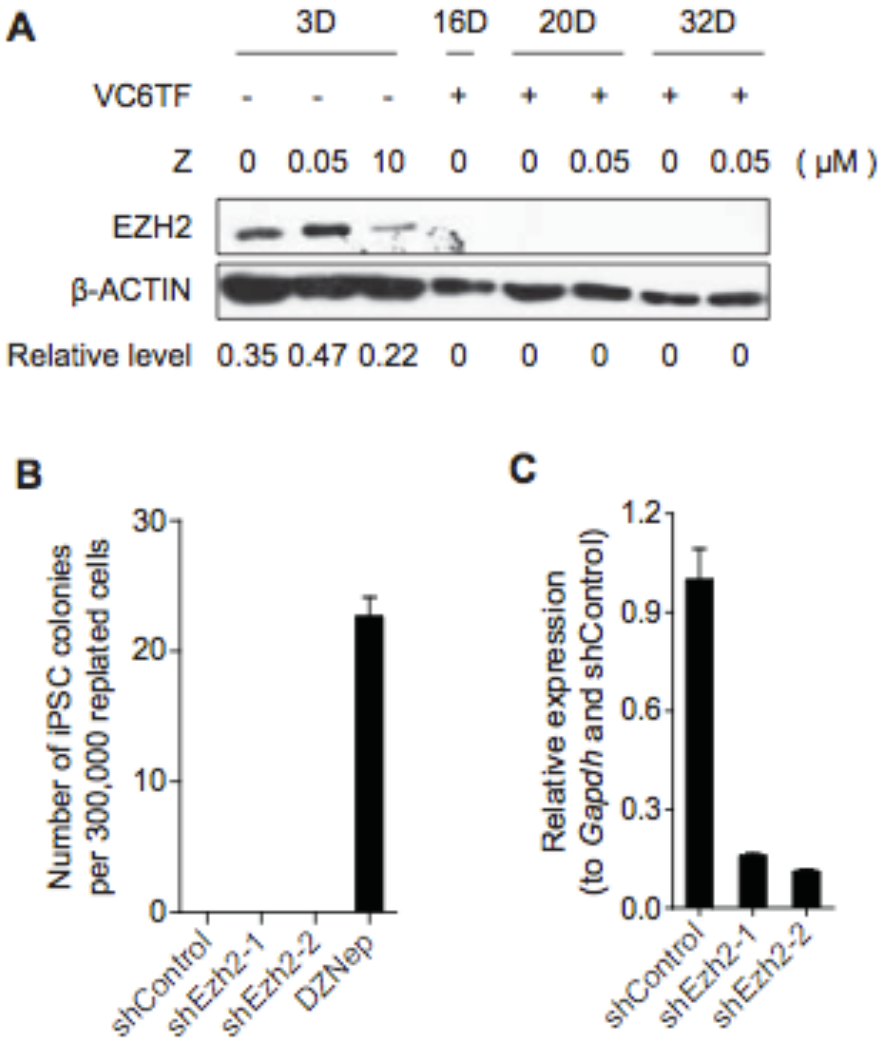


Fig. S19

Part 10: Investigate role of small molecules

VC6TFZ: Gene expression during chemical reprogramming

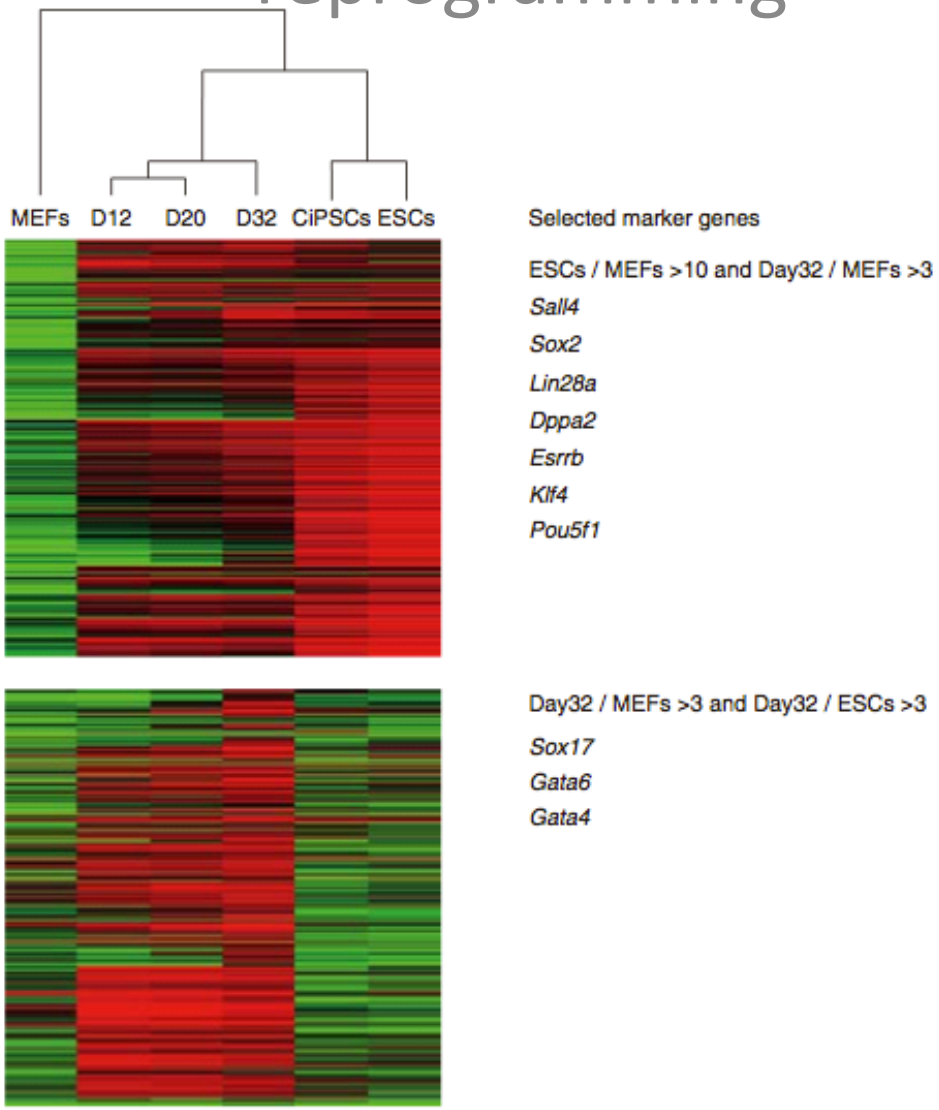


Fig. S21

Part 10: Investigate role of small molecules

VC6TFZ: Gene expression during chemical reprogramming

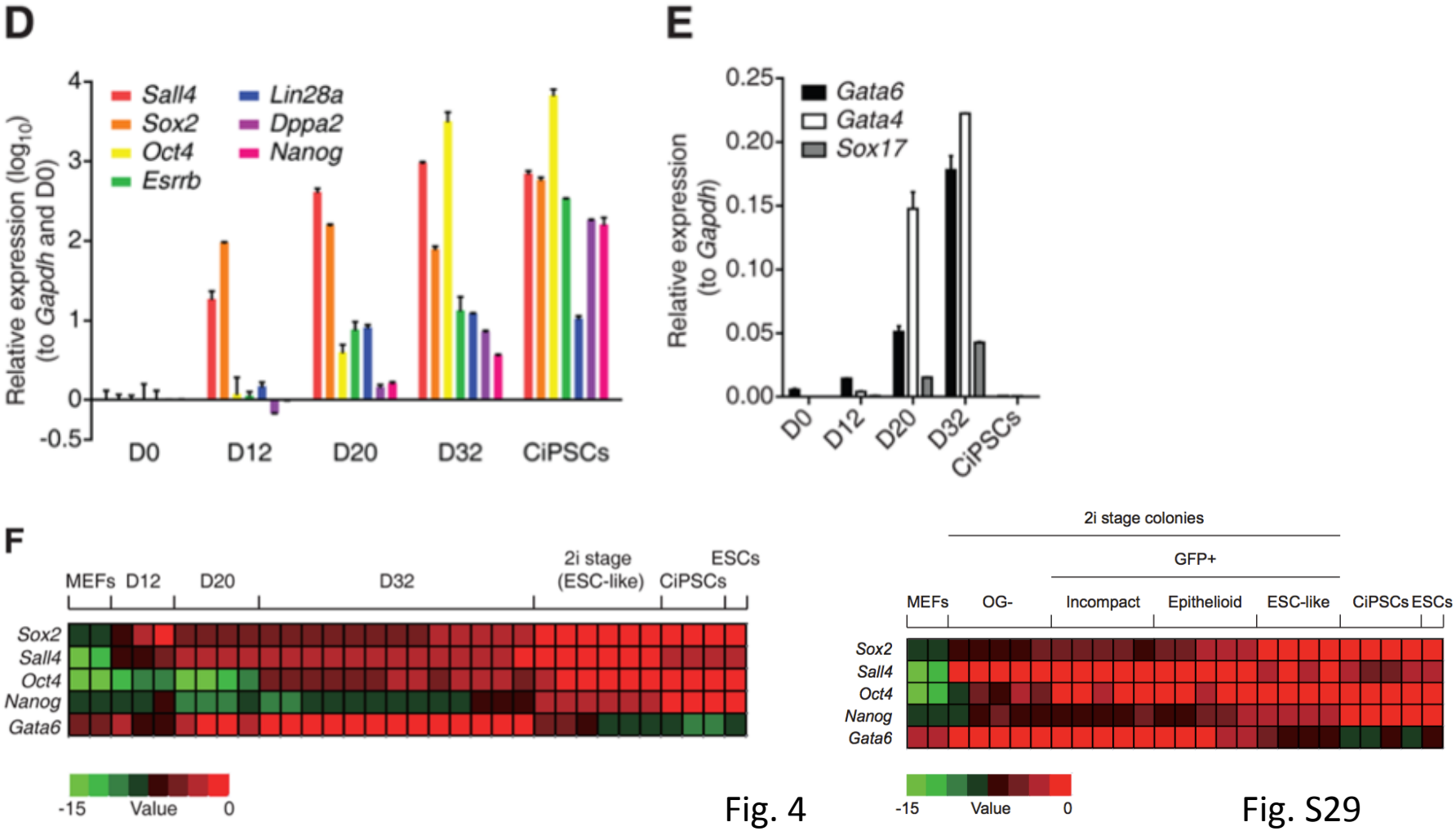
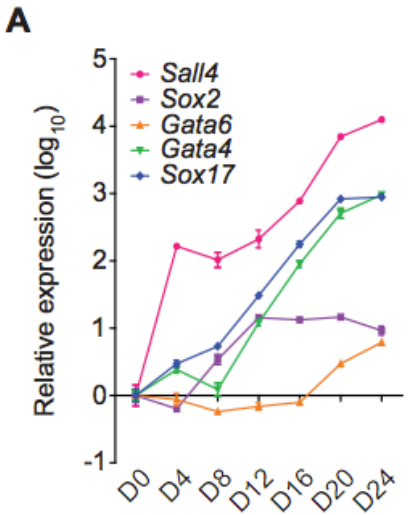


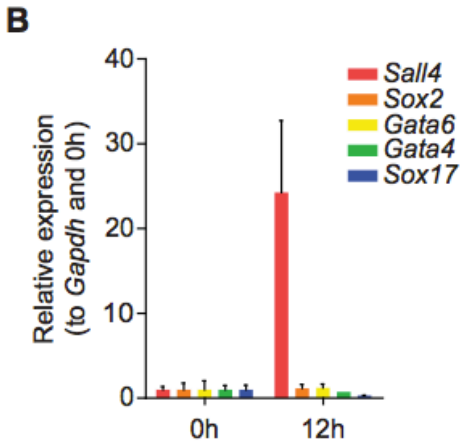
Fig. 4

Fig. S29

Part 10: Investigate role of small molecules

VC6TFZ: Gene expression during chemical reprogramming





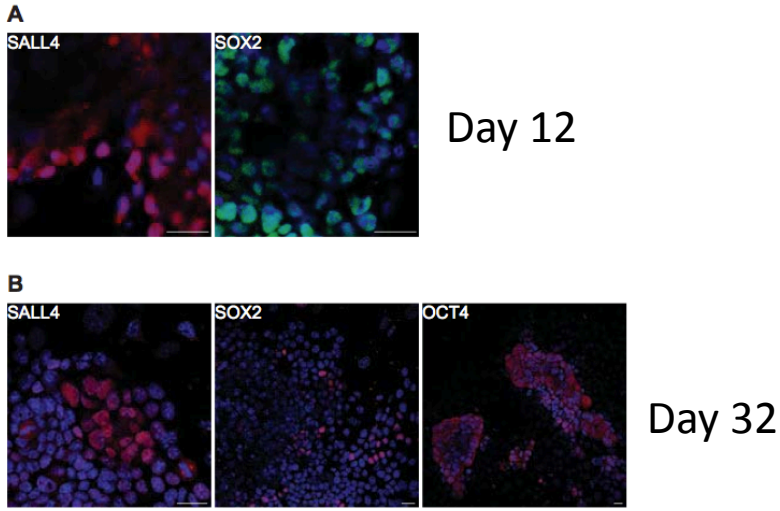


Fig. S22 & S23

Part 10: Investigate role of small molecules

VC6TFZ: effect of specific chemicals on gene expression

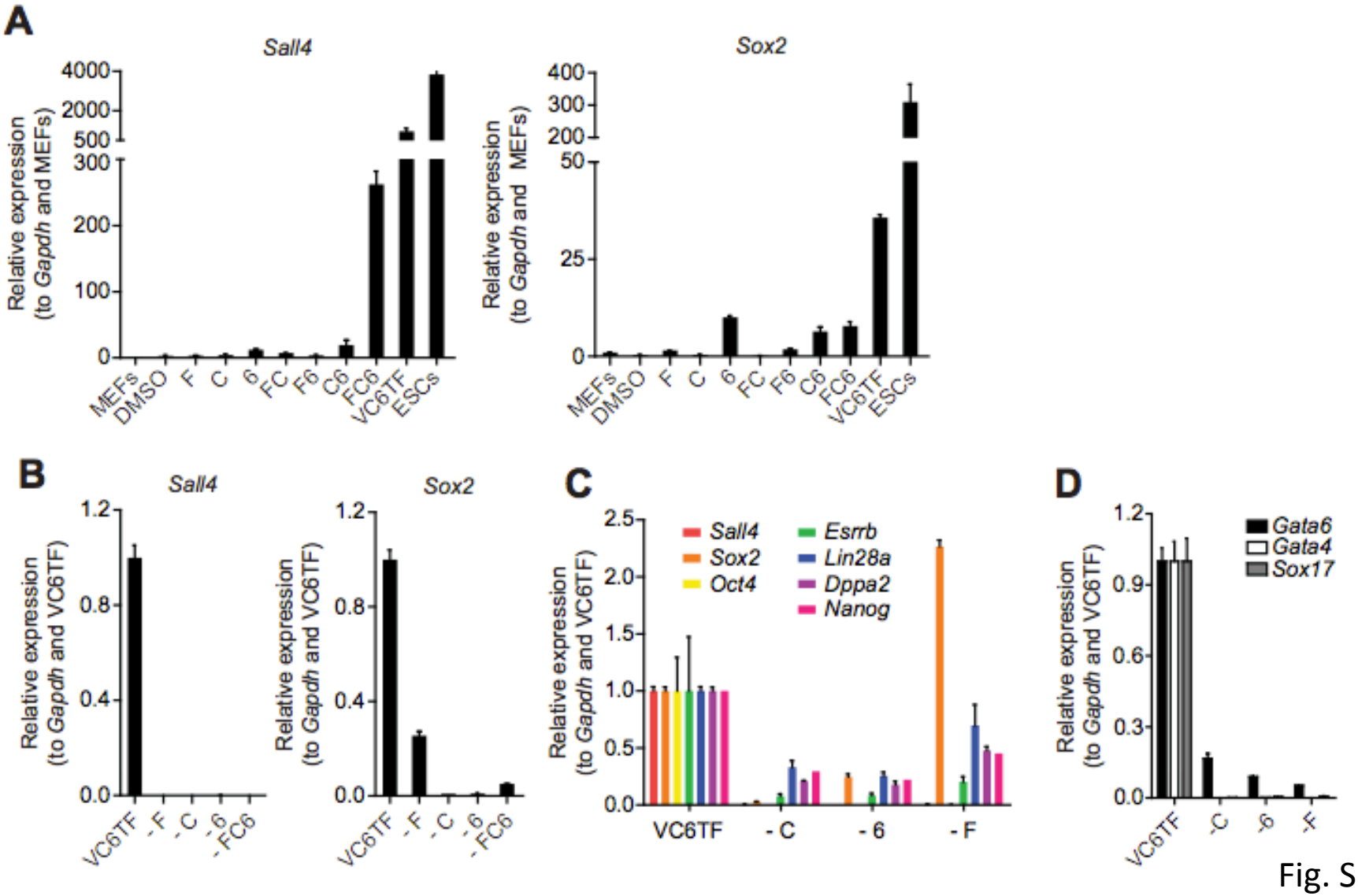


Fig. S24

Part 10: Investigate role of small molecules

Overexpression of Sall4 and Sox2: Oct-4 promoter-driven luciferase reporter

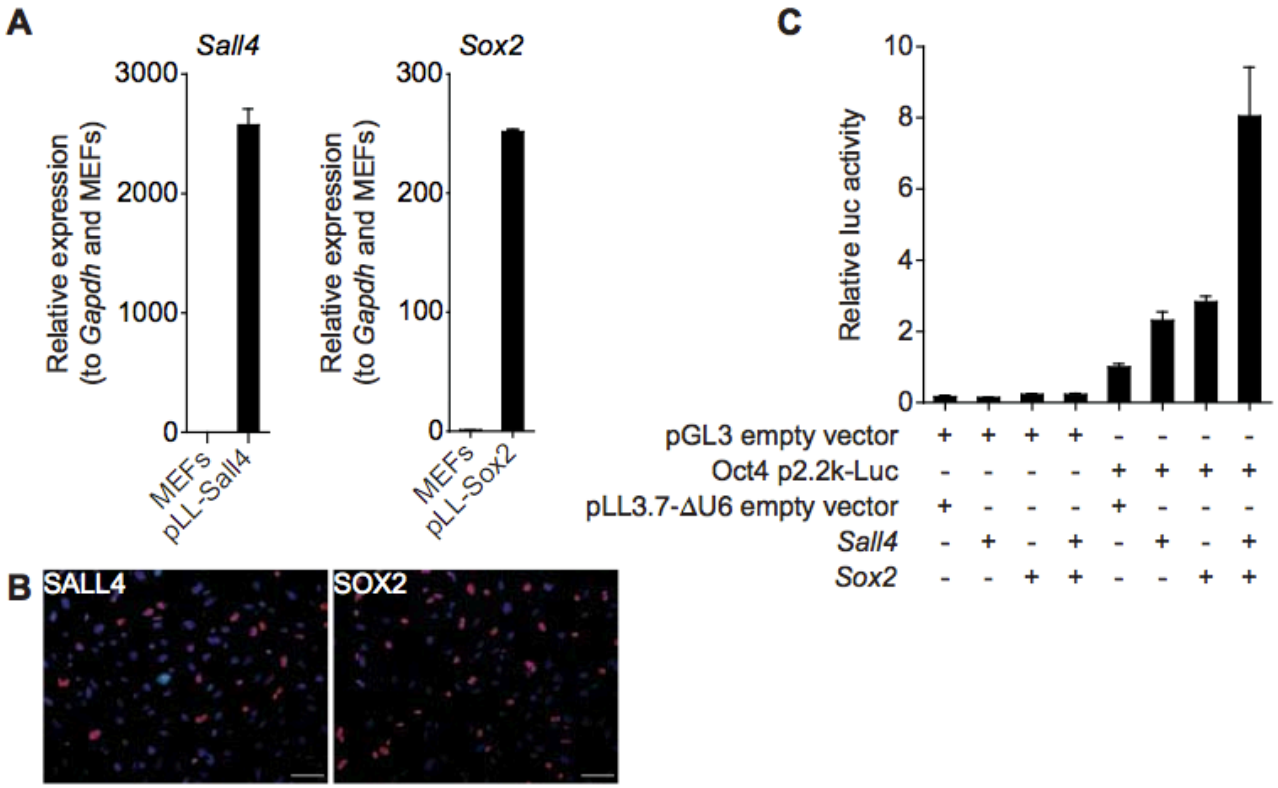


Fig. S25

Part 10: Investigate role of small molecules

VTZ: overexpression of Sall4 and Sox2

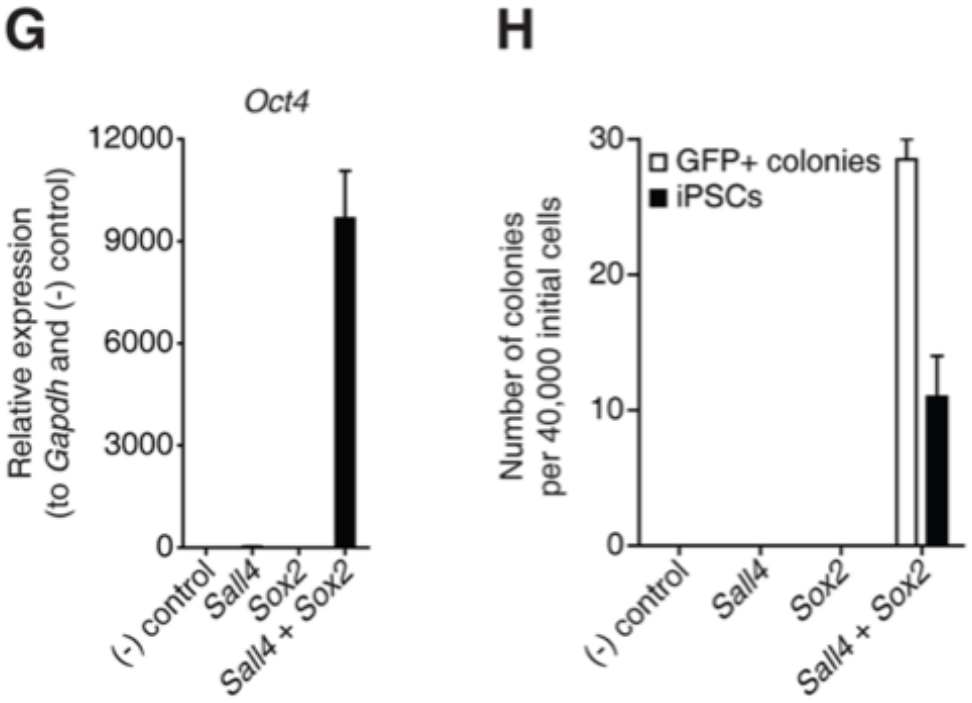


Fig. 4

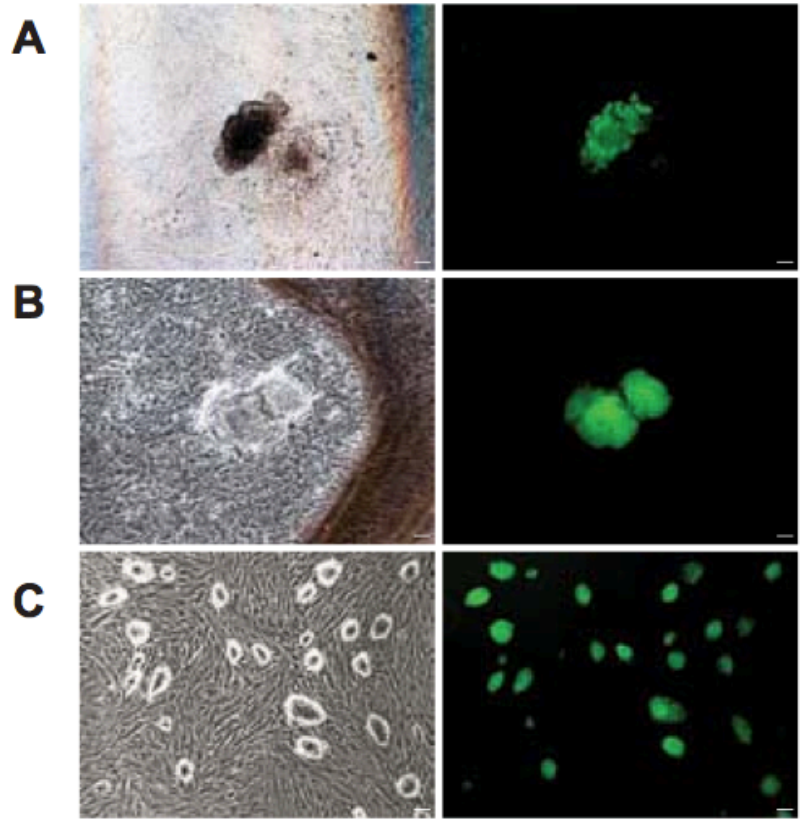


Fig. S26

Part 10: Investigate role of small molecules

VC6TFZ: Effects of knockdown on gene expression

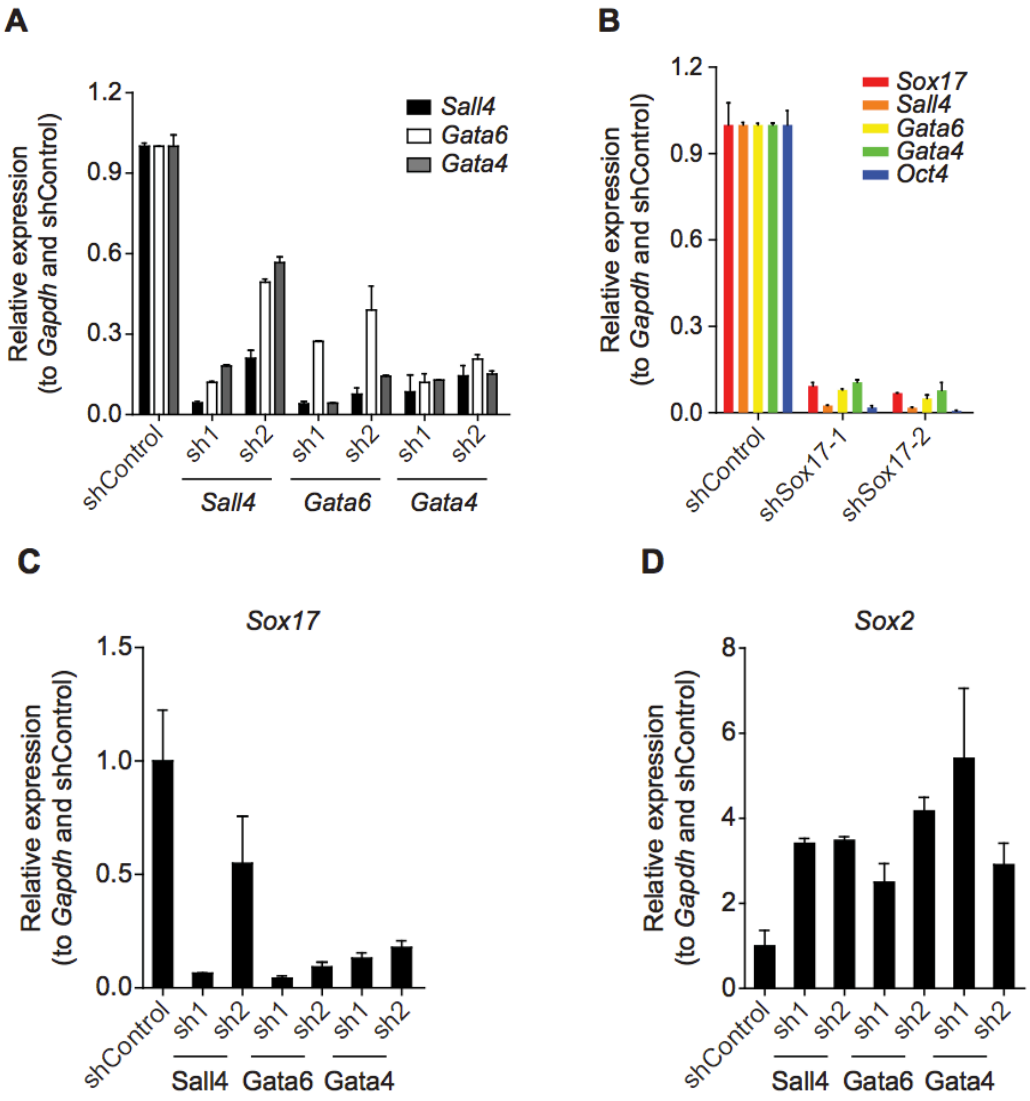


Fig. S27

Part 10: Investigate role of small molecules

VC6TFZ: Effects of knockdown on expression of Oct4 and iPSC formation

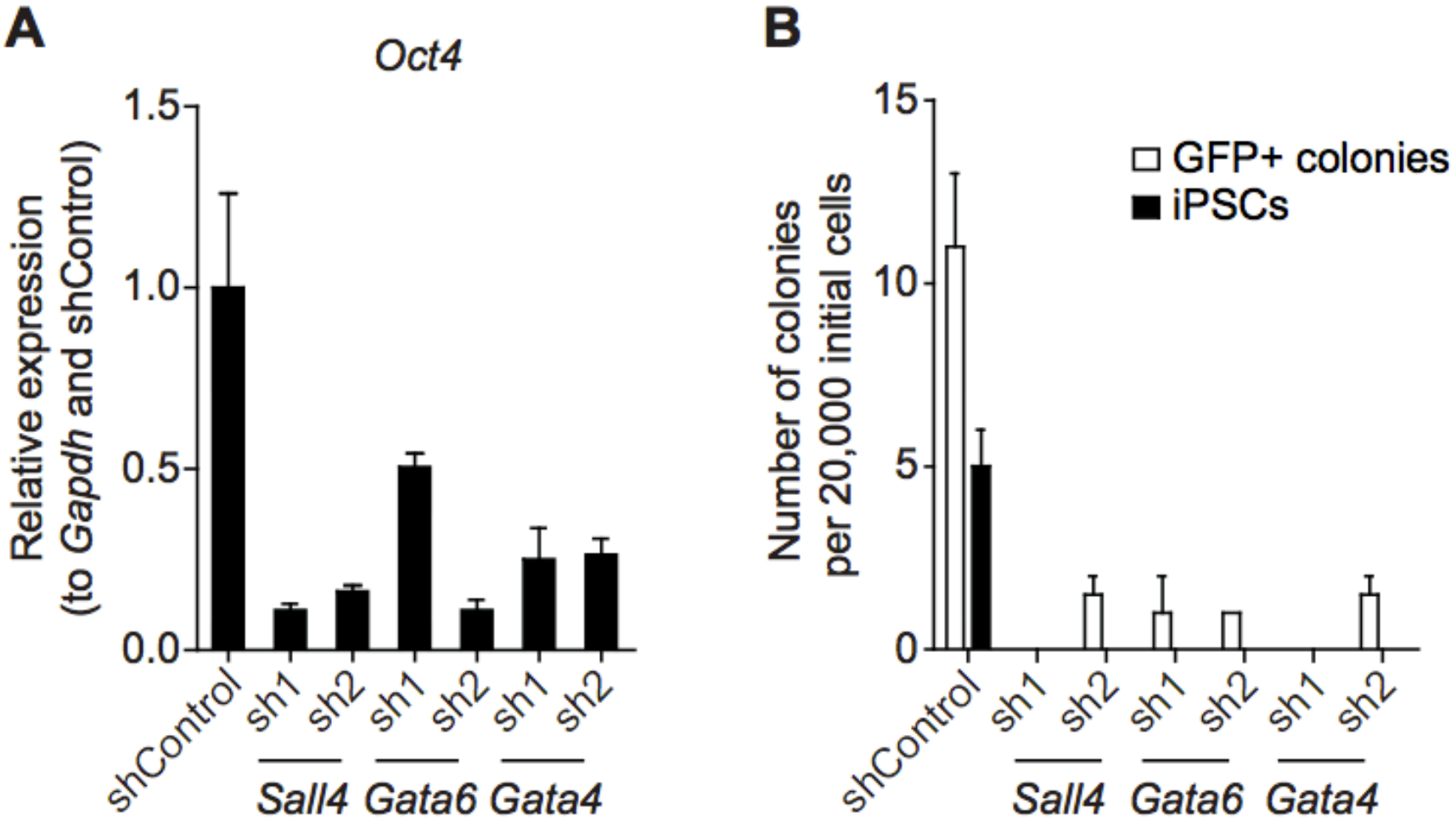


Fig. S28

Part 10: Investigate role of small molecules

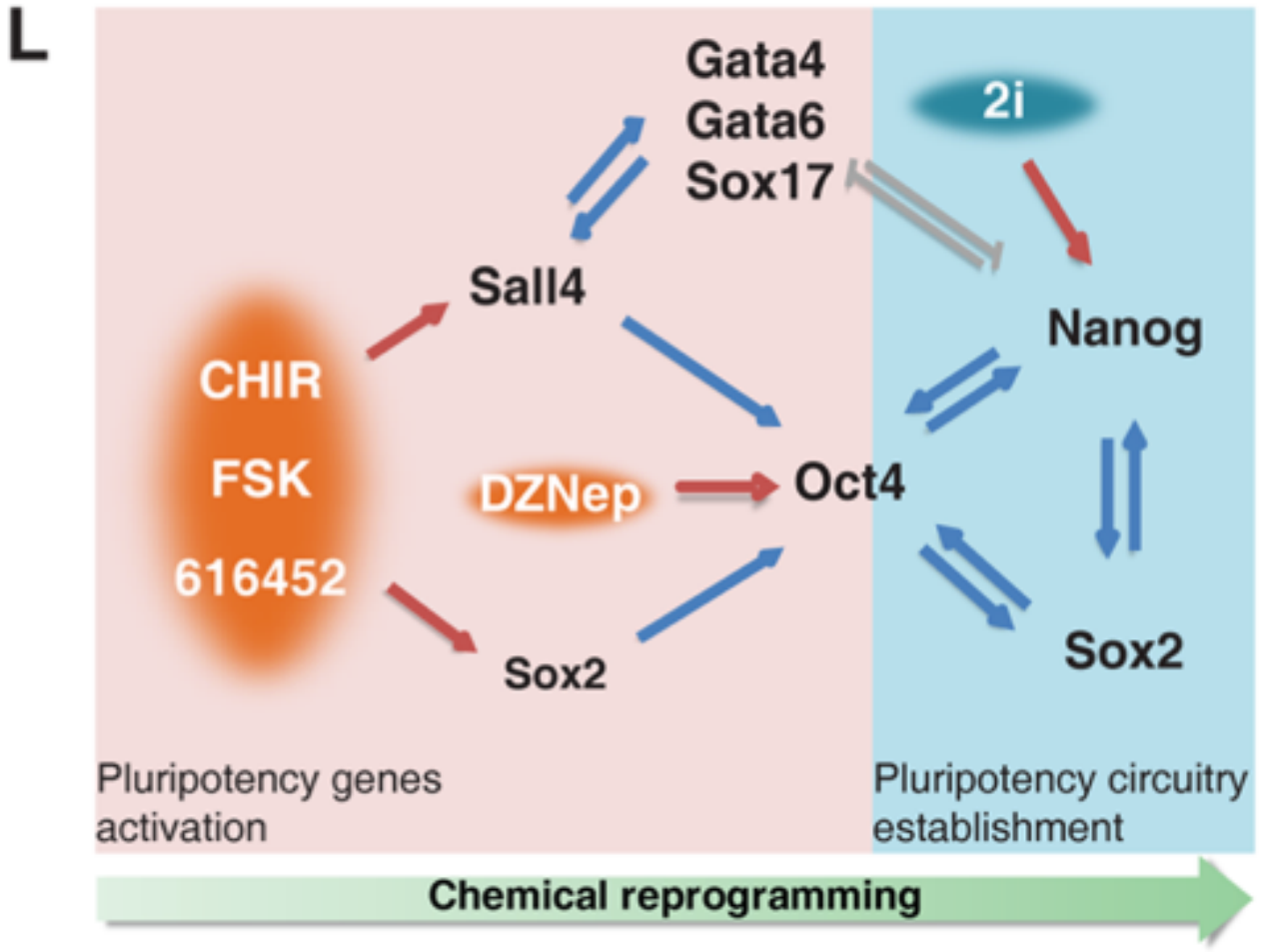


Fig. 4

Summary

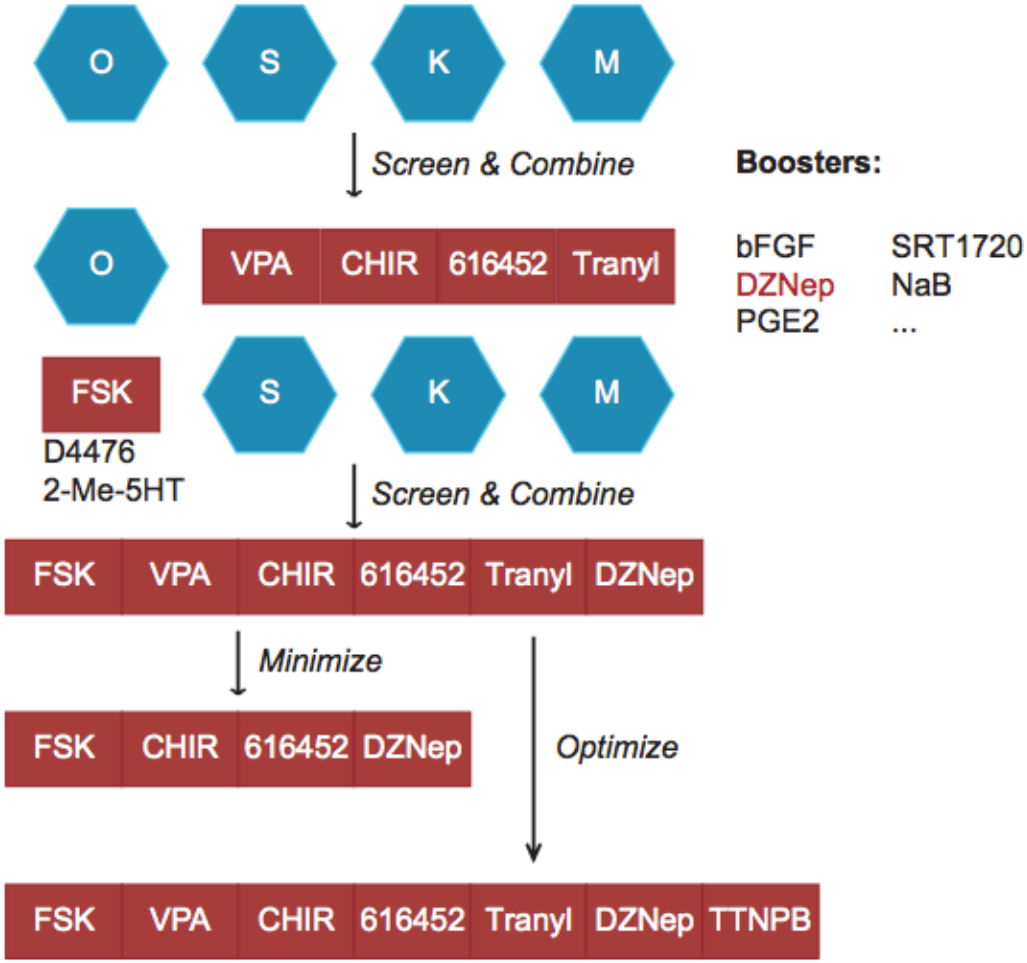


Fig. S30

What's next

- Human somatic cells
- Improve efficiency
- Differentiation of CiPSCs
- Direct reprogramming

Additional information

Part 1: Find Oct4 Substitute

Characterization of iPSC colonies induced from SKM or SK-infected MEFs with FSK treatment

Methods:

- Immunofluorescence
  - -Primary antibodies: SSEA-1, OCT4, NANOG, UTF1
  - -Secondary antibodies: Rhodamine-conjugated
- Chimera:
  - -Blastocyst injection:
    - •Injection needle
    - •10-15 CiPSCs into embryo cavity of F2 or CD-1 female mice at 3.5 days post coitum
    - •Transferred into 2.5 day pseudopregnant females
  - -Eight cell embryo injection:
    - •XYClone laser system
    - •Collected from female mice at 2.5 days; 7-10 CiPSCs injected into each embryo
    - •Transferred into .5 day pseudopregnant females
  - -Chimeric mice identified by coat color
  - -Assessed for germline transmission by mating with ICR mice
- RT-PCR
  - -Isolate RNA
  - -Convert to cDNA
  - -Carry out PCR
  - -Analysis of data using delta-delta Ct method
- Scatter plot – DNA microarray
  - -Total mRNA was labeled with Cy5, hybridized to a mouse Oligo Microarray
  - -Red line = boundary for two-fold change
  - -R = Pearson's coefficient

Fig. S1

Cell culture

- Cells used in reprogramming were passage 1-5
- Cells cultured in DMEM/High glucose containing 10% fetal bovine serum

- ESCs, iPSCs and CiPSCs maintained on feeder layers of mitomycin C-treated (halts division) MEFs in ESC culture medium (KnockOut DMEM containing 10% knockout serum replacement, 10% FBS, 2mM GlutaMAX-I, 1% nonessential amino acids, 0.1 mM 2-mercaptoethanol, 1% penicillin-streotinycin and 1,000 U/ml leukemia inhibitory factor)

- For CiPSC induction, LIF-free ESC culture medium supplemented with 20-100ng/ml bFGF

Part 2: Test small molecule cocktail

VC6TF: Characterization of GFP+ clusters; day 24

Methods:

- Bisulfite genomic sequencing
  - -Genomic DNA modified by bisulfite treatment and purified
  - -Amplified fragments cloned into pEASY-blunt vector
  - -Ten randomly picked clones from each sample were sequenced
- RNA-seq:
  - -RNA sequencing libraries constructed
  - -Fragmented and randomly primed 200bp paired-end libraries were sequenced using Illumina HiSeq 2000

Fig. S3

Part 3: Screen for late reprogramming molecule

VC6TFZ: GFP positive cells induced

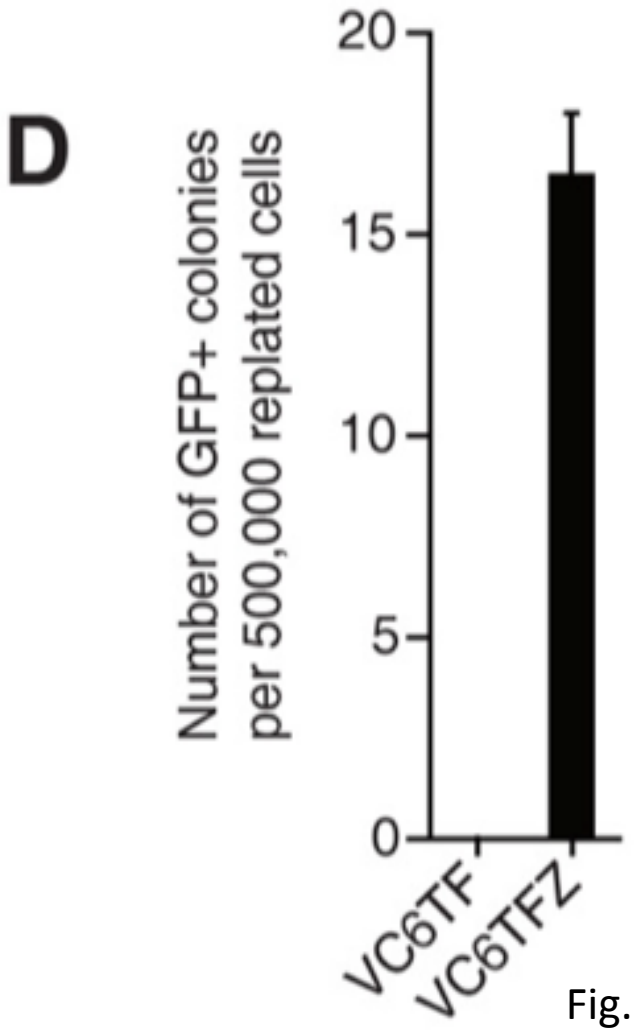


Fig. 1

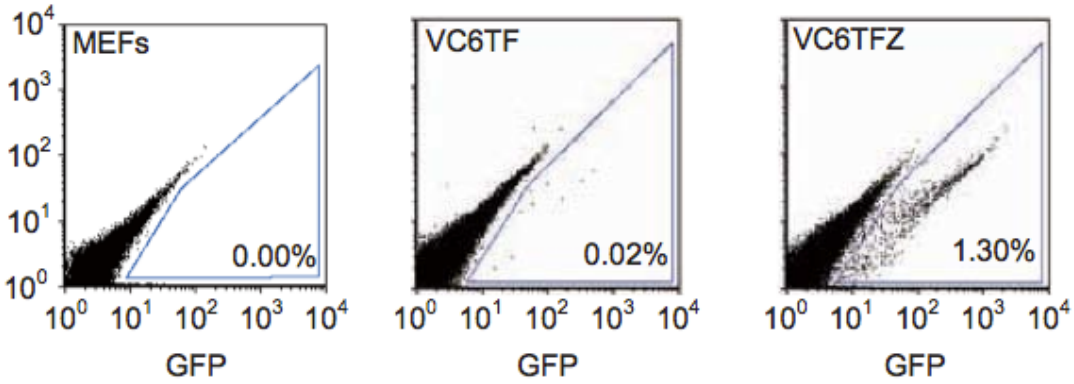
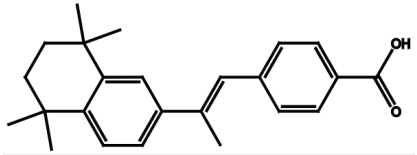
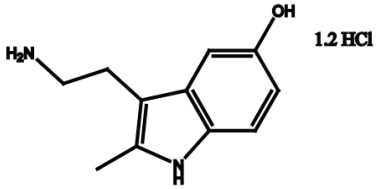


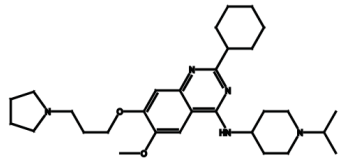
Fig. S5

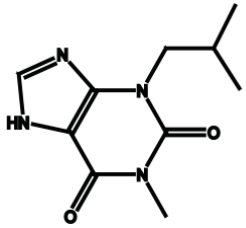
Methods:
Flow cytometry analysis:
•Cultured cells trypsinized into single cells and resuspended in PBS containing 3% FBS
•Using endogenous Oct4-GFP, FACS analyses preformed with FACSCalibur instrument

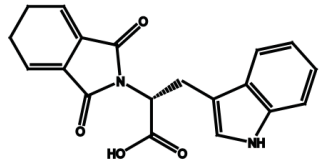
Part 6: Screen for reprogramming booster

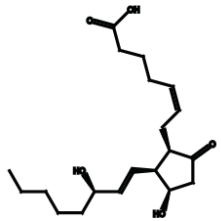
TTNPB 

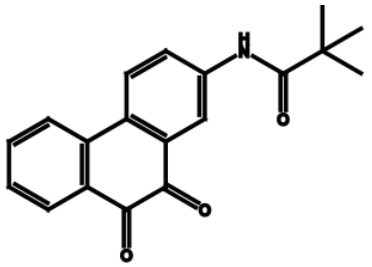
2-Me-5HT 

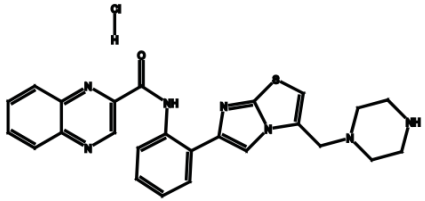
UNC0638 

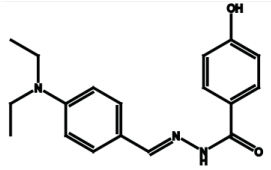
IBMX 

RG108 

PGE2 

SF1670 

SRT1720 

DY131 

[Table S1 (B)]

Part 8: Characterize CiPSC lines

VC6TFZ: Histone H3 modifications at Oct4, Sox2 and Nanog promoter regions

Methods:
Chromatin immunoprecipitation (ChIP):
•EZ-Magna ChIP A/G kit
•Anti-H3K27me3, Anti-H3K9me2, Anti-H3K4me3, Anti-H3K9ac antibodies used
•Following immunoprecipitation, DNA analyzed by real-time PCR

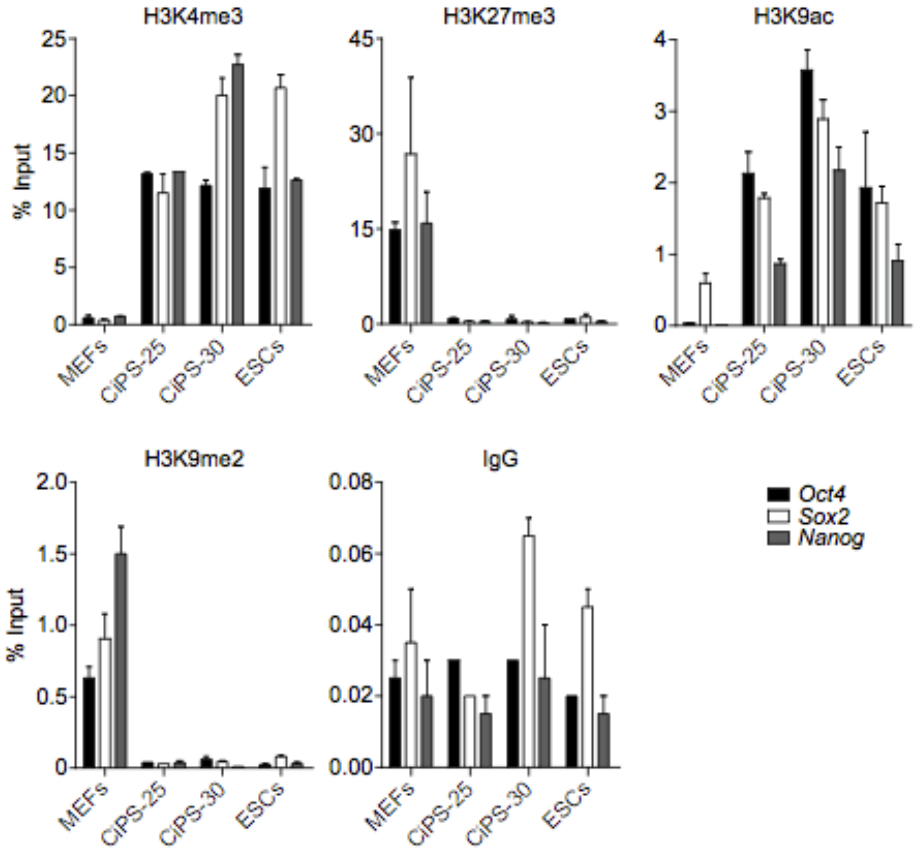
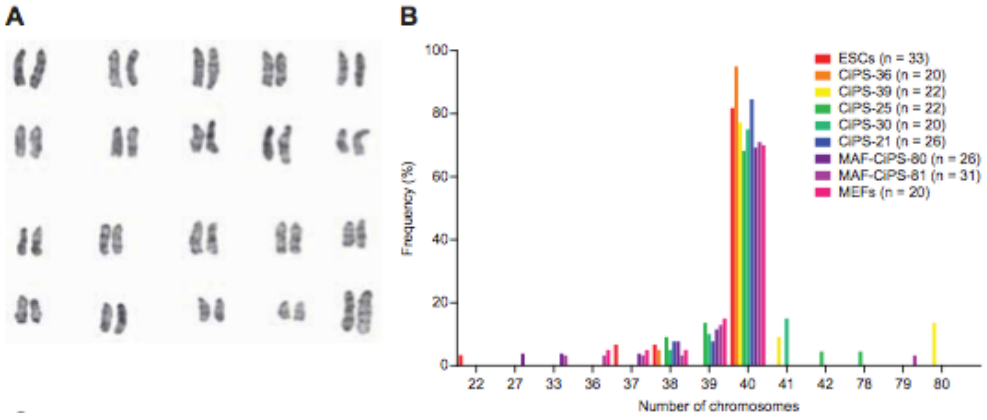
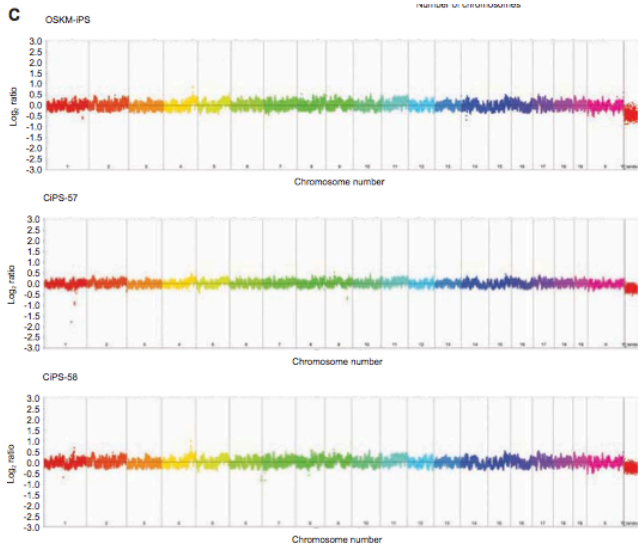


Fig. S13

Part 8: Characterize CiPSC lines

VC6TFZ: genetic integrity of CiPSCs





Comparitive genomic hybridization analysis:

- Genomic DNA extracted and hybridized to mouse whole-genome tiling arrays

Fig. S14

Part 8: Characterize CiPSC lines

VC6TFZ: pluripotency of CiPSCs

Methods:

Teratoma formation:

- 10^5 iPS cells were injected into the kidney capsule of one severe combined immunodeficient beige mouse
- Teratomas were recovered 4 weeks after grafting
- Control mice were injected with 1 million MEFs and failed to form teratoma
- Embedded in paraffin and processed with hematoxylin and eosin staining

Fig. 3

Part 10: Investigate role of small molecules

Overexpression of Sall4 and Sox2: Oct-4 promoter-driven luciferase reporter

Methods:

- MEFs plated 40,000 cells/well; 24 well plate
- Transiently transfected with Oct4 promoter reporters using Lipofetamine LTX and Plus Reagent
- pRL-TK plasmids cotransfected in each well as internal references
- Total DNA concentrations for all transfections were equalized by adding empty pLL3.7-ΔU6 vector
- At 48 hours, cells washed and lysed
- Luciferase activity measured with Dual-luciferase Reporter Assay system and normalized to Renilla luciferase activity
- Empty expression vector plasmids used as negative control

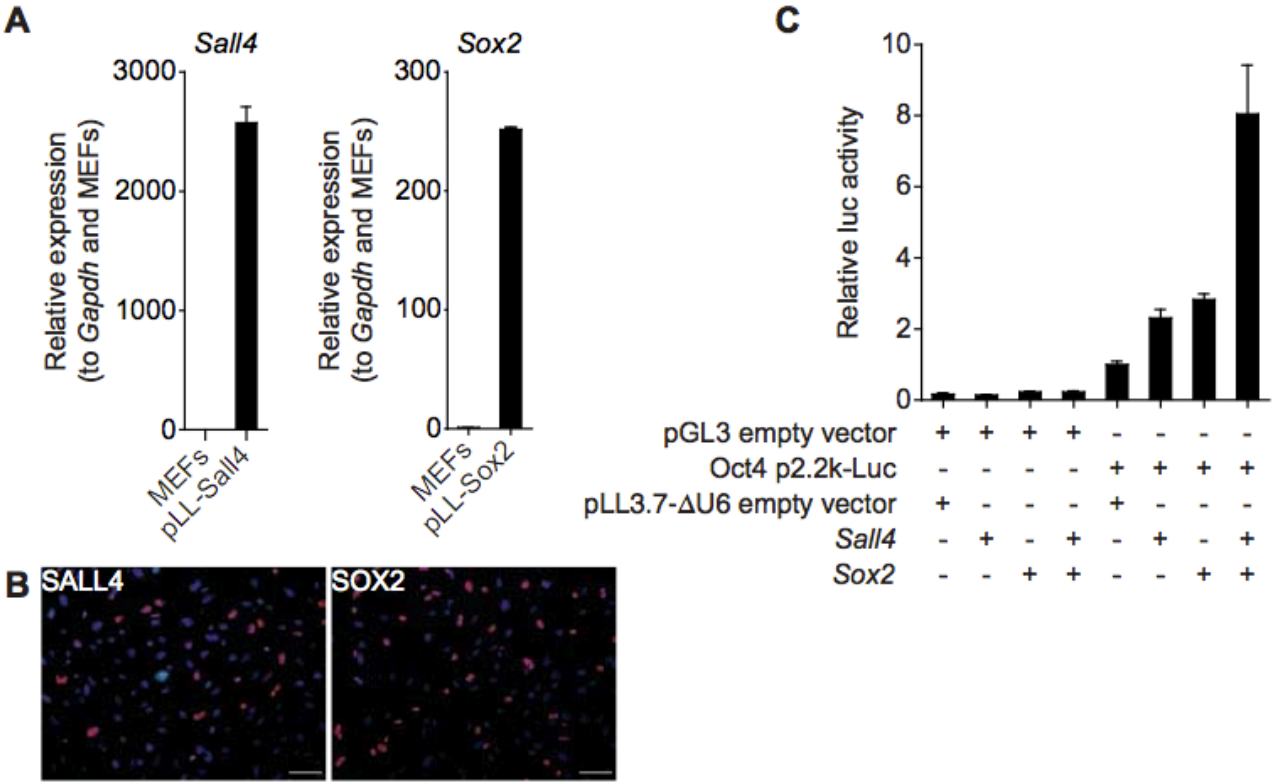
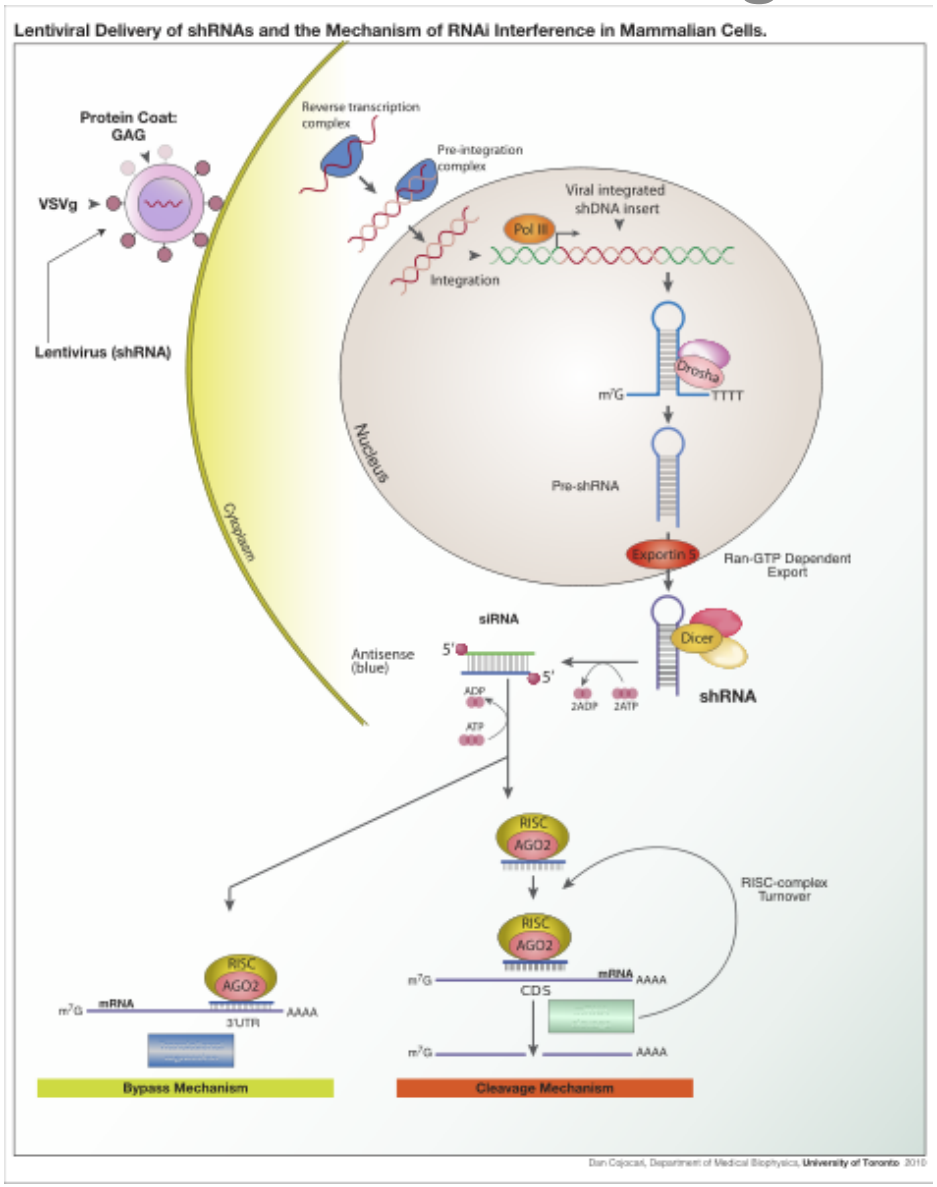


Fig. S25

Part 10: Investigate role of small molecules

VC6TFZ: Effects of knockdown on gene expression



shRNA silencing

Fig. S27