学位論文
Doctoral Thesis

Replacement of Sox2 in reprogramming by identification of the biphasic role of calcineurin pathway

(二方向性のカルシニューリン経路によるSox2非依存的リプログラミングの同定)

コデール　シェリフ　マフルース

Sherif Mahrous Khodeer

熊本大学大学院医学教育部博士課程医学専攻
発生・再生医学研究者育成コース

指導教員

江良　択実　教授
熊本大学大学院医学教育部博士課程医学専攻幹細胞誘導学

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By

Sherif Mahrous Khodeer
(B.Sc, M.Sc)

Doctoral PhD student, Cell Modulation Department,
Institute of Molecular embryology and Genetics (IMEG)
Graduate School of Medical Sciences,
Kumamoto University, Japan

Supervisor

Prof. Takumi Era
Professor and Head of Cell Modulation Department,
Institute of Molecular embryology and Genetics (IMEG)
Graduate School of Medical Sciences,
Kumamoto University, Japan

Institute of Molecular embryology and Genetics (IMEG)
Graduate School of Medical Sciences,
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著 者 名 ： **コデール　シェリフ　マフルース**
（単名） **Sherif Mahrous Khodeer**

指導教員名 ： 熊本大学大学院医学教育部博士課程医学専攻幹細胞誘導学　**江　良　択　実**　教授

審査委員名 ： 多能性幹細胞学担当教授　丹羽　仁史
病態生化学担当教授　山縣　和也
分子生理学担当教授　富澤　一仁
神経分化学担当准教授　太田　訓正

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Abstract of the Thesis

Background and Purpose: Somatic cell reprogramming using defined factors (Oct4, Sox2, Klf4 and c-Myc) known as OSKM to produce induced pluripotent stem cell (iPS cell) raised new hopes for successful clinical trials of stem cell therapy. Despite that, it would be crucial to understand the detailed molecular and epigenetic modifications for ensuring the safety of their application. Here, we aimed in our study to dissect the role of calcineurin and their downstream NFAT targets in reprogramming.

Methods: To study the role of calcineurin/NFAT pathway, we used chemical and genetic inhibitors to inhibit the calcineurin activity and their downstream targets NFATc isforms during each phase of reprogramming. We studied the accompanied change at the level of RNA expression by qPCR, histone modifications by ChIP-qPCR, protein level by western blot, cytoplasmic/nuclear translocation by immunostaining, enzymatic activity by ELISA, protein-protein interaction by immunoprecipitation, cell cycle analysis by flow cytometry , estimation reprogramming efficiency by alkaline phosphatase staining, overexpression and knockdown by retroviral and lentiviral infections.

Results: Calcineurin exhibits a dual opposing role in reprogramming. In the early phase, calcineurin in required for maintaining proper cell cycle proliferation, and knockdown of calcineurin results in arresting cells in G1 phase with delaying in mesenchymal and epithelial transition (MET) rate. In the late phase, calcineurin possesses a negative role mediated by transiently expressed NFATc2, which translocates to the nucleus where it recruits Suv39h1, hdac3 and ezh2 over Sox2 and Klf2 loci and repress their expression by increasing the relative enrichment of the repressive mark H3k9me3 resulting in decreasing reprogramming efficiency. We also identified Gnaq as an upstream regulator of calcineurin. By knockdown of calcineurin, we can replace Sox2 in reprogramming process.

Conclusions: Overall results show that calcineurin can regulate the reprogramming in stage specific manner, and by critically analyzing the calcineurin and its downstream targets, we can replace Sox2 in reprogramming process. We also clarified the new regulatory Gnaq/calcineurin/NFATc2 loop, which could be useful for biological studies.

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Sherif Mahrous Khodeer

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Summary

Induction of pluripotency with defined factors (Oct4, Sox2, Klf4, and c-Myc) generating induced pluripotent stem cell (iPS) opens new era of stem cell biology and raised hopes for clinical trials of regenerative medicine. Despite iPS cell provides a vital tool for studying the disease through establishing iPS-disease specific models, defining the precise role of several epigenetic and transcriptional modifications and their impact on biological processes, much efforts are needed to make iPS a safe strategy for that.

Here in our study, we focused on Calcineurin/NFAT and its role in reprogramming process. We identified that calcineurin has a dual contrary role in reprogramming. In the early phase, the calcineurin seems to have a positive effect by maintaining proper cell division, and downregulation or inhibition of calcineurin activity in the early phase cause a negative effect on cell proliferation, and delaying the MET which are considered the two critical events on early phase of reprogramming.

In the late phase, calcineurin possesses a negative role through its downstream target NFATc2 which expressed on the late phase. The NFATc2 has a negative effect by binding and repressing the expression of endogenous Sox2 and Klf2 which is mediated by recruiting Ezh2, Hdac3 and Suv39h1 to their regulatory sequence , hence downregulating their expression. The Suv39h1 exhibits its negative effect on endogenous Sox2 and Klf2 expression by increasing the level of H3K9me3; a known

repressive mark; over SRR2 a major regulatory domain for Sox2 , and Klf2 promoter, resulting in downregulating their expression and hence decreasing the reprogramming efficiency.

Moreover, we also identified Gnaq; a member of G protein coupled receptor; an upstream activator of calcineurin which was shown to be bound by almost all the reprogramming factors. The Gnaq/calcineurin/NFATc2 was shown to form a negative inhibitory axis over Sox2- SRR2 enhancer and Klf2 promoter mediated by recruiting Suv39h1.

Finally, we also succeeded to replace Sox2 in reprogramming cocktails by either inhibiting calcineurin chemically, genetically or by knockdown of NFATc2

List of Reference Articles

1st Author Article

Sherif Khodeer, Takumi Era. Identifying the Biphasic Role of Calcineurin/NFAT Signaling Enables Replacement of Sox2 in Somatic Cell Reprogramming. Stem Cells. Accepted Author Manuscript. doi:10.1002/stem.2572

List of abbreviations

ALP	Alkaline phosphatase
Annexin V	Annexin A5
BrdU	5-bromo-2'-deoxyuridine
Cdk4	Cyclin-Dependent Kinase 4
c-Myc	V-Myc Avian Myelocytomatosis Viral Oncogene Homolog
CSA	Cyclosporine
Dox	Doxcycline
E-cad	E-cadherin (Cadherin 1)
Eed	Embryonic Ectoderm Development
Epcam	Epithelial Cell Adhesion Molecule
Esrrb	Estrogen Related Receptor Beta
Ezh1or 2	Enhancer of zeste homolog 1or 2
Gnaq	G Protein Subunit Alpha Q
Gnaq $_{q209L}$	G Protein Subunit Alpha Q mutant active
Hdac	Histone Deacetylase
Klf2	Kruppel-Like Factor 2
Klf4	Kruppel-Like Factor 4
Luci	luciferase
MLL	Mixed-lineage leukemia 1
Nanog	Homeobox Transcription Factor Nanog
NFATc	Nuclear factor activated T cell
Oct4	Octamer-Binding Protein 4
p16	Cyclin-Dependent Kinase Inhibitor 2A (CDKN2A)
p19	Cyclin-Dependent Kinase Inhibitor 2D (CDKN2D)
p21	Cyclin-Dependent Kinase Inhibitor 1A (CDKN1A)
p27	Cyclin-Dependent Kinase Inhibitor 1B (CDKN1B)
Pdgfra	Platelet Derived Growth Factor Receptor Alpha
Ppp3ca (CnAα)	Protein Phosphatase 3 Catalytic Subunit Alpha
Ppp3cb	Protein Phosphatase 3 Catalytic Subunit Beta
Ppp3cc	Protein Phosphatase 3 Catalytic Subunit Gamma
Ppp3r1	Protein Phosphatase 3 Regulatory Subunit B, Alpha
Ppp3r2	Protein Phosphatase 3 Regulatory Subunit B, Beta
Rb	Retinoblastoma
Rex-1	Reduced Expression Protein 1 (ZFP42)
Sall4	Spalt-Like Transcription Factor 4
SET	Drosophila Su(var)3-9 and 'Enhancer of zeste' proteins
Setdb1 or 2	SET Domain Bifurcated 1or 2
Slug	Snail Family Zinc Finger 2

Sox2	SRY (Sex Determining Region Y)-Box 2
SSEA1	Stage-Specific Embryonic Antigen 1
Suv39h1or 2	Suppressor Of Variegation 3-9 Homolog 1 or 2
Suz12	Suppressor of Zeste 12 Protein Homolog
Tbx3	T-Box Protein 3
Thy1	Thymocyte differentiation antigen 1
Utf1	Undifferentiated Embryonic Cell Transcription Factor 1
WDR5	WD repeat-containing protein 5
Zeb2	Zinc Finger E-Box Binding Homeobox 2

Background & Objectives

Background and Objectives

Ectopic expression of four transcription factors (Oct4, Sox2, Klf4 and c-Myc) successfully enables reprogramming somatic cells and induces the pluripotency to produce ESC-like cells known as induced pluripotent cells (iPSCs) *[1, 2].* These iPSCs hold a great promise for remodeling patient-specific diseases, and provide a proper tool for drug screening and studying the early embryonic events. In addition to raising the potentiality of successful trails for cell transplantation, and paving the lands for enlightened future of regenerative medicine *[3].*

Reprogramming models

Ever since establishing iPSCs , great efforts have been made aiming to decipher the detailed molecular and epigenetic modifications linked to reprogramming process. Several models have been proposed to reveal the cell demeanor along reprogramming , Hanna and colleagues reported two phases explicated by early stochastic with variable latency, and late deterministic with constant latency *[4]*. Other studies supporting the previous model from another side taking cellular and morphological changes in account ; explained by down regulation of somatic cell genes and subsequent upregulation of pluripotency markers *[5, 6]*. However, two other groups dissected the phase of reprogramming into three phase, the early Initiation phase accompanied with downregulation of mesenchyme lineage markers snail 1, zeb1, zeb2 and slug , and upregulation of epithelial lineage markers occludin, epcam and e-cadherin, followed by maturation phase mediated by

upregulation of pluripotency markers, then the stabilization phase characterized by removing of epigenetic memory , Telomeres elongation, and the self-renewal and pluripotency marker's expression of reprogrammed cells become independent on transgene [7, 8]. In 2012, Buganim and his colleagues intensify the importance of endogenous Sox2 expression as a rate limiting step to terminate the early hierarchical phase and triggers subsequent of events leads to acquiring ESC identity [9]. Surprisingly, shu and his colleagues assumed seesaw model, stressing on the possibility of lineages modifies such as Gata3, Gata6, Sox7 to facilitate reprogramming process [10]. Moreover, Rais and his colleagues identify NurD complex and its Mbd3 subunit as a blocker for reprogramming and its depletion enables nearly absolute reprogramming efficiency [11] .Despite that, this concept is critically denied by another group affirming the importance of NuRD complex for successful somatic reprogramming [12] .

Epigentic modifications.

Chromatin modifcations and reprogramming.

While on the epigenetic levels, the reprogramming process includes global dynamic changes on both histone marks, and DNA methylation level. On the onset of reprogramming, our starting somatic cells or terminally differentaited cells charaterized by closed chromatin structure ; known as heterochromatin, but we ends with iPS cell which featured by its open chromatin structure; known as euchromatin [13].

On the chromatin level, it is subjected to post-translational modifications including methylation, demethylation, acetylation, deacetylation, phosphorlation, dephosphorylation, deimination, β-N-

acetylglucosamination, ADP ribosylation, ubiquitylation, sumoylation, histone proline isomerization [14]. Among these modifications, there are several important histone marks which has been identified for their role in regulating the expression profile as for example; tri methylation of lysine 4 on histone 3 known as H3K4me3 as an activation mark, while tri methylation of lysine 27 on histone 3 known as H3K27me3 and tri methylation of lysine 9 on histone 3 known as H3K9me3 as a repessive marks. The activation mark H3K4me3 is mediated by Trithorax members harboring SET domain including MLL family and WDR5. The H3K4me3 is usually linked to the promoter and enhancer of trancrpitional active genes. While H3K27me3 is miantained by Polycomb group known as PcG. The PcG complex comprised two complexs polycomb repressive complex 1 and 2 known as PRC1 and PRC2. The PRC1 is associated with maintaining the repressive mark, which PRC2 is consisdered the catalytic domain consists of four subunits Ezh1, Ezh2, Eed, and Suz12. The repressive mark H3K27me3 is mainly deposited on the promoter and emhancer of transcriptional inactive genes. Despite that, there is group of genes associated with development process called bivalent genes and considered an important feature of pluripotent cells including ESCs and iPSCs, which possess both activation mark H3K4me3 and repressive mark H3K27me3 on their regulatory regions. These genes are transcriptioanl poised in the pluripotent cells , and once these cells go for differentaition, these genes are depleted from the repressive mark and become transcriptionally active.

Another repressive mark is H3K9me3 which is catalyzed by either Suv39h1, Suv39h2, Setdb1 or Setdb2 considered a marker for

heterochromatin , and linked to repressed genes. Besides, H3K9me3 is considred as abarrier for somatic cell reprogramming [15], and reduction of H3K9me3 marks enhances the reprogramming effiency and improves mamilian cloning effeciency [16].

Acetylation of chromation is mediated by several histone acetylases and has also been reported in regulation of several biological processes. The acetylated chromatin is considered as a marker for open chromatin structure and actively trsancriped genes. The acetylation is counteracted by deacetylation process to repress gene expression to maintain specific biological fucntion [14]. The deacetylated histone is considered as abarrier for reprogramming, and inhibition of histone deacetylases Hdacs either by chemical or genetic inhibitors enhance the reprogramming process [6, 17].

DNA methylation and reprogramming.

The epigentic signature can be regulated on the DNA methylation level. The methylation of DNA occurs at the fifith cytosine residue to generate 5mC. Despite 5mC marks can be located throughtout the whole genome, those occurred both CpG are strongly associated with gene repression. The 5mC can be converted to 5hmC through TET family which is considered as a trasit step for DNA demethylation [18]. The DNA methylation is aslo considered as abarrier for reprogramming, and using small chemical inhibitors like 5-azacytidine enhance the reprogramming process [19].

Cell signallings and reprogramming.

A wide variety of signaling pathways and their roles have been investigated in context of reprogramming, including TGF-beta *[20, 21]*,

BMPs *[8, 15]*, Yap/TAZ *[22]*, PI3K/AKT *[23]*, FGF *[24]*, Wnt *[25]*, LIF/STAT3 *[26]* and MAPK *[27]*, Stressing on the importance of tying the signaling pathways and their downstream targets to fine-tune the reprogramming process.

However, the precise mechaism still poorly understood due to the complexity of tracing interwoven steps wiring the microenvironment, cell-cell communication *[28, 29]*, stimulated signaling pathways and their cross talk *[20, 21]*, functional transcriptional and miRNA machinary *[30, 31],* besides the epigenetic modifications including DNA methylation *[19]*, histone modifications *[15]*, X-chromosomal inactivation *[32]* and telomerase enlongation *[33, 34]* in the course of reprogramming process.

Calcineurin/NFAT siganlling pathway

Among of the signaling pathways which are poorly understood in somatic cell reprogramming is calcineurin/NFAT signaling pathway. Calcineurin is the Ca2+/calmodulin-dependent serine phosphatase which consists of two subunits, the catalytic subunits which encoded by three genes (Ppp3ca ; CnA α subunit, Pppcb; CnA β subunit, and Pppcc; CnA γ subunit), while the regulatory subunits encoded by two genes (Ppp3r1; CnB1 subunit, and Ppp3r2; CnB2 subunit). The calcineurin exerts its dephosphorylation effect on its downstream target known as nuclear factor activating T-cell (NFAT) family. There are 5 different isoforms of NFAT including NFATc1, c2, c3 and c4 , while NFAT5 does not have calcineurin binding site, so it is not subjugated to regulation by calcineurin. Upon dephosphorylation, the NFAT family members translocate into the nucleus where it binds to their targets and regulate the transcription. The calcineurin/ NFAT signaling pathways has been

implicated in different cellular and physiological process including T-cell differentiation, osteoclast differentiation, cardiac valve development, skeletal muscle differentiation *[35]*, pancreatic beta-cell growth and functions *[36]*.

Great contributions have been done over the last decade to elucidate the precise role of each of NFAT isoforms in the early embryonic development by producing several knockout mice. NFATc1−/− mice exhibit defective heart valve development and abnormalities in the cardiac septum, while NFATc2−/− mice form small multinucleated muscle cells due to a defect in the recruitment. On the other hand, NFATc3/c4 double null mice display disorganization of blood vessels and poor vessel wall integrity, leading to early embryonic lethality reviewed in *[37]*.

Aim of the work.

Recently, calcineurin/NFAT signaling pathway was firstly reported to regulate the early events of lineage specification in mouse ESCs, and inhibition of calcineurin activity enables to maintain the pluripotent state , indicated the possibility of different downstream arms of calcineurin to negatively interfere in the ESC molecular circuitry *[38]*. In this study, we decided to elucidate the role calcineurin/NFAT signaling pathway and its associated cellular, epigenetic and molecular events during reprogramming process.

Materials & Methods

Materials and Methods

MEF Isolation

Pregnant BC57BL/6J females were sacrificed on 13.5 or 14.5 d.p.c and embryos were dissected as previously reported [1].

Cell culture

MEFs were cultured in Dulbecco's modified essential medium (DMEM, Life Technologies) supplemented with 10% fetal bovine serum (FBS), 0.5% penicillin and streptomycin (Nacalai Tesque, Japan). For culturing mouse iPS cells, the cells were cultured on MMC-treated MEF feeder cells pre-coated with 0.1% gelatin and the culture medium was supplemented with 0.1 mM 2-mercaptoethanol (SIGMA), and homemade Leukemia inhibitory factor (LIF). Mouse CCE ESCs were cultured in feeder free condition using DMEM+10%FBS and 2ME plus 1unit of LIF, while iPS cells were cultured in feeder condition using DMEM+10%FBS and 2ME plus 1unit of LIF.

Mitomycin c treatment

ICR isolated MEFs were cultured till passage four, and then treated with mitomycin C at final concentration 10µg/ml for 3 hours. Then the cells were washed 2 times with 1xPBS. The cells were treated with 0.25% trypsin and incubated at 37ºC for 5 minutes. The cells were precipitated, and suspended in fresh medium then counted, and stored at different cell numbers in freezing solution at -80 ºC.

Reprogramming induction

Early passage - till passage 2 - MEFs were plated as single cells at $1x10^5$/ well of 6 well plate or 5-6 $x10^5$/ 10 cm dish depending on the purpose of experiment. The cells were infected with equal ratio of the retroviruses expressing the four reprogramming factors (oct4, sox2, klf4, and c-myc),

and incubated at 37ºC for 8-12 hours with 8 µg/ml of polybrene, then the medium changes next day.

Retrovirus and Lentivirus preparation

293T cells were plated at $3x10^6$ / 10 cm dish, and incubated at 37ºC till reaching to 80% confluence. On the day of transfection, 9ml of fresh medium was added supplemented 50µM chloroquine. Then mixture of 10 µg gene of interest retroviral encoding plasmid plus 10 µg of (gag-pol-env) plasmid , and mixture of 10 µg gene of interest lentiviral encoding plasmid plus 5 µg of (Gag-Pol) and 5 µg of (Vsvg- Rev) suspended in 500µl MQ and 500 µl 2x HBS soln was supplemented with 50µl of 2M $Cacl_2$ with vigorous vortex, and then incubated in ice for 20 minutes, then this mixture was added drop by drop to the dish till covering the surface area for transfection. The medium was changed after 12-14 hrs with 10ml DMEM+5%FBS, then after 7 hours , fresh medium 5ml of IMDM+10% FBS, the supernatant was harvested after 48 and 36 hours and filtered by 0.45µm Millipore filter, then used freshly or stored at -80 ºC for future use.

For dox inducible system, the M2rtTA purchased from addgene (Plasmid #20342) was used and infected with equal ratio to the virus encoding gene of interest.

Plasmids

PMXs retroviral encoding the four reprogramming factors (PMXs-Oct4, PMXs-Sox2, PMXs-Klf4, PMXs-c-Myc) kindly provided by professor Shinji Masui (Department of life science frontiers, CIRA, Kyoto university).For immunoprecipitation experiment, the reprogramming cDNA was amplified using EcoRI/XhoI restriction site flanking primers, then digested and ligated to Mie-3XFLAG-GFP plasmid. NFATc1, ca-NFATc1, and NFATc2 was

kindly provided by professor Katsuhiko Mikoshiba (Department of Pharmacotherapeutics, Showa Pharmaceutical University, Machida, Tokyo), and agreement for using ca-NFATc1 was approved by professor (Neil Clipstone, Loyola University Chicago), while the agreement for using NFATc2 was provided by professor (Timothy Hoey, OncoMed Pharmaceuticals, Redwood City). The cDNA for NFATc3 (clone ID: 5830424L08) and NFATc4 (clone ID: K820003H17) were purchased from DNAForm. Then the cDNA was amplified using the listed primer. For making the ca-NFATc3, 400-1076 a.a fragment was amplified and cloned, and for making ca-NFATc4 318-901 a.a fragment was amplified and cloned. Mouse NFATc2 was isolated and amplified from spleen CDNA. The NFATc1, NFATc2, NFATc3, NFATc4, and their constitutive active form ca-NFATc1, ca-NFATc2 (331-921a.a), ca-NFATc3, and ca-NFATc4 were amplified using (PrimeSTAR GXL, Takahara), and digested and then cloned to retroviral Mie-DsRed, or Mie3XFLAG-GFP plasmid according to the purpose of experiment except for NFATc2 which was digested by EcoRI/XhoI and cloned directly. The plasmid encoding Gnaq and $Gnaq_{Q209L}$ were kindly provided from (Chieko Aoyama, Dokkyo Medical University School of Medicine), then the cDNA was amplified and cloned to Mie3XFLAG-GFP plasmid. Constitutive active form of calcineurin CnAα was amplified from spleen cDNA and cloned to either Mie3xFLAG-GFP or FUW tet-on plasmid. Dox inducible system for NFATc2 and ca-NFATc2 was made by amplifying the cDNA using EcoRI restriction site flanking primers, ligated to FUW tet-on plasmid obtained from addgene (Plasmid #20726) and then positively- oriented clone was selected.

Knockdown experiment

The shRNA oligos were designed harboring the BamHI/EcoRI protruding ends in case of using RNAi-Ready pSIREN-RetroQ-ZsGreen Vector plasmid obtained from (clontech, Z2455N), BamHI/HindIII protruding ends in case of using pQCXIX-U6-BamHI-EcoRI-HindIII-puro [2], AgeI/EcoRI protruding ends in case of using pLKO-Tet-On lentivirus system purchased from addgene (Plasmid #21915), after purchasing from (Eurofins), the oligos were dissolved in 1xTE buffer , and then annealing by ramping PCR, then ligated to linear vector using Ligation high version 2 (Toyobo), and then transformed in to DH5α , then the digested and positive clones were sequenced, the target sequence for knockdown were listed , and the targets sequences sticky ends are changed depending on the used vector (supplementary table 4).

FACS analysis

Cells were washed two times with 1xPBS, and trypsinized, precipitated, and counted. Then $1x10^6$ cells were washed again with 1x Hanks buffer and stained with (10 ml of anti-SSEA1-FITC) or (1:800 of anti-SSEA1-bition) antibody for 30min on Ice, and then cells were washed once with 1x Hanks buffer, and in case of biotin conjugated Ab, the cells were stained with (1:400 streptavidin-APC) 15 minutes on Ice, then 7AAD (1:200) , and SSEA1 positive fraction was analyzed using FACS canto. For sorting, the sorted cells using FACS AriaIII were washed with warm medium and counted and plated at 5000 cells on feeder layer using medium supplemented with LIF.

Teratoma experiment

iPS cells were suspended at $1x10^6$ cells/100µl in DMEM containing 10% FBS. This 100 µl of iPS cells were subcutaneously injected into both sides of dorsal flank of SCID mice. Four to six weeks after the injection, tumors were surgically dissected from the mice and fixed in 1xPBS containing 4% formaldehyde, and embedded in paraffin. Sections were cut at a thickness of 3 mm using a Leica RM2255 (Leica, Wetzlar, Germany) and stained with hematoxylin and eosin.

Immunostaining

Alkaline phosphatase staining was performed using the Leukocyte Alkaline Phosphatase kit (SIGMA). For Immunostaining, cells were fixed with PBS containing 4% paraformaldehyde for 30 min at 4℃. For the Oct4, sox2 and Nanog, samples were treated with 0.2% Triton X-100 for 15 min at room temperature (RT), but this step was omitted for SSEA1 cell surface marker. The cells were washed three times with PBS containing 2% FBS and then incubated overnight at 4℃ in PBS containing 2% FBS with primary antibodies, next day the cells were washed 3 times, and incubated with secondary antibody conjugated with proper flurochrome for 1 hours at R.T in the dark, then washed three times and finally stained with1 µg/ml Hoechst 33258 (Invitrogen). Specific antibodies are listed in supplementary table 3.

Westernblot

The cells were lysed with proper volume of lysis buffer and them the total protein concentration was measured according to lowery method. Then equal concentration of samples was loaded and the gel was washed and blotted to PVDF membrane either 2 hours or O.N at 4ºC. The membrane was blocked with 5% skimmed milk for 1 hour, and then incubated with the

1st antibody O.N at 4ºC with mild shaking. Next day the membrane was washed 3 times each 10 minutes using 1xTBST buffer, and then incubated with the 2nd antibody for 1hours at R.T with mild shaking. Then washed three times with 1XTBST and incubated with Western Lightning® Plus-ECL (PerkinElmer) and bands were detected using (ImageQuant LAS 4000).

Cell fractionation

The cells were fractioned as previously described [3].

Calcineurin assay

Cellular calcineurin activity was measured using calcineurin cellular assay kit (ENZO Life Sciences) according to the manufacturer's protocol. Briefly, cells were suspended in the kit`s lysis buffer. Then the supernatant was taken and desalted to remove free phosphate and the protein concentration was measured and normalized. Then lysate was incubated with the calcineurin substrate RII phosphopeptide mixed with assay buffer and EGTA buffer at 30°C for 30 min to determine total phosphatase activity and phosphatase activity without calcineurin, respectively. Then, 100 µl BIOMOL GREEN was added to each well to stop the reaction and visualize the released phosphates. The color was developed at room temperature for 30 min before the detection of the absorbance value at 620 nm. Each sample was measured as a triplicate to minimize the error.

RNA extraction and qPCR

Total RNA was purified with Sepasol® Super G reagent (Nacalai Tesque, Japan). Total RNA was transcribed to DNA with Superscript III (Invitrogen) and randam primers (Invitrogen). RT-PCR and Quantitative PCR (qPCR) was performed with QuickTaqTM (TOYOBO, Japan) and THUNDERBIRDTMqPCR Mix (TOYOBO) as described previously [4]. The

data were analyzed with StepOnePlus real-time PCR system (Applied Biosystems). The sequences of primers are listed in supplementary table 3.

Karyotype analysis

G band analyses of chromosome were performed by Nihon Gene Research Laboratories. Inc. (Sendai, Japan), according to the manufacturer's protocol.

ChIP q-PCR

The cells were refreshed with 9ml medium and 243µl of 37% formaldehyde (final concentration 1%), and incubated at RT for 10 minutes with swirling, then 1,350 µl of 1M glycine (final concentration 150mM to reverse crosslinking and incubate at RT for 5 minutes, then the cells were washed 2 times with 10 ml 1xPBS , counted, harvested in 4ml 1xPBS containing protease inhibitors, scarped and collected in 15 ml conical tube, and centrifuged 5 min at 4ºC at 1,200 r. p.m. The supernatant was aspirated and the cells were suspended in 400 µl lysis buffer containing protease inhibitors with pipetting up and down, and incubated on ice for 15 minutes, then sonication six cycles (15sec 30% output and 1 min ice) to get genomic size in range 600bps, then centrifuged 10 min 132,000 r.m.p at 4ºC ,and transfer the chromatin soln to new eppendorf. The chromatin was cleared by incubated with prewashed 40µl magnetic beads, and incubated at 4º for 30 min with rotation, and then the chromatin was purified with magnetic stand and dispended in 10 aliquots 40 µl each, one was kept as input, and the used one was diluted 10 fold with 360 IP buffer (total 400µl) , and then incubated with the 1st antibody at 4ºC O.N with rotation. Next day, the prewashed magnetic beads were added to the O.N mixture and incubated at 4ºC with rotation for 2 hours, then washed 3 times with rotation at 4ºC for

5 min firstly with 500µl low salt buffer, secondly with 500µl high salt buffer, and finally with 500µl LiCl buffer. Then the magnetic beads were eluted using 200µl and 8µl 5M NaCl , and incubated at 65ºC O.N , then 1µl RNase was added and incubated at 37ºC for 30 min, then 1µl (10mg/ml) proteinase K was added and incubated at 55 ºC for 1hr, then phenol chloroform extraction and the pellet was suspended in 50µl 1xTE buffer , and DNA concentration was measured by Nanodrop 200c (Thermo scientific Invitrogen) and used for q-PCR . For Micro-Chip, The experiment was done according to previously reported method [4].

Brdu incorporation

Cells were supplemented with 30µM BrdU, and incubated at 37ºC for 6 hours in dark. Then the BrdU was removed and cells were washed with 1xPBS, and cells trypsinized, harvested and centrifuged. The cells were fixed using ice cold 70% ethanol and incubated at 4ºC for 1hour. The pellet was suspended in 500µl of 2N HCl/0.5% Tritionx-100 and incubated at RT for 30 minutes, then centrifuged and the pellet was suspended in 500µl 0.1M sodium tertaborate for 2 minutes. The pellet was centrifuged at washed with 150µl 1xPBS/BSA. The pellet was suspended in 50µl 0.5% Tween 20/1%BSA/1XPBS, and incubated with 1st antibody anti BrdU-biotin and incubated for 1 hours at RT. Then washed and suspended in 50µl 0.5% Tween 20/1%BSA/1XPBS and incubated with 2nd antibody (SA-APC) and incubated at RT for 30 minutes. The cells were centrifuged and resuspended in 500 µl 1xPBS, and 5µg RNase, 2.5 µl 7AAD, and incubated in dark for 30 minutes at RT and then analyzed by FACS Canto.

Apoptosis

The cells were trypsinized, harvested and collected, and washed 2 times with 1xPBS, and then suspended in 200µ 1x binding buffer, 1µl Annexin V-alexa647, and PI 0.5 µl and incubated at RT for 30 minutes in the dark. The cells were centrifuged and suspended in 500µl 1x binding buffer, and then analyzed by FACS.

Cell cycle

The cells were trypsinized, harvested and collected, and washed 2 times with 1xPBS, and then fixed with 70% ethanol and incubated at 4ºC for 30 minutes. Then the cells centrifuged and supernatant was aspired and suspended in 200µ 1 1%BSA/Hanks buffer 0.5µl PI or 7AAD and 1µl RNase and incubated at RT for 30 minutes and then analyzed by FACS.

Immunoprecipitation

The cells were washed 2 times with 1xPBS ice cold and then harvested and centrifuged, and supernatant was aspirated. The cells were suspended in 400µl lysis buffer (50mM Tris-HCl PH8, 1%NP-40, 0.25% sodium deoxycholate, 150mM NaCl, 1mM EDTA, and protease inhibitor. And incubated on ice for 15 minutes, then centrifuged. The cells supernatant was transferred to new eppendorf and cleared with 40µl prewashed magnetic beads, and incubated with target antibody at 4ºC O.N with rotation, and then 40µl prewashed magnetic beads was added and incubated for 2 hrs at 4ºC with rotation, then washed 3 times IP dilution buffer and then suspended in 2x Laemmli sample, and used for western blot.

List of primers Used

Gene	primers
cloning both NFATc1 and ca-NFATc1	F-GGCCCTCGAGATGCCAAGCACCAGCT
	R-GGCCCTCGAGTTAGAAAAAGCACCC
cloning ca-NFATc2	F-GGCCGAATTCCCGGTGTCTGCCGCCCC
	R-GGCCCTCGAGTCATAATATGTTTTGTAT
cloning NFATc3	F-GGCCGAATTCATGACTACTGCAAACTGTG
	R-GGCCCTCGAGTCACTGAGCACTGTGAGAG
cloning ca-NFATc3	F-GGCCGAATTCTTTACCTGGAGCAAACCAA
cloning NFATc4	F-GGCCGAATTCATGGGGGCCGCAAGCTGC
	R-GGCCCTCGAGTCAGGCAGGAGGCTCTTCT
cloning ca-NFATc4	F-GGCCGAATTCGTGGGTGCTCCACCAACC
EcoRI WT h-nfatc2	F-GGCCGAATTCATGAACGCCCCCGAGCG-3`
	R- GGCCGAATTCTCATAATATGTTTTGTAT-3`
cloning full PPP3ca EcoRI	F-GGCCGAATTCTGTGCAGTCGGACGGGACGA
cloning full PPP3ca XhoI	R-GGCCCTCGAGCCATCATGCCCTGCAGCTCAA
Cloning CA mPPP3ca XhoI	R-GGCCCTCGAGGTTTCTGATGACTTCCTTCCG
pLKO sequencing Forward	F-GGCAGGGATATTCACCATTATCGTTTCAGA
pLKO sequencing Reverse	R- ATTCTTTCCCCTGCACTGTACCCC
cloning EcoRI-m-OCT4-	F-GCCGAATTCGCTGGACACCTGGCTTCAGACTTC
cloning XhoI-m-OCT4-R	R-GCCCTCGAGTCAGTTTGAATGCATGGGAGAGC
cloning EcoRI-m-Sox2	F-GCCGAATTCATGGAGACGGAGCTGAAGCCGC
cloning XhoI-m-Sox2	R-GCCCTCGAGTCACATGTGCGACAGGGGCAGTG
cloning EcoRI-m-KLF4	F-GCCGAATTCGCTGTCAGCGACGCTCTGCTCCCG
cloning XhoI-m-KLF4	R-GCCCTCGAGTTAAAAGTGCCTCTTCATGTGTAAG
cloning EcoRI-m-CMYC	F-GCCGAATTCCCCCTCAACGTGAACTTCACCAAC
cloning XhoI-m-CMYC	R- GCCCTCGAGTTATGCACCAGAGTTTCGAAGCTG
cloning both Gnaq and $Gnaq_{Q209L}$ XhoI/ BamHI	F- CCGGCTCGAGATGACTCTGGAGTCCATCATGGCG
	R- CCGGGGATCCTCACACCAGATTGTACTCCTTCA

Mouse qPCR p16	F-GCTCTGGCTTTCGTGAACAT
	R-CGAATCTGCACCGTAGTTGA
Mouse qPCR p19	F-CCAAGATGCCTCCGGTACTA
	R-CCCTCTCTTATCGCCAGATG
Mouse qPCR p21	F-CGGTGGAACTTTGACTTCGT
	R-GACCCAGGGCTCAGGTAGAC
Mouse qPCR p27	F-CAGCTTGCCCGAGTTCTACT
	R-GGTCCTCAGAGTTTGCCTGA
Mouse qPCR Ehmt2 [5]	CAGCAAGGAAGAGGATGG
	AGCAGCATACGAATCACAT
Mouse qPCR Suv39h1 [5]	F-GAGATACCAGCCTAACATCAA
	R-TTAATACCAGCCAGCATCAA
Mouse qPCR Suv39h2 [5]	F-ATGGCAAGATTACCTCAACA
	R-CTTCAGCAGGACAACACT
Mouse qPCR Setdb1 [5]	F-AAGGAAGGATATGAGAGTGATG
	R-ACTGAACTGGTGCTGAAG
Mouse qPCR Lsd1 [5]	F-GGCATTATGGAGAACATTAGTG
	R-CCAACGAGATACCACAGTT
Mouse qPCR Phf8 [5]	F-AAGAGATTGATGTGATTGATGTG
	R-AGCCTGGTATCGGAGAAT
Mouse qPCR Kdm3a [5]	F-GAAGTCCTTAGAACCATCCAA
	R-CCTGCTCCTCTGATACCT
Mouse qPCR Kdm3b [5]	F-CTCTTCATCCTCAGCAGTAG
	R-ATCTCCTTCACCTCCTTCT
Mouse qPCR Kdm3c [5]	F-CACTGTTCACGGTCATTATAC
	R-TATCTTCCTTCATCTTCTCTTCAT
Mouse qPCR Kdm4a [5]	F-CGCTTCTACCAGTGTGAG
	R-TCTGTCCATCTGACTTGAAC
Mouse qPCR Kdm4b [5]	F-CACTAACTTCGCCACACT

	R-CTTGCTTCCATTGCTCATAG
Mouse qPCR Kdm4c [5]	F-CATTGACGAAGAAGTTGAAGAA
	R-GCTGCTATCAGGCTTGTA
Mouse qPCR Kdm4d [5]	F-CCTAAGTCCATTACCTCATAGTT
	R-AGCAGGAGTTAGCAGTTATC
Mouse qPCR Ppp3ca [6]	F-GAGGAGGCCAAGGGCTTAGA
	R-GCGAGAGCCTTGTTGATGGA
Mouse qPCR Ppp3cb [6]	F-CATAAGAAACAAGATCCGAGCAATT
	R-TGGGAGTCAGGCCCTTGAG
Mouse qPCR Ppp3cc	F-GGGTCCTCTCTGGAGGAAAG
	R-TAGACCTCGGGCTTCTTCAA
Mouse qPCR Ppp3r1	F-GGTGGGCAACAATCTGAAAG
	R-CGACAGCACAGAATTCCTCA
Mouse qPCR Ppp3r2	F-ATCTCCAATGGGGAGCTCTT
	R-CAAGATGCTCTTGTCCACCA
Mouse qPCR Eras	F-ACTGCCCCTCATCAGACTGCTACT
	R-CACTGCCTTGTACTCGGGTAGCTG
Mouse qPCR endo-Sox2	F-AAGGGTTCTTGCTGGGTTTT
	R-AGACCACGAAAACGGTCTTG
Mouse qPCR endo-Klf4	F-CCAGCAAGTCAGCTTGTGAA
	R-GGGCATGTTCAAGTTGGATT
Mouse qPCR endo-Oct4	F-AAGCCCTCCCTACAGCAGAT
	R-CTGGGAAAGGTGTCCCTGTA
Mouse qPCR Nanog	F-CCAGGTTCCTTCCTTCTTCC
	R-GGTGAGATGGCTCAGTGGAT
Mouse qPCR Klf2	F-GCCTGTGGGTTCGCTATAAA
	R-AAGGAATGGTCAGCCACATC
Mouse qPCR ESSRB	F-TTTCTGGAACCCATGGAGAG
	R-AGCCAGCACCTCCTTCTACA

Mouse qPCR Tbx3	F-AGGAGCGTGTCTGTCAGGTT
	R-GCCATTACCTCCCCAATTTT
Mouse qPCR Klf5	F-CAAGCCGTTCCAGTGCAT
	R-GTCTGCGGTTTAAAGGATGG
Mouse qPCR REX1	F-ACGAGTGGCAGTTTCTTCTTGGGA
	R-TATGACTCACTTCCAGGGGGCACT
Mouse qPCR Sall4	F-CTCATGGGGCCAACAATAAC
	R-CGGAGATCTCGTTGGTCTTC
Mouse qPCR Ezh1	F-GTCCACGGTGAAGAAGAG
	R-GTCCTCCTCCTCATCAGA
Mouse qPCR Ezh2	F-TTGCTGCTGCTCTTACTG
	R-CTCTGTCACTGTCTGTATCC
Mouse qPCR Eed	F-AGTTCTGAGTGCTGATTATGAT
	R-TTGAGTTGATTCTCCACAGTT
Mouse qPCR Suz12	F-ACAACAGACAGAAGCCAGAG
	R-CACCGTCAGTTTCCAAAG
Mouse qPCR Utx	F-ACAGGAAGTGGAAGTAATGG
	R-GAGTGGAGTTAGATAGTTGGTT
Mouse qPCR Jmjd3	F-TGCTCAGTCAACATCAACA
	R-CCAGGAACCAGTCAAGTAG
Mouse qPCR HDAC1	F-TGCTGTGAACTACCCACTGC
	R-CACTGCACTAGGCTGGAACA
Mouse qPCR HDAC2	F-GAGGGATATTGGTGCTGGAA
	R-GCGCTAGGCTGGTACATCTC
Mouse qPCR HDAC3	F-TGCTTCAATCTCAGCATTCG
	R-GTAGCCACCACCTCCCAGTA
Mouse qPCR HDAC4	F-GAAGGGCAAAGAGAGTGCTG
	R-GGAAATGCAGTGGTTCAGGT
Mouse qPCR HDAC5	F- GGACGCCTCCCTCCTACAAATTG

	R- AGTTGGG TTCCGAGGCCGTTTTAC
Mouse qPCR HDAC7	F- GTGGCGAGGGCTTCAATGTCAACG
	R- TCGGGCAATGGGCATCACCACTA
Mouse qPCR GNAO1	F-GGTGTGTGACGTGGTGAGTC
	R-GAGATCGGTTGAAGCACTCC
Mouse qPCR GNAS	F-CAGAGAGACCCCCAGTTGAG
	R-GAAGATCCGTCCCCTCTCTC
Mouse qPCR Gnaq	F-GCACAATTGGTTCGAGAGGT
	R-ATTCCCGTCGTCTGTCGTAG
Mouse qPCR Gnai1	F-GATGATGCTCGCCAACTTTT
	R-GTACTCCCGGGATCTGTTGA
Mouse qPCR Gnai2	F-CTGCAGATCGACTTTGCTGA
	R-GATGACACCGGACAGGTCTT
Mouse qPCR Plcb1	F-CATTCCCCAAGAGGACTTCA
	R-CGTCAGGTACGGTTTGCTTT
Mouse qPCR Plcb3	F-AGCCACTGAGCGCATATTTT
	R-TCCATACATCCAGCTCCACA
Mouse qPCR Itpk1	F-TGGGAGTGTCACTGTTTGGA
	R-AACTGTGGCAATGTGGTTCA
Mouse qPCR Itpr2	F-CCCCATGTCCTCATATTTCG
	R-CTGCTGAGCACCATCTGTGT
Mouse qPCR Itpka	F-CATCAAGAAAGCCGATGGAT
	R-TATCCCGGATCTGCTGTAGG
Mouse qPCR Itpkb	F-ACTGGAGCGCTTTGGAACTA
	R-AGCTCACAGCCTGTCTCCAT
Mouse qPCR Itpkc	F-TTCCAGGACTCCACATGACA
	R-CCATCACTCCCAGGTTGTCT
Mouse qPCR Ocln	F-CGGTACAGCAGCAATGGTAA
	R-CTCCCCACCTGTCGTGTAGT
Mouse qPCR Cdh1	F-CCCAGAGACTGGTGCCATTT

	R- TGGCAATGGGTGAACCATCA
Mouse qPCR EpCam	F- ATTTGCTCCAAACTGGCGTC
	R- TCGTACAGCCCATCGTTGTT
Mouse qPCR endo c-Myc	F- GCCTAACCTCACAACCTTGG
	R- CCTATTTACATGGGAAAATTGGA
Mouse qPCR NFATc1	F- GGGTCAGTGTGACCGAAGAT
	R- GGAAGTCAGAAGTGGGTGGA
Mouse qPCR NFATc2	F- CTGCTCATTATTCCCCCAGA
	R- GCATCCATGAGAACAGCAGA
Mouse qPCR NFATc3	F- TGGATCTCAGTATCCTTTAA
	R- CACACGAAATACAAGTCGGA
Mouse qPCR NFATc4	F- ACCCTCCGGTACAGAGGACT
	R- GGCTGCCCTCAGTCTCATAG
Mouse qPCR Slug	F- CCTTTCTCTTGCCCTCACTG
	R- ACAGCAGCCAGACTCCTCAT
Mouse qPCR PDGFRa	F- GTTGCCTTACGACTCCAGATG
	R- TCACAGCCACCTTCATTACAG
Mouse qPCR Thy1	F- CGCTCTCCTGCTCTCAGTCT
	R- GTTATTCTCATGGCGGCAGT
Mouse qPCR Zeb1	F- GCATCCAAAGAGCAAGAAGC
	R- ACTGGGCTGCTCAAGACTGT
Mouse qPCR Zeb2	F- GACCACCGACTCAAGGAGAC
	R- GGCATGAAAATGGAGTGGAT
Mouse qPCR GAPDH	F- CTTTGTCAAGCTCATTTCCTGG
	R- TCTTGCTCAGTGTCCTTGC
Mouse qPCR B-actin	F-ACCTTCTACAATGAGCTGCG
	R-CTGGATGGCTACGTACATGG
RT-PCR for endogenous Oct3/4 [1]	F-TCTTTCCACCAGGCCCCCGGCTC
	R-TGCGGGCGGACATGGGGAGATCC
RT-PCR for endogenous Sox2	F-TAGAGCTAGACTCCGGGCGATGA

[1]	R-TTGCCTTAAACAAGACCACGAAA
RT-PCR for Nanog [1]	F-CAGGTGTTTGAGGGTAGCTC
	R-CGGTTCATCATGGTACAGTC
RT-PCR for Esrrb	F-TAGGGGTTGAGCAGGACAAG
	R-CTACCAGGCGAGAGTGTTCC
RT-PCR for Sall4	F-ACACAAGAAAAGTTGCACTAAAACC
	R-CCTTTGGGTAAATAGCTTATGTCCT
RT-PCR for UTF1 [1]	F-GGATGTCCCGGTGACTACGTCTG
	R-GGCGGATCTGGTTATCGAAGGGT
pMx-S1811 m-Oct4-virus Geno [1]	F-GCTTGGATACACGCCGC
	R-TTCATGTCCTGGGACTCCTC
m-Sox2-virus Geno [1]	R-TTGCTGATCTCCGAGTTGTG
m-KLF4-virus Geno [1]	R-AACCGCTCCACATACAGTCC
m-c-MYC-virus Geno [1]	R-GGAAGACGAGGATGAAGCTG
pMXs-TgUS RT exo [1]	F-GTGGTGGTACGGGAAATCAC
pMXs-Oct3/4-TgDS [1]	R-TAGCCAGGTTCGAGAATCCA
pMXs-Klf4-TgDS [1]	R-GGGAAGTCGCTTCATGTGAG
pMXs-Sox2-TgDS [1]	R-GGTTCTCCTGGGCCATCTTA
pMXs-c-Myc-TgDS [1]	R-AGCAGCTCGAATTTCTTCCA
ChIP-qPCR nfatc2 binding site SRR2 of SOX2	F-CAGTCCAAGCTAGGCAGGTT
	R-CTGTGCTCATTACCACGTGAA
ChIP-qPCR nfatc2 binding site SRR2 of SOX2 [7]	F-TCCAAGCTAGGCAGGTTCCCCT
	R-CACAATGGCTGCCCGAGCCC
ChIP-qPCR nfatc2 new SRR2 of SOX2	F-CAGGTTCCCCTCTAATTAATGC
	R-CTGTGCTCATTACCACGTGAA
ChIP-qPCR nfatc2 on klf2 region 1-2	F-CTCCCTCCTCCAGAATCCTT
	R-CAAAAGTGAGCCATGTGGTG
ChIP-qPCR nfatc2 on klf2 region 3	F-CTCACATTCTGCCTCCATGA
	R-AGCAGAAACATTGGCGAACT
ChIP-qPCR nfatc2 on klf2	F-AGCACTGTGACCCCAGACTC

region 4	R-CAACAAACCAGGCACAACAG
ChIP-GNAI1-R1 OSKM	F-GGAGGCGAGGTCTGCTAGT
	R-CTAGCGGTCGCAGGGATT
ChIP-GNAI1-R2 OSKM	F-TCCGACAGAAACGTTTGACA
	R-AAGGCGATCCATTACCACAG
ChIP-GNAI2-R1 OSKM	F-CTCCGGCTCCCAAATCTAAT
	R-ACCGGCTTCACTACAACACC
ChIP-GNAI2-R2	F-GCGACTTCAGAGGCTTCCT
	R-TAGCCGAAGGCAAGTGAAGA
ChIP-GNAQ-R1 OSKM	F-CCTGAGCTCGTCCCTGAC
	R-AGAGTCATTCTTCCGAAGTGC
ChIP-GNAQ-R2 OSKM	F-TGGTGACAGGCGTCTCTATG
	R-TCTGGGTCGCCTCATTAAAC
ChIP-ITPK1-R1 OSKM	F-GCGCCTTGTGTCTATCTTGG
	R-AATTGCAACGAATTGCCTCT
ChIP-ITPK1-R2 OSKM	F-GTGCTTTGGATCCTCTGAGC
	R-TTCTCCTCCCAATGCCTAAA
ChIP-ITPKB-R1 OSKM	F-CCGGGACTAAGCCGAGAG
	R-GGCTATTGAGGGCATAGCAG
Chip-ITPKB-R2 OSKM	F-CAAGTGAGGAACGCAGAACA
	R-CTGGCTGGGCCTAAAAGAG
ChIP-ITPr2-R1 OSKM	F-GAGATTATGAAGTTGCTGAGAAGC
	R-CAACTCGGGTCCCTGTCC
ChIP-ITPr2-R2 OSKM	F-GTTCCTGGCGAAGTTCTGTC
	R-GACCTAGAGGCTCCGCTCA
ChIP-NFATc1-R1 OSKM	F-CCTGGTTTGGACAGGGGTA
	R-CCAGTCCCTTGTGTCCTCAT
ChIP-NFATc1-R2 OSKM	F-GGGCAAAGGTGTACAAGAGG
	R-CGTTTCGTCCCTGACTGTTT
Chip-NFATc2-R1 OSKM	F-ATCAGCGCGCAGAGCTAC

	R-GGCTATTGAGGGCATAGCAG
ChIP-NFATc2-R2 OSKM	F-TGCAGGTGAACCAGAAAGTG
	R-GAACGAGCCCTACCCAAAC
ChIP-PLCB1-R1 OSKM	F-CTCCCGAGGCTCTACAGTCA
	R-CTTAGTAAGCGGGACGACAC
ChIP-PLCB1-R2 OSKM	F-GGTCCCAAACCTTGCAGTTA
	R-GACCACCTGTCGGCTGTACT
ChIP-Ppp3CA-R1 OSKM	F-GTCTTAATCCCGACCGTGTG
	R-GCCTCCCGGTTCTTCTTTTA
ChIP-Ppp3CA-R2 OSKM	F-CCTCGGTCTCCGAACTCACT
	R-GAGGCTGGACTGGGAGTAGG
ChIP-Ppp3CB-R1 OSKM	F-CCTCCTCTTTGTAAGATGGCTTT
	R-GAAAACCGTATGGGACTGGA
ChIP-Ppp3CB-R2 OSKM	F-ACCCTCTTTCCCTTCAGAGG
	R-AGCCTCGGTAATTTTGCTCA
ChIP-Ppp3R1-R1 OSKM	F-GCACGGTACGCAAAAAGC
	R-AGGCTGACATCCGGGAAC
ChIP-Ppp3R1-R2 OSKM	F-CCTTGAGGTTGCGGTCAG
	R-GGAGTCCCGGTTGGAGAT
ChIP IL2 [6]	F-ATGGGAGGCAATTTATACTG
	R-CCATTCAGTCAGTGTATGGG

List of target sequence used

Gene name	Target sequence
PPP3ca-1	Top 5`- GATCCGTATTTCACGTTTAAACAAGATTCAAGAGATCTTGTTTAAACGTGAAATACTTTTTTACGCGTG-3`
	Bottom 5`- AATTCACGCGTAAAAAAGTATTTCACGTTTAAACAAGATCTCTTGAATCTTGTTTAAACGTGAAATACG-3`
PPP3ca-2	Top 5`- GATCCGCTAACTAAGGACTATTTATTGTTCAAGAGACAATAAATAGTCCTTAGTTAGTTTTTTACGCGTG-3`
	Bottom 5`- AATTCACGCGTAAAAAACTAACTAAGGACTATTTATTGTCTCTTGAACAATAAATAGTCCTTAGTTAGCG-3`
PPP3ca-3	Top 5`- GATCCGCAGTAATAGCAGCAATATCCTTCAAGAGAGGATATTGCTGCTATTACTGCTTTTTTACGCGTG-3`
	Bottom 5`- AATTCACGCGTAAAAAAGCAGTAATAGCAGCAATATCCTCTCTTGAAGGATATTGCTGCTATTACTGCG-3`

PPP3ca-4	Top 5`- GATCCGCCGTTCCATTTCCACCAATTCAAGAGATTGGTGGAAATGGAACGGCTTTTTTACGCGTG-3`
	Bottom 5`- AATTCACGCGTAAAAAAGCCGTTCCATTTCCACCAATCTCTTGAATTGGTGGAAATGGAACGGCG-3`
PPP3ca-5	Top 5`- GATCCCACAGAGTATTTCACGTTTAACTCGAGTTAAACGTGAAATACTCTGTGTTTTTG-3`
	Bottom 5`- AATTCAAAAACACAGAGTATTTCACGTTTAACTCGAGTTAAACGTGAAATACTCTGTGG-3`
PPP3ca-6	Top 5`- GATCCGCCAAGGCGATTGATCCCAATTCAAGAGATTGGGATCAATCGCCTTGGTTTTTTACGCGTG-3`
	Bottom 5`- AATTCACGCGTAAAAAACCAAGGCGATTGATCCCAATCTCTTGAATTGGGATCAATCGCCTTGGCG-3`
PPP3ca-7 [8]	Top-5'-GATCCGTTACAATCTTCTCGGCACCTTCAAGAGAGGTGCCGAGAAGATTGTAATTTTTTACGCGTG-3`
	Bottom-5'-AATTCACGCGTAAAAAATTACAATCTTCTCGGCACCTCTCTTGAAGGTGCCGAGAAGATTGTAACG-3`
PPP3ca-7 PLKO-tet	Top-5`CCGGGTTACAATCTTCTCGGCACCCTCGAGGGTGCCGAGAAGATTGTAATTTTT-3`
	Bottom -5`AATTAAAAATTACAATCTTCTCGGCACCCTCGAGGGTGCCGAGAAGATTGTAAC-3`
PPP3cb-1	Top-5'- GATCCGAAATTGCACTAAGAATTATCTTCAAGAGAGATAATTCTTAGTGCAATTTCTTTTTTACGCGTG-3`
	Bottom 5`- AATTCACGCGTAAAAAAGAAATTGCACTAAGAATTATCTCTCTTGAAGATAATTCTTAGTGCAATTTCG-3`
PPP3cb-2	Top-5'- GATCCGCAAATATTTAATATATAGACCTTCAAGAGAGGTCTATATATTAAATATTTGTTTTTTACGCGTG-3`
	Bottom 5`- AATTCACGCGTAAAAAACAAATATTTAATATATAGACCTCTCTTGAAGGTCTATATATTAAATATTTGCG-3`
PPP3cb-3	Top-5'- GATCCGTAAATGTTCTGAGTATTTGTTTCAAGAGAACAAATACTCAGAACATTTACTTTTTTACGCGTG-3`
	Bottom 5`- AATTCACGCGTAAAAAAGTAAATGTTCTGAGTATTTGTTCTCTTGAAACAAATACTCAGAACATTTACG-3`
Suv39h1	Top 5`-GATCCGCCTTTGTACTCAGGAAAGAATTCAAGAGATTCTTTCCTGAGTACAAAGGCTTTTTTG-3`
	Bottom 5`-AATTCAAAAAAGCCTTTGTACTCAGGAAAGAATCTCTTGAATTCTTTCCTGAGTACAAAGGCG-3`
HDAC3	Top 5`-GATCCGTGTTGAATATGTCAAGAGTTTTCAAGAGAAACTCTTGACATATTCAACACTTTTTTG-3`
	Bottom 5`-AATTCAAAAAAGTGTTGAATATGTCAAGAGTTTCTCTTGAAAACTCTTGACATATTCAACACG-3`
Gnaq	Top5`-GATCCGCTTGTGGAATGATCCTGGAATTCAAGAGATTCCAGGATCATTCCACAAGCTTTTTTG-3`
	Bottom 5'-AATTCAAAAAAGCTTGTGGAATGATCCTGGAATCTCTTGAATTCCAGGATCATTCCACAAGCG-3`
NFATc2	Top5`-GATCCGCCCTATCGAAGAAGAACCGATTTCAAGAGAATCGGTTCTTCTTCGATAGGGTTTTTTA-3`
	Bottom 5'-AGCTTAAAAAACCCTATCGAAGAAGAACCGATTCTCTTGAAATCGGTTCTTCTTCGATAGGGCG-3`
NFATc3-1	Top 5`- GATCCGCTCACATTGTCCTTGAAGTTTTCAAGAGAAACTTCAAGGACAATGTGAGCTTTTTTG-3`
	Bottom 5`- AATTCAAAAAAGCTCACATTGTCCTTGAAGTTTCTCTTGAAAACTTCAAGGACAATGTGAGCG-3`
NFATc3-2	Top 5`- GATCCGTGGGAAACGAGCTTTGCTTTTTCAAGAGAAAAGCAAAGCTCGTTTCCCACTTTTTTG-3`
	Bottom 5`- AATTCAAAAAAGTGGGAAACGAGCTTTGCTTTTCTCTTGAAAAAGCAAAGCTCGTTTCCCACG-3`
NFATc4-1	Top 5`- GATCCGCCAGACTCTAAAGTGGTGTTTTCAAGAGAAACACCACTTTAGAGTCTGGCTTTTTTG-3`
	Bottom 5`- AATTCAAAAAAGCCAGACTCTAAAGTGGTGTTTCTCTTGAAAACACCACTTTAGAGTCTGGCG-3`
NFATc4-2	Top 5`- GATCCGCGAGGTGGAGTCTGAACTTAATTCAAGAGATTAAGTTCAGACTCCACCTCGTTTTTTG-3`
	Bottom 5`- AATTCAAAAAACGAGGTGGAGTCTGAACTTAATCTCTTGAATTAAGTTCAGACTCCACCTCGCG-3`
luciferase	Top 5`-GATCCGTAAGGCTATGAAGAGATACTTCAAGAGAGTATCTCTTCATAGCCTTACTTTTTTA-3`
	Bottom 5`-AGCTTAAAAAAGTAAGGCTATGAAGAGATACTCTCTTGAAGTATCTCTTCATAGCCTTACG-3`

List of reagents used

Reagent	Manufacture and catalog number
NFATc1	SantaCruz Sc-7294
NFATc2	SantaCruz Sc-7296
NFATc3	SantaCruz Sc-8321
NFATc4	SantaCruz Sc-13036
CnAα	Cell signaling #32614
CnAα/β	Millipore 07-1491
Alpha-tub	Sigma-Aldrich T5168
Hisotone 3	Cell signaling #4620
Hisotone 3	Cell signaling # 9715
B-actin	Santa Cruz Sc-47778
p16	Santa Cruz Sc-1207
p19	Santa Cruz Sc-22784
p21	Santa Cruz Sc-397
P27kip1	Cell signaling #2552
P27	Santa Cruz Sc-528
CDK4	MBL K0065-3
CyclinD1	MBL K0062-3
Rb	Santa Cruz Sc-50
Rb	Abcam ab6075
p-Rb807/811	Cell signaling # 9308
Anti-FLAG M2	Sigma F1804
H3K4Me1	Cell signaling # 5326
H3K4Me2	Cell signaling # 9725
H3K4Me3	Cell signaling # 9751
H3K9Me1	Cell signaling # 7538
H3K9Me2	Cell signaling # 4658
H3K9Me3	Millipore # 07-442

H3K27Me1	Cell signaling #7693
H3K27Me2	Cell signaling # 9728
H3K27Me3	Cell signaling # 9733
H3K36Me1	Cell signaling #5928
H3K36Me2	Cell signaling # 2901
H3K36Me3	Cell signaling # 4909
H3K79Me1	Cell signaling # 9398
H3K79Me2	Cell signaling # 8427
H3K79Me3	Cell signaling # 4260
FK506	Abcam ab120223
Cyclosporin A CSA	Abcam ab120114
Annexin V	Beckman Coulter
Sox2	SantaCruz Sc-17320
SSEA1-FITC	BD pharmingen MC-480
SSEA1-biotin	eBioscience eBioMC-480
Streptoavidin-allophycocyanin SA-APC	eBioscience
7-amino-actinomycin D 7AAD	Beckman Coulter
2nd Antibody anti-HRP	BioRad
2nd Antibody anti- HRP	BioRad
2nd Antibody anti- HRP	BioRad
Anti-BrdU-bition	Abcam ab2284
Propidium iodide (PI)	WAKO, Japan
Hoechst 33258	Invitrogen
Alkaline phosphatase kit	Sigma
Calcineurin assay kit	ENZO Life Sciences
Suv39h1	Santacruz Sc23961
Nanog	Benthyl A300-397A2
ChIP-Grade protein magnetic beads	Cell signaling #9006
Anti-V5	Nacalai tesque (V5005)

Results

Results

8.1 Calcineurin/NFAT signaling is activated during reprogramming.

To address the role of calcineurin/NFAT signaling in the context of reprogramming, we first examined the expression patterns of different components of calcineurin, and its downstream targets NFATc1-4 (Fig. 1A-1C). Genes encoding several subunits of calcineurin (i.e. *Ppp3ca, Ppp3cb, and Ppp3r1*) were upregulated during reprogramming from day 2 until day 14. The protein levels of *CnAα* remained constant during reprogramming, but were reduced in mouse ESCs (Fig. 1B).

Expression of NFATc1–4 at both the mRNA and protein levels differed throughout the course of reprogramming. For example, although NFATc1 mRNA and protein expression was very low throughout the entire reprogramming process, there was a significant increase in NFATc3 and NFATc4 protein levels until day 8, after which levels decreased until day 14 (Fig. 1B). Immunoblot analysis revealed fluctuations between phosphorylated and dephosphorylated states, indicating that NFATc3 and NFATc4 were subject to post-translational modification during reprogramming (Fig. 1B). There was a corresponding increase in *NFATc3* mRNA expression on day 2, which decreased gradually until day 8 to levels that were maintained until day 14 (Fig. 1C). Interestingly, NFATc2 mRNA and protein was transiently expressed from day 6 until day 14, but NFATc2 was not detected in either mouse embryonic fibroblasts (MEF) or ESCs (Fig. 1B, 1C).

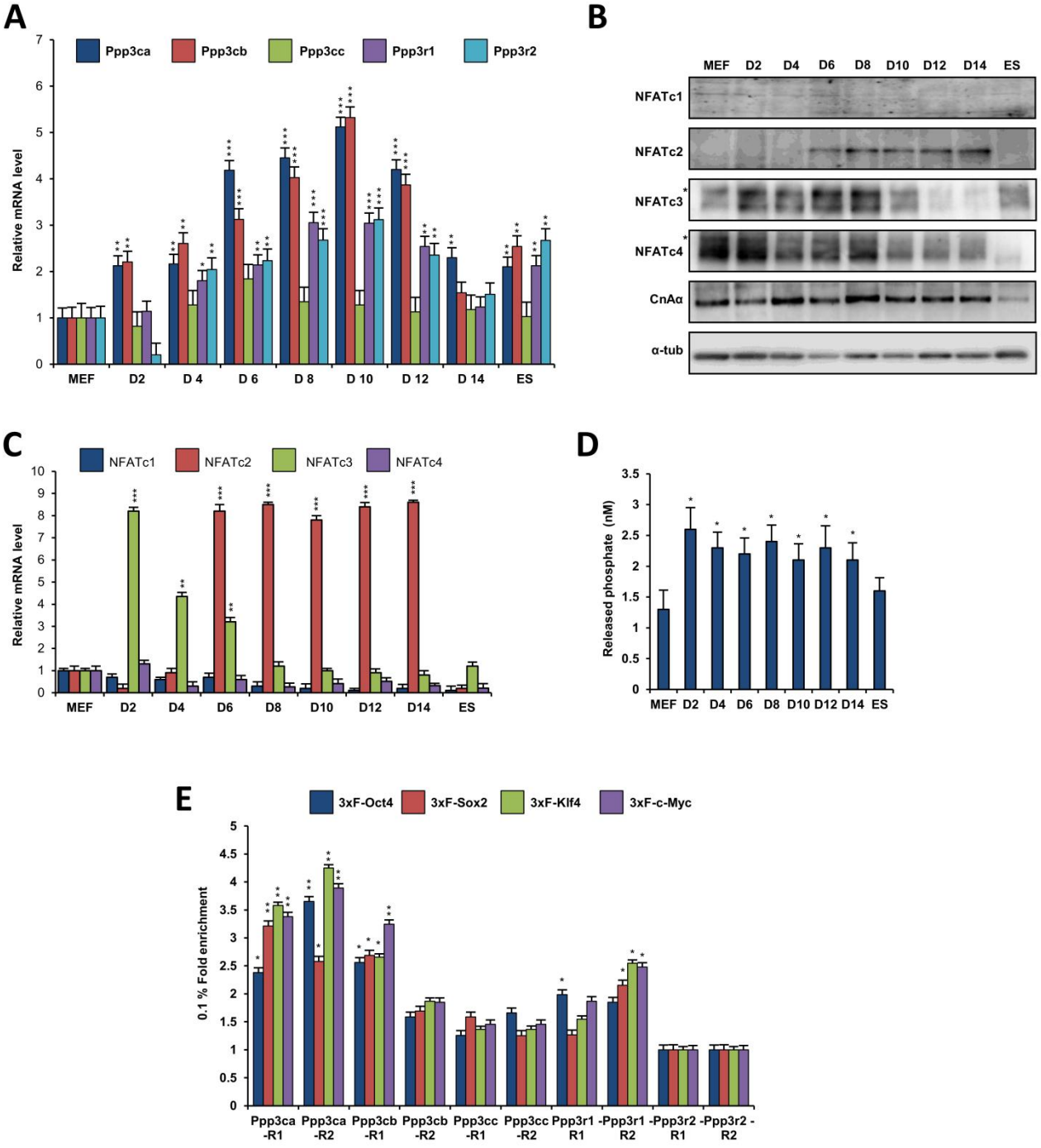


Figure 1. Calcineurin/NFAT signaling is activated during reprogramming. (A): Expression of calcineurin subunits during reprogramming by q-PCR. **(B):** Immunoblot analysis for NFATc1, 2, 3 and 4 isoforms and catalytic subunits

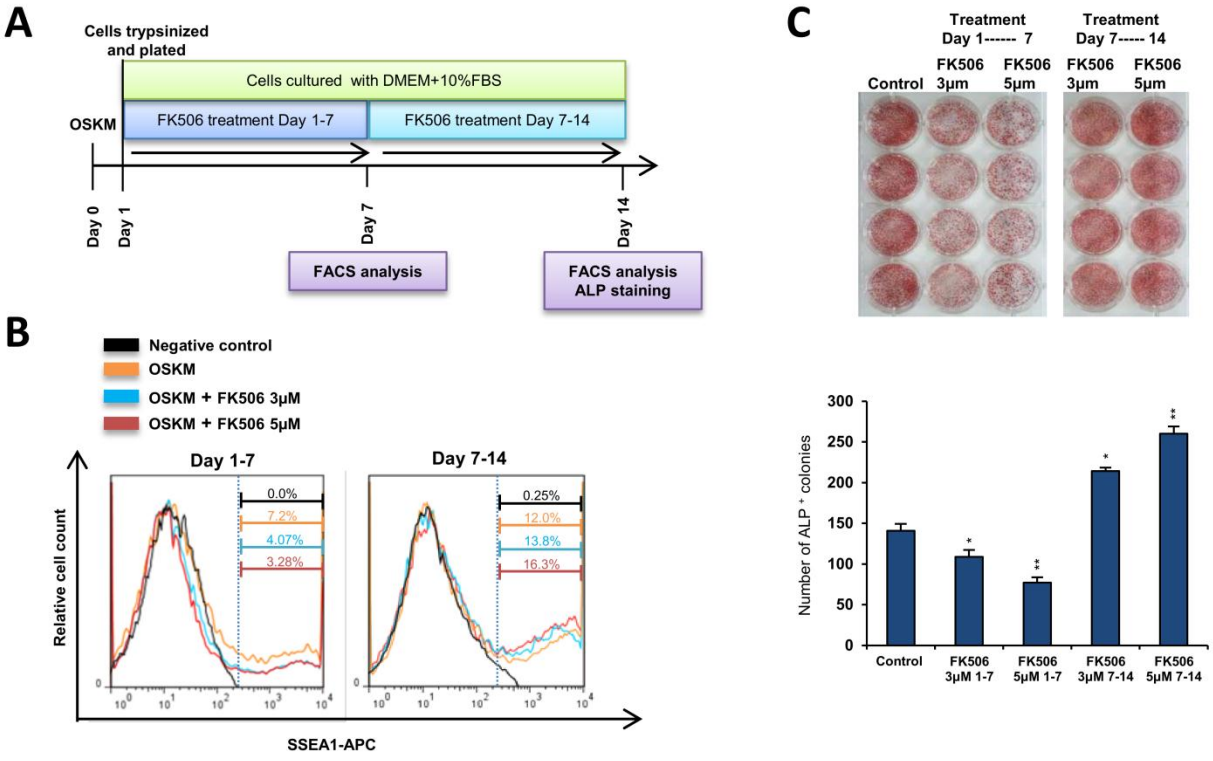
of calcineurin CnAα. The asterisk (*) indicate the phosphorylated forms of protein, α-tub, α-tubulin used as internal control. **(C):** Expression of *NFATc1, 2, 3 and 4* isoforms during reprogramming by q-PCR. **(D):** The calcineurin activity during reprograming was estimated by ELISA. **(E):** Binding of 3xflag-Oct4, 3xflag-Sox2, 3xflag-Klf4 and 3xflag-c-Myc at Regions 1 and 2 (R1 and R2, respectively) of the calcineurin subunits determined by ChIP-qPCR. 3xflag empty vector and IgG used as negative control. Samples from MEF and E14tg2a mouse ES are used as a negative and positive control, respectively, and the relative expression levels are normalized to the housekeeping gene, *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)*

Calcineurin activity increased significantly from day 2 to day 14 during reprogramming compared with activity in MEF (Fig. 1D). To elucidate the mechanism, we did chromatin immunoprecipitation (ChIP) coupled with quantitative polymerase chain reaction (qPCR) revealed significant enrichment of the genes encoding the different calcineurin subunits (Fig. 1E). These results suggested that the expression of calcineurin subunits is upregulated by the four reprogramming factors, whereby the calcineurin activity is enhanced during reprogramming process.

8.2 Biphasic effects of calcineurin/NFAT signaling during reprogramming

To ask whether modulating calcineurin signaling could affect the reprogramming process, we Firstly tested the effects of different concentrations of two known but unrelated pharmacological inhibitors of calcineurin phosphatase activity, namely cyclosporin A (CSA) and FK506. Although both CSA and FK506 inhibited calcineurin phosphatase activity, we chose to use 3 μM FK506, a concentration that exhibits minimal side effects.

In our first experience, we noticed an opposing role of calcineurin inhibition on reprogramming process throughout the morphological change so we divided the reprogramming process into two phases: (i) an early phase from day 1 to day 7; and (ii) a late phase from day 7 to day 14 (Fig. 2A). The effects of calcineurin inhibition with 3 and 5 μM FK506 were evaluated during the early, late, and entire reprogramming process. FK506 treatment in the early phase inhibited reprogramming efficiency, as measured by expression of stage-specific embryonic antigen1 (SSEA1) (an early reprogramming marker) and colony formation of iPSCs. In contrast, FK506 treatment during the late phase enhanced reprogramming efficiency (Fig. 2B, 2C).



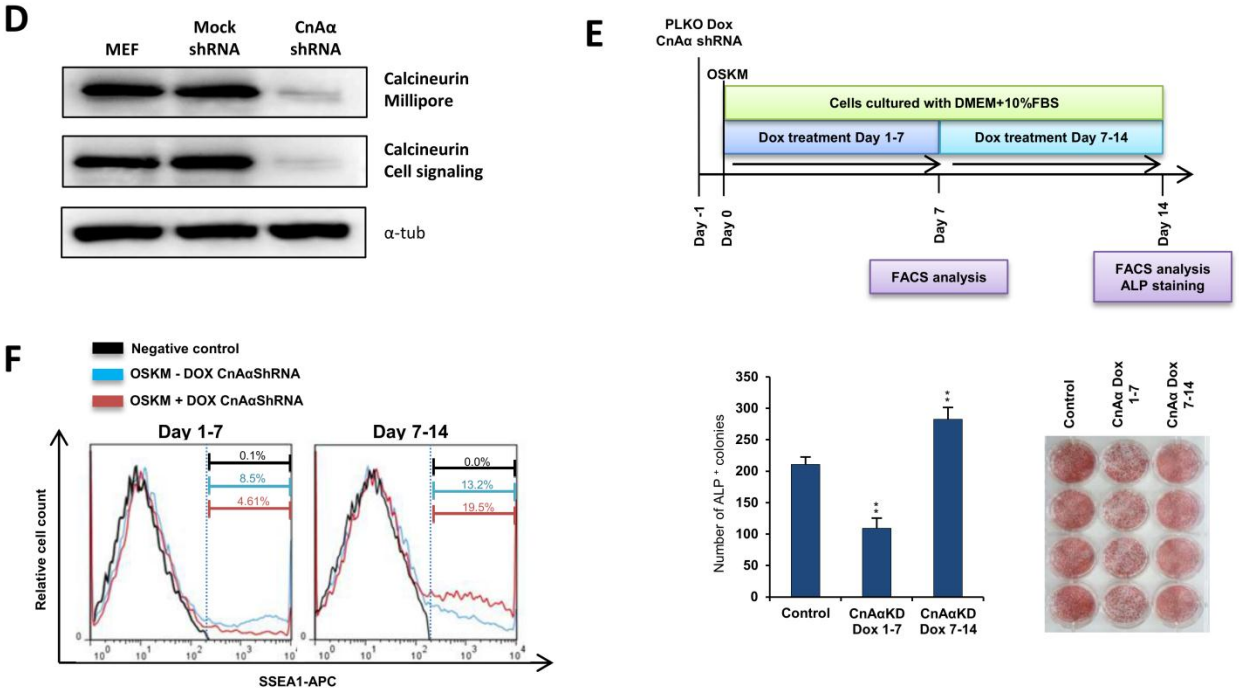


Figure 2. Calcineurin signaling exhibits biphasic role during reprogramming process. (A): Experimental design showing the timing of FK506 treatment during reprogramming. **(B):** SSEA1 expression analyzed by fluorescence-activated cell sorting (FACS) on either day 7 or 14 of reprogramming with two different concentrations of FK506 (3 and 5 μM). **(C):** Reprogramming efficiency across two timeframes of reprogramming (days 1-7 and 7-14) and with two different concentrations of FK506 treatment; upper panel shows ALP staining, whereas quantification of the number of ALP-positive colonies is shown in the lower panel. **(D):** calcineurin knockdown efficiency estimated by immunoblot **(E):** The experimental design for periodic knockdown of CnAα during the early (days 1–7) and late (days 7–14) phases of reprogramming using Dox-inducible shRNA (upper panel). Reprogramming efficiency was measured after counting the number of ALP positive colonies (lower panel). **(F):** SSEA1 expression determined by FACS analysis after periodic knockdown of CnAα during the early and late phases of reprogramming.

To prove this hypothesis, we used doxycycline (DOX)-inducible short hairpin RNA (shRNA) expression to knockdown the catalytic subunit (CnAα) (Fig. 2D). Suppression of CnAα expression in the early and late phase of reprogramming decreased and increased, respectively, both the SSEA1-positive fraction and the number of alkaline phosphatase (ALP)-positive colonies (Fig. 2E, 2F). These results show that the calcineurin pathway has opposite effects during the early and late stages of the reprogramming process.

8.3 Effects of calcineurin knockdown during the early phase of reprogramming on cell cycle regulators

During early phase of reprogramming, cell proliferation and MET were reported as critical events to achieve successful reprogramming [5, 6]. Both cell senescence and apoptosis are evoked by upregulation of different cell cycle regulators, including p53, p21, and p16 upon ectopic expression of the four reprogramming factors, [13, 14].

We analyzed cell proliferation and apoptosis using bromodeoxyuridine (BrdU) incorporation and annexin V staining. The percentage of apoptotic cells remained unchanged compared with control (Fig. 3A). However, Suppression of CnAα reduced the rate of proliferation, as demonstrated by low BrdU uptake, on both days 2 and 4 during reprogramming, (Fig. 3B). Most cells after CnAα knockdown were arrested in the G1 phase, revealing that after downregulation of this calcneurin catalytic subunit, the cells become much more resistant to reprogramming by entering cell cycle arrest (Fig. 3C). Consistent with these observations,

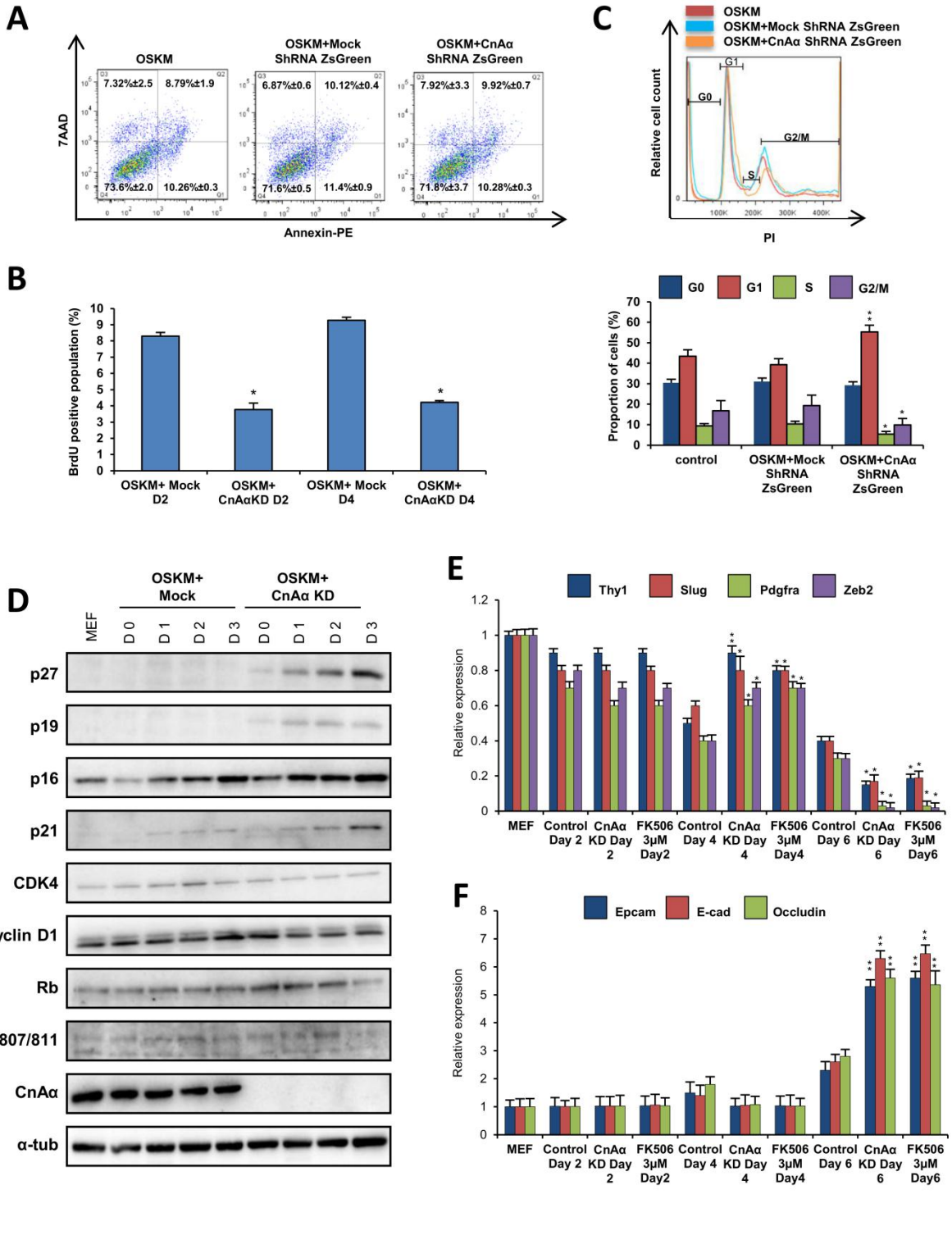

A
OSKM
OSKM+Mock ShRNA ZsGreen
OSKM+CnAα ShRNA ZsGreen
7AAD
Annexin-PE
7.32%±2.5
8.79%±1.9
73.6%±2.0
10.26%±0.3
6.87%±0.6
10.12%±0.4
71.6%±0.5
11.4%±0.9
7.92%±3.3
9.92%±0.7
71.8%±3.7
10.28%±0.3
B
BrdU positive population (%)
OSKM+ Mock D2
OSKM+ CnAαKD D2
OSKM+ Mock D4
OSKM+ CnAαKD D4
C
OSKM
OSKM+Mock ShRNA ZsGreen
OSKM+CnAα ShRNA ZsGreen
Relative cell count
G1
G0
G2/M
S
PI
G0
G1
S
G2/M
Proportion of cells (%)
control
OSKM+Mock ShRNA ZsGreen
OSKM+CnAα ShRNA ZsGreen
D
OSKM+ Mock
OSKM+ CnAα KD
MEF
D 0
D 1
D 2
D 3
p27
p19
p16
p21
CDK4
Cyclin D1
Rb
pRb 807/811
CnAα
α-tub
E
Thy1
Slug
Pdgfra
Zeb2
Relative expression
MEF
Control Day 2
CnAα KD Day 2
FK506 3μM Day2
Control Day 4
CnAα KD Day 4
FK506 3μM Day4
Control Day 6
CnAα KD Day 6
FK506 3μM Day6
F
Epcam
E-cad
Occludin
Relative expression

Figure 3. Calcineurin knockdown during the early phase of reprogramming impairs cell proliferation and delays MET. **(A):** Cell proliferation determined by BrdU incorporation on days 2 and 4 of reprogramming with mock shRNA or *CnAα* shRNA. **(B):** The apoptosis level was estimated on day 3 or reprogramming using Annexin V staining analyzed by FACS. **(C):** The percentage of cells in the different phases of the cell cycle at day 3 of reprogramming analyzed by FACS. **(D):** Immunoblot analysis of protein level for several cell cycle regulators after ectopic expression of *OSKM* with either mock shRNA or *CnAα* shRNA. Day 0 represents 8 hours after *OSKM* infection, whereas days 1, 2 and 3 indicated 24, 48, 72 hours after infection, respectively. **(E):** Expression of mesenchymal **, (F):** epithelial markers, as determined by qPCR, on days 2, 4, and 6 of reprogramming after treatment with 3 μM FK506 and *CnAα* knockdown.

several cell cycle regulators, including p16, p19, p21, and p27, were elevated from day 0 to day 3 following CnAα knockdown (Fig. 3D).

We next investigated the MET process with CnAαKD. The slow rate of downregulating mesenchymal markers Platelet-Derived Growth Factor Receptor Alpha (*PDGFRa),* Thymocyte antigen 1 *(Thy1),* Snail Family Zinc Finger 2 *(Slug), and* Zinc Finger E-Box Binding Homeobox 2 *(Zeb2)* (Fig. 3E), and upregulating epithelial markers *Occludin, E-cadherin (E-cad), and Epithelial Cell Adhesion Molecule (Epcam)* continues until day 4 (Fig. 3F), then this pattern was altered by increasing MET rate especially on day 6. These results suggested that during reprogramming the rate of MET was delayed after inhibiting calcineurin activity with FK506 treatment or shRNA. Taken together, these results demonstrate that calcineurin pathway plays a crucial role in maintaining proper cell proliferation and MET in the early

phase of reprogramming, and inhibiting calcineurin activity can impair cell cycle regulators, leading to a decline in MET rate.

8.4 Inhibitory role of calcineurin mediated by NFATc2 expression

To investigate the inhibitory role of calcineurin in the late phase of reprogramming, we first examined the expression of several pluripotency markers after depletion of calcineurin activity either by knockdown using shRNA or pharmacological inhibition with FK506. These treatments upregulated the expression of pluripotency markers during progression through the late phase of reprogramming from day 8 to day 14; in particular, *endo-Sox2* and *Klf2* appeared to be the most significantly upregulated pluripotency markers as early as day 8 of reprogramming compared with control (Fig. 4A,4B).

Next, we attempted to identify the downstream molecules of calcineurin. Because NFATc2 was transiently expressed at both RNA and protein levels from day 8 to day 14 during reprogramming, it is reasonable to assume that it is a maindownstream molecule of calcineurin in the late phase of reprogramming. To prove this hypothesis, we suppressed *NFATc2* expression (Fig. 4C), and found that knockdown of *NFATc2* increased the SSEA1-positive fraction and the number of ALP-positive colonies (Fig. 4D).

Consistent with our hypothesis, we found that NFATc2 is localized in both the nucleus and cytoplasm, but inhibition of calcineurin by either FK506 or shRNA changes its localization from the nucleus to cytoplasm (Fig. 4E). We next assessed the effect of *ca-NFATc2* overexpression on positive and negative SSEA1 fraction using dox system. The SSEA1 positive population preferentially forms ALP positive colonies compared to SSEA1 negative one, but inducting the overexpression of *ca-NFATc2*

results in decreasing number of ALP positive colonies in SSEA1 positive population (Fig. 4F).

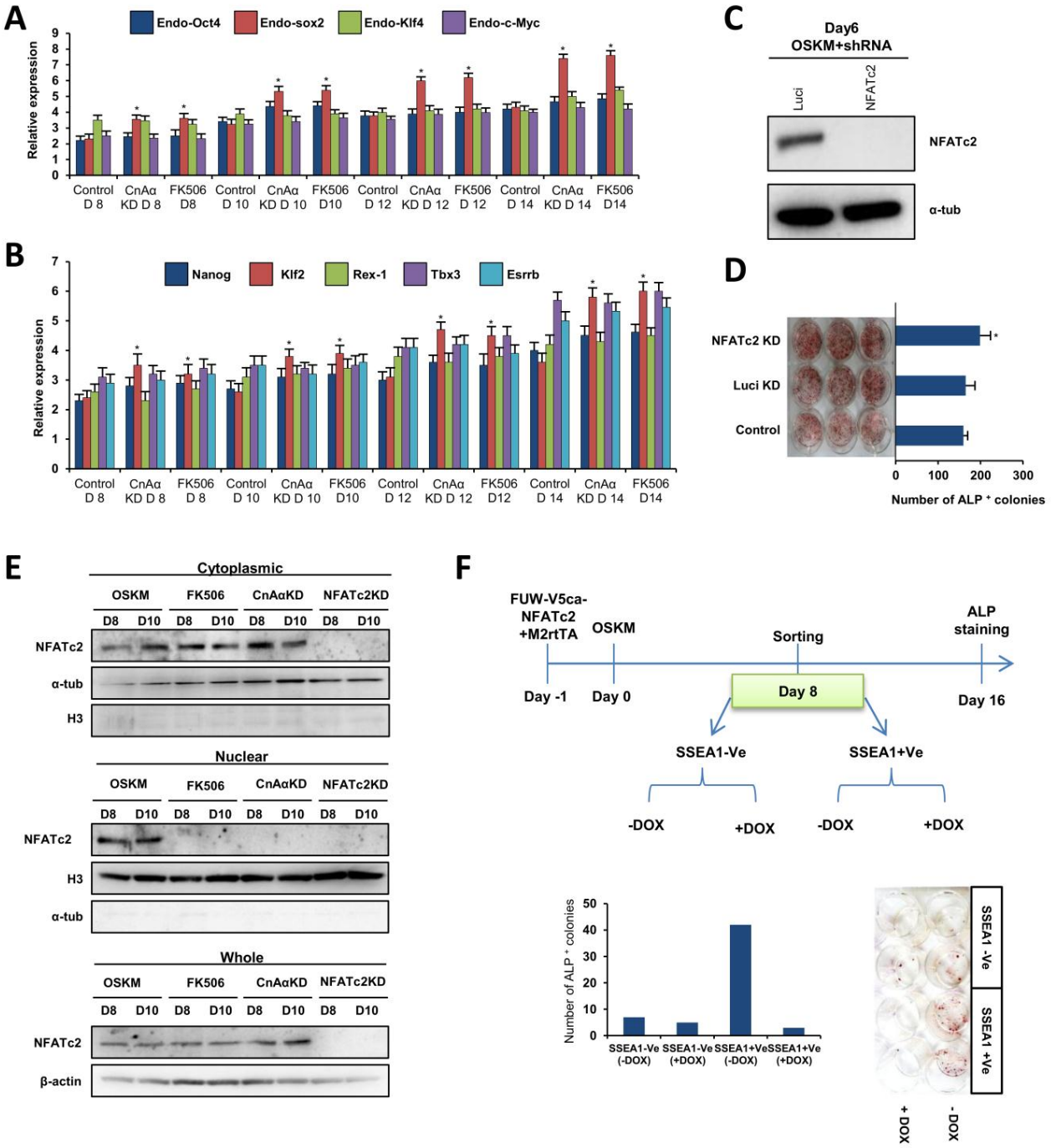


Figure 4. Inhibitory role of calcineurin was mediated by NFATc2. **(A, B):** Suppression *CnAα* expression or inhibition of CnAα activity with Fk506 treatment affected the expression of pluripotency markers, as determined by qPCR. **(C):** Knockdown (KD) efficiency of *NFATc2* analyzed by immunoblotting. **(D):** Increased reprogramming efficiency after *NFATc2* KD quantified by ALP staining on day 14. **(E):** Immunoblot analysis of the NFATc2 localization during reprogramming α-tubulin (α-tub), histone 3 (H3), and β-actin were used as internal loading controls for cytoplasmic, nuclear, and whole cell lysates, respectively. **(F):** Experimental design to investigate the potential of reprogramming in SSEA1-positive and -negative cells. Cells were sorted by FACS on day 8 and treated with or without 1μg/mL dox for another 8 days. Reprogramming efficiency was measured by determining the number of ALP-positive colonies on day 16 (lower panel).

Based on these findings, we concluded that calcineurin activity in the late phase of reprogramming mediates the translocation of NFATc2 to the nucleus, where it inhibits the expression of pluripotency markers, resulting in decreased reprogramming efficiency.

8.5 NFATc2 binding to the Klf2 promoter and Sox2 regulatory region 2, and induction of heterochromatin formation

To investigate how NFATc2 inhibits reprogramming and represses the expression of pluripotency markers, specifically *Sox2* and *Klf2*, we first scanned the regulatory regions of both genes with the binding motif GGAAA of NFATc isoforms [9]. *Sox2* has two proximal enhancer regions, *Sox2* regulatory region 1 (SRR1), located 4 kb upstream of the transcription start site (TSS), and *Sox2* regulatory region 2 (SRR2), located 4 kb downstream of the TSS [15], while *Klf2* can be regulated through the promoter region [16]. Only one binding motif was identified for NFATc2 at SRR2, whereas four binding motifs were identified at Klf2 promoter, R1: -

348 to -353, R2:-1561 to -1566, R3: -1785 to -1790, and R4: -1824 to -1829, and R1 exhibited the maximum enrichment of NFATc2.

ChIP-qPCR analysis revealed significant binding of NFATc2 at both SRR2 and the *Klf2* promoter, which was enhanced following overexpression of *NFATc2* and *ca-NFATc2*. However, binding decreased in the case of NFATc2, but not ca-NFATc2, after pharmacological or genetic inhibition of calcineurin activity (Fig. 5A).

We next investigated the enrichment of epigenetic marks tri-methylated lysine 9 of histone 3 (H3K9me3) and tri-methylated lysine 27 of histone 3 (H3K27me3) as repression marks over *Sox2* and *Klf2* loci. Overexpression of *NFATc2* and *ca-NFATc2* increased H3K9me3 and H3K27me3, and this enrichment was significantly reduced by *NFATc2* knockdown or inhibition of calcineurin activity (Fig. 5B).

Previous studies revealed the possibility of direct and indirect interactions of NFATc isoforms with histone deacetylases (HDAC) class I and class II, respectively [17-19]. We found NFATc2 binds to Enhancer Of Zeste 2 Polycomb Repressive Complex 2 Subunit known as Ezh2 *(*a catalytic subunit of H3K27methyltransferase)*,* Suppressor Of Variegation 3-9 Homolog 1 known as Suv39h1 *(*a methyltransferase for H3K9), and Hdac3*,* but no other *Hdacs* on day 6 of reprogramming (Fig. 5C). After knockdown Suv39h1 expression, both endo-*Sox2* and *Klf2;* which was inhibited by overexpression of *NFATc2*; were significantly upregulated (Fig. 5D). Furthermore, Ezh2 and Suv39h1 exhibited a significant enrichment pattern that was sensitive to calcineurin activity over both SRR2 and the

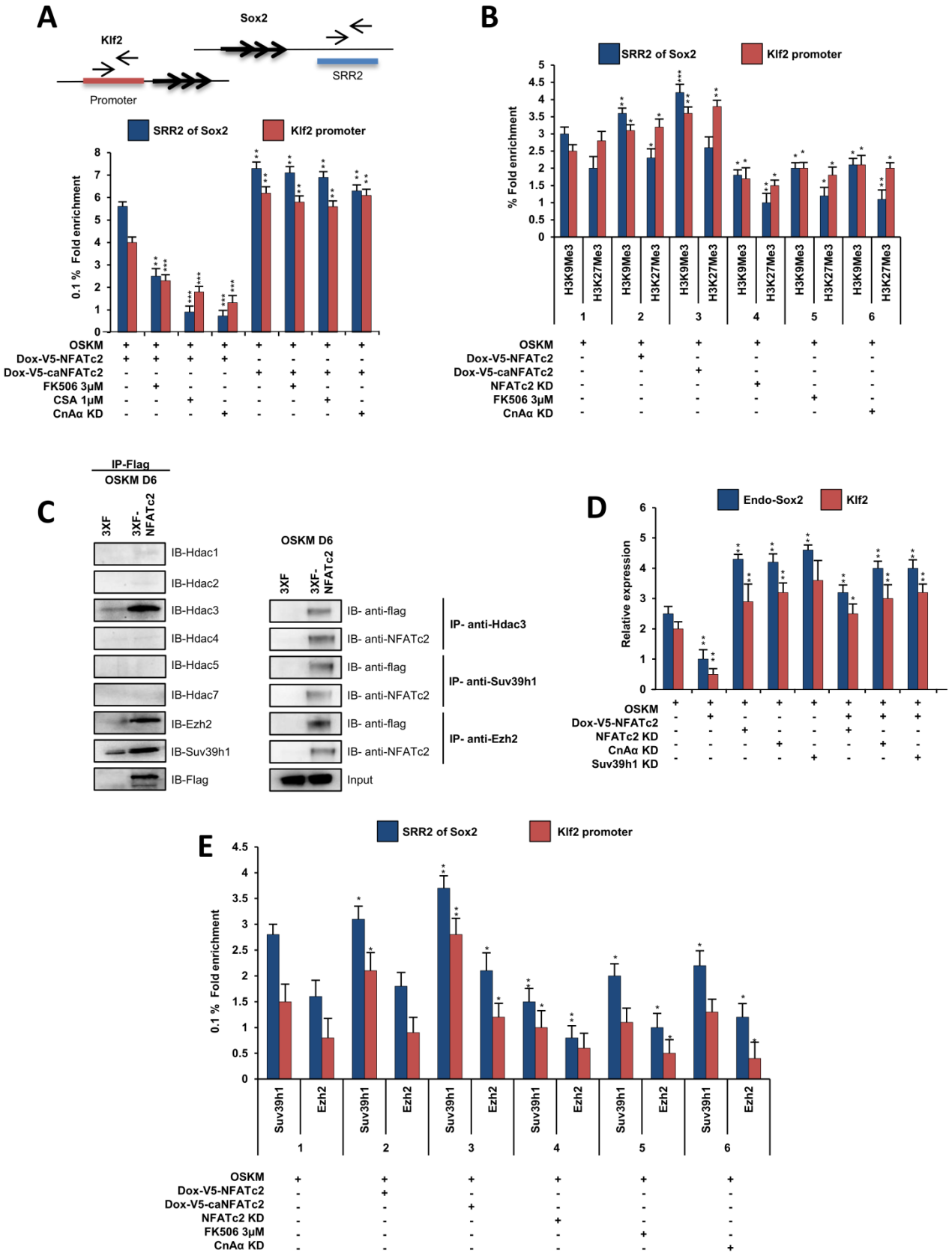

A
Sox2
Klf2
SRR2
Promoter
SRR2 of Sox2
Klf2 promoter
0.1 % Fold enrichment
OSKM
Dox-V5-NFATc2
Dox-V5-caNFATc2
FK506 3µM
CSA 1µM
CnAα KD
B
SRR2 of Sox2
Klf2 promoter
% Fold enrichment
H3K9Me3
H3K27Me3
1
2
3
4
5
6
OSKM
Dox-V5-NFATc2
Dox-V5-caNFATc2
NFATc2 KD
FK506 3µM
CnAα KD
C
IP-Flag
OSKM D6
3XF
3XF-NFATc2
IB-Hdac1
IB-Hdac2
IB-Hdac3
IB-Hdac4
IB-Hdac5
IB-Hdac7
IB-Ezh2
IB-Suv39h1
IB-Flag
IB- anti-flag
IB- anti-NFATc2
IP- anti-Hdac3
IP- anti-Suv39h1
IP- anti-Ezh2
Input
D
Endo-Sox2
Klf2
Relative expression
OSKM
Dox-V5-NFATc2
NFATc2 KD
CnAα KD
Suv39h1 KD
E
SRR2 of Sox2
Klf2 promoter
0.1 % Fold enrichment
Suv39h1
Ezh2
1
2
3
4
5
6
OSKM
Dox-V5-NFATc2
Dox-V5-caNFATc2
NFATc2 KD
FK506 3µM
CnAα KD

Figure 5. NFATc2 binds directly to the Klf2 promoter and SRR2, and suppresses their expression. **(A):** Binding of V5-tagged NFATc2 and ca-NFATc2 at the *Sox2* enhancer and *Klf2* promoter on day 10 of reprogramming, as determined by ChIP-qPCR. Enrichment of *NFATc2* but not *ca-NFATc2* was reduced by FK506 and CSA treatment, as well as *CnAα* KD. Cells were infected with *OSKM* on day 0 and all other plasmids on day -1, and dox was added from day 5 at 1µg/mL concentration to start the overexpressions of *V5-NFATc2* and *V5-ca-NFATc2*, 3 µM FK506 treatment was started from day 5. **(B):** Relative enrichment of H3K9Me3 and H3K27Me3 over the *Klf2* promoter and SRR2 enhancer on day 10, as determined by ChIP-qPCR**. (C):** *NFATc2* association with hdac3, Ezh2, and Suv39h1. Immunoblotting was performed using antibodies against the indicated targets after immunoprecipitation. **(D):** Expression of endogenous *Sox2* and *Klf2* on day 10 of reprogramming, as determined by qPCR. MEF infected with *all indicated plasmids* (V5-NFATc2, NFATc2KD, CnAαKD, and Suv39h1KD) at day -1 of reprogramming, and the dox was added from day 5 at 1µg/mL. **(E):** Relative enrichment of Suv39h1 and Ezh2 on day 10 of reprogramming, as determined by ChIP-qPCR. Enrichment was normalized to IgG, used as a control, relative expression levels are normalized to *GAPDH* and data are the mean±S.D deviation of triplicate samples.

Klf2 promoter (Fig. 5E). These results indicate that Suv39h1 is a repressive epigenetic factor recruited by NFATc2 to the *Sox2* and *Klf2* loci.

Furthermore, we did not find any significant changes in the expression of any of the H3K9 methylase and demethylase components. Taken together, these results suggest that NFATc2 binds directly to the *Sox2* SRR2 region and the *Klf2* promoter and recruits different repressive epigenetic modifiers including Suv39h1, Ezh2, and Hdac3 to repress their expressions.

8.6 G-Protein-coupled receptor signaling pathway located upstream of calcineurin/NFAT triggers its activation during reprogramming

We next attempted to identify the upstream molecules of the calcineurin/NFAT signaling pathway. Based on previous ChIP-seq data

[20], we speculated that genes encoding G-protein-coupled receptors (GPCR) are reasonable candidates for upstream molecules in the calcineurin/NFAT signaling pathway.

We confirmed this hypothesis, we performed ChIP-qPCR using flag-tagged Oct4, Sox2, Klf4, and c-Myc. Among GPCR targets, only Guanine Nucleotide Binding Protein (G Protein) known as *Gnaq,* Guanine Nucleotide Binding Protein (G Protein) Alpha Inhibiting Activity Polypeptide 1 known as *Gnai1,* Phospholipase C β1 known as *Plcb1,* Inositol 1,4,5-Trisphosphate Receptor, Type 2 known as *Itpr2*, and Inositol-Trisphosphate 3-Kinase A known as *Itpka* loci were enriched with the reprogramming factors and this enrichment is consistent with their upregulated expression upon initiation of reprogramming.

Next, we investigated whether Gnaq/calcineurin/NFATc2 can form an inhibitory axis over *Sox2* and *Klf2* loci to regulate their expression during reprogramming (Fig. 6A). Overexpression of *Gnaq* and *Gnaq (q209l)*, a constitutive active form of Gnaq, upregulated calcineurin activity (Fig. 6B) and downregulated of *endo-Sox2* and *Klf2* expressions with decreasing the enrichment of repressive mark, H3K9Me3, over both *Sox2* SRR2 enhancer and *Klf2* promoter (Fig. 6C, 6D). Overexpression of *Gnaqs* reduced reprogramming efficiency (Fig. 6E). All these events initiated by overexpression of Gnaqs were restored after Fk506 treatment, as well as knockdown of *Gnaq*, *CnAα*, *NFATc2*, or *Suv39h1*. It should be noted that Gnaq knockdown alone significantly downregulated calcineurin activity during reprogramming (Fig. 6B).

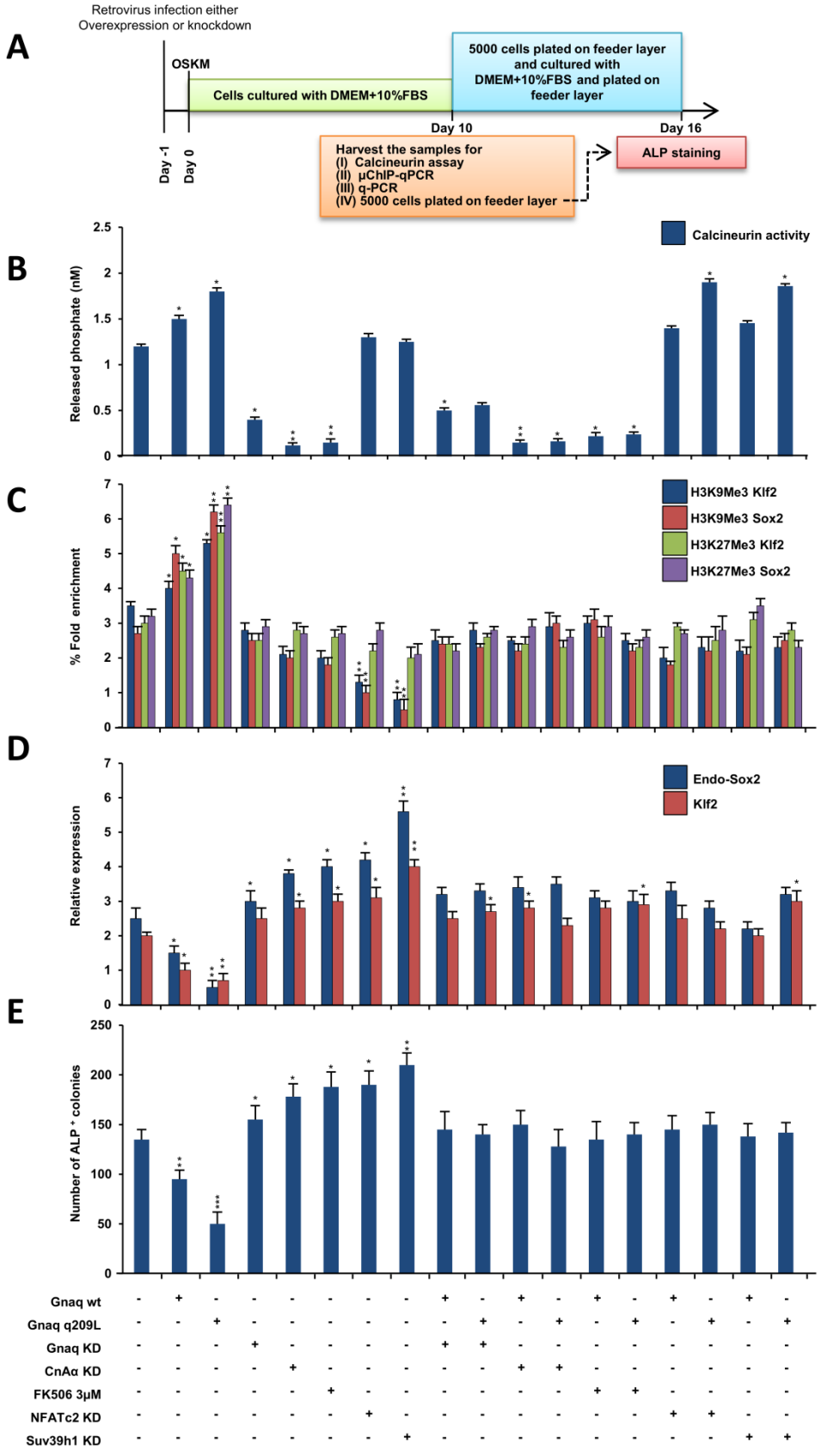
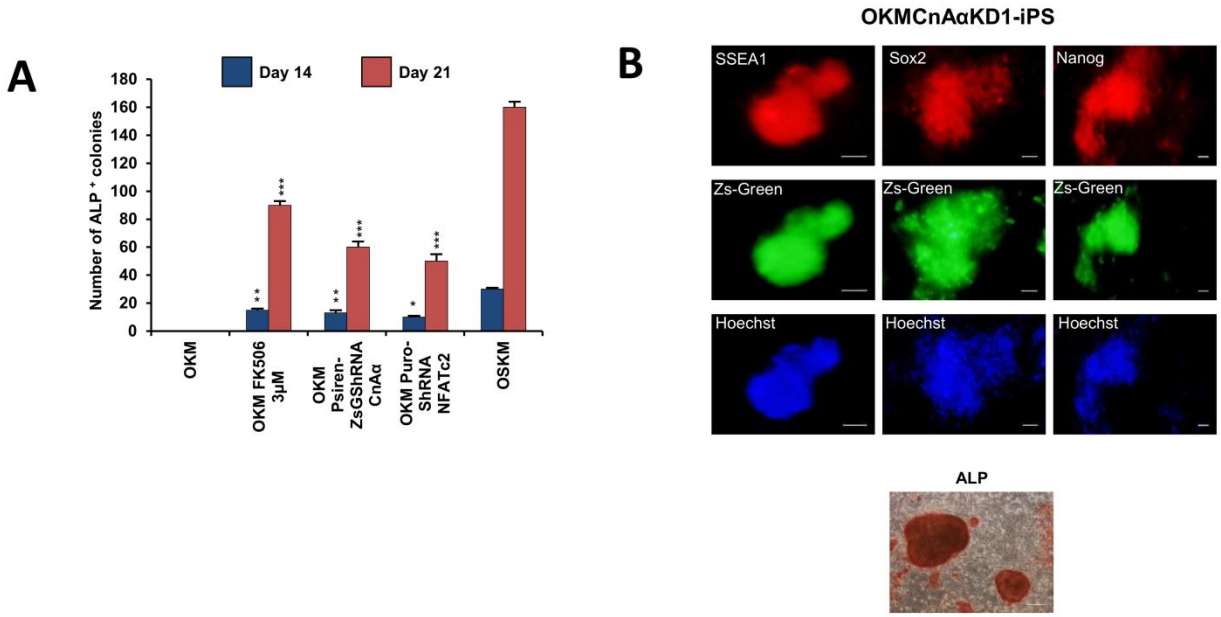

A
Retrovirus infection either
Overexpression or knockdown
OSKM
Cells cultured with DMEM+10%FBS
5000 cells plated on feeder layer and cultured with DMEM+10%FBS and plated on feeder layer
Day 10
Day 16
Day -1
Day 0
Harvest the samples for
(I) Calcineurin assay
(II) µChIP-qPCR
(III) q-PCR
(IV) 5000 cells plated on feeder layer
ALP staining
B
Calcineurin activity
Released phosphate (nM)
C
H3K9Me3 Klf2
H3K9Me3 Sox2
H3K27Me3 Klf2
H3K27Me3 Sox2
% Fold enrichment
D
Endo-Sox2
Klf2
Relative expression
E
Number of ALP+ colonies
Gnaq wt
Gnaq q209L
Gnaq KD
CnAα KD
FK506 3µM
NFATc2 KD
Suv39h1 KD

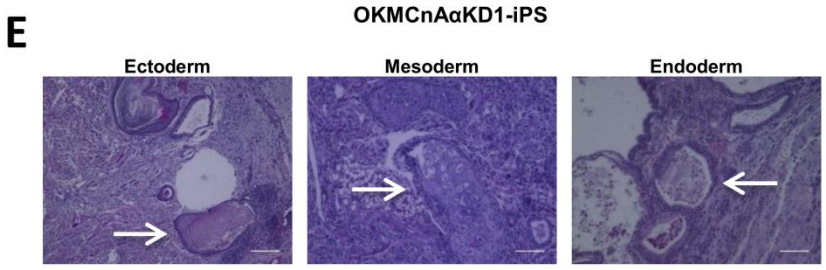
Figure 6. GPCR signaling is located upstream of calcineurin/NFAT and triggers activation of this pathway during reprogramming. (A): Experimental design of Fig. 6. The cells were infected with the virus carrying the indicated DNA and *OSKM* on day -1, and day 0, respectively. FK506 treatment started from day 5. On day 10, the cells were harvested from all conditions and divided into the four parts for the following experiments. **(B):** Calcineurin activity of the reprogramming cells with the indicated treatments. **(C):** Histone enrichment of H3K9Me3 and H3K27Me3 analyzed by ChIP-qPCR for *Sox2* enhancer and *Klf2* promoter. IgG was used as internal control. **(D):** Expression of endogenous *Sox2* and *Klf2* using qPCR. **(E):** Reprogramming efficiency with the indicated treatments measured by the number of ALP-positive colonies on day 16. For ChIP-qPCR the enrichment was normalized to IgG, for q-PCR the relative expression levels are normalized to *GAPDH* and data are the mean±S.D deviation of triplicate samples.

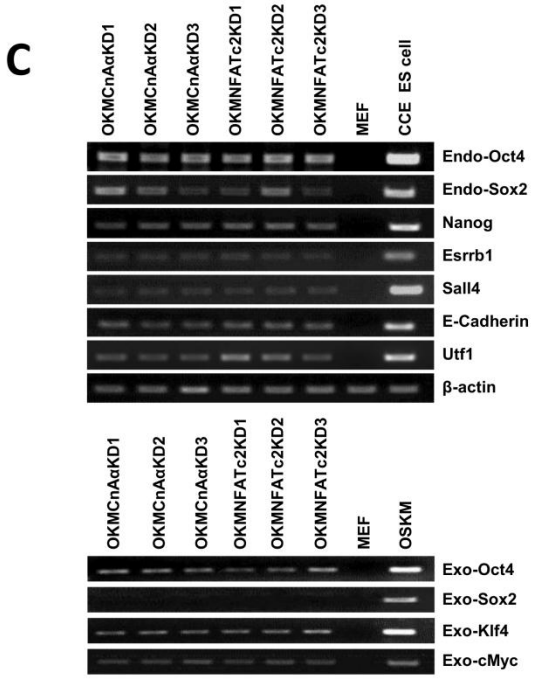
Taken together, these results suggest that Gnaq is one of the main sources of calcineurin activation, which is upregulated during reprogramming and triggers the cascade of mediator's activation responsible for the repression of *Klf2* and *Sox2* expressions by increasing the repressive marks over their regulatory region, thus decreasing the reprogramming efficiency.

8.7 Replacement of Sox2 in reprogramming by inhibition of calcineurin or NFATc2

We next investigated whether calcineurin/NFAT inhibition could replace Sox2 in the context of reprogramming. We induced cellular reprogramming without Sox2 together with FK506 treatment or knockdown of calcineurin or NFATc2. On day 14 of reprogramming, few ALP-positive colonies could be identified after inhibition of calcineurin or NFATc2, with the number of colonies increasing at day 21. However, the number of colonies in each case was lower than that seen in the presence of the four


A
Day 14
Day 21
Number of ALP+ colonies
180
160
140
120
100
80
60
40
20
0
OKM
OKM FK506 3μM
OKM Psiren-ZsGShRNA CnAα
OKM Puro-ShRNA NFATc2
OSKM
B
OKMCnAαKD1-iPS
SSEA1
Sox2
Nanog
Zs-Green
Hoechst
ALP


E
OKMCnAαKD1-iPS
Ectoderm
Mesoderm
Endoderm


C
OKMCnAαKD1
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MEF
CCE ES cell
Endo-Oct4
Endo-Sox2
Nanog
Esrrb1
Sall4
E-Cadherin
Utf1
β-actin
OSKM
Exo-Oct4
Exo-Sox2
Exo-Klf4
Exo-cMyc

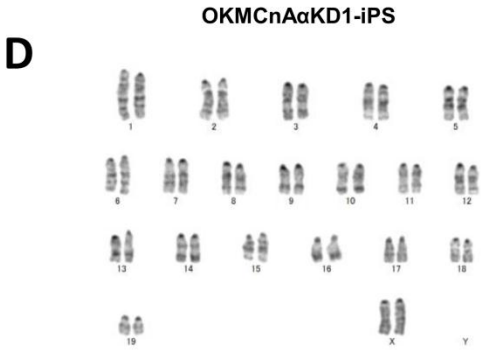
D
OKMCnAαKD1-iPS

Figure 7. Inhibition of calcineurin or NFATc2 can successfully replace Sox2 in reprogramming. (A): Reprogramming efficiency quantified by counting ALP-positive colonies after treatment with FK506 or knockdown of *calcineurin* or *NFATc2* without *Sox2* on day 14 and day 21 of reprogramming. The *OKM* combination was used as negative control, whereas *OSKM* was used as a positive control. **(B):** Expression of pluripotency markers *Nanog, Sox2*, and SSEA1 in (OKMCnAαKD1-iPS) clone established by *OKM* plus knockdown of *calcineurin*. Panels show the immunostainings of the indicated markers with Hoechst 33258 (blue), whereas the green fluorescence shows the Zs-green marker of *calcineurin* knockdown. Scale bar: 50μm, and 100μm for ALP. **(C):** Histological analysis of teratoma formed from the OKMCnAαKD1-iPS clone. The teratoma contained three germ layers; arrows indicate a keratinocyte in the ectoderm, cartilage in the mesoderm, and intestinal epithelium in the endoderm. Sections of the teratoma were stained by hematoxylin and eosin. **(D):** Expression of pluripotent markers by RT-PCR in iPS clones established by OKM plus knockdown either *calcineurin* or *NFATc2*, (OKMCnAαKD1–3 and OKMNFATc2KD1–3; respectively). MEF was used as a negative control, whereas CCE ESC was used as a positive control. β-actin was used as internal control. The lower panel, shows exogenous expression of *Oct4, Sox2, Klf4* and *c-Myc*. MEF was used as a negative control, whereas *OSKM* was used as a positive control. **(E):** Normal karyotype of OKMCnAαKD1-iPS clone.

reprogramming factors OSKM (Fig. 7A). The colonies exhibited morphological features and other characteristics of iPSCs, such as immunostaining for Sox2, Nanog, and SSEA1 (Fig. 7B), expression of pluripotency markers by RT-PCR (Fig. 7C), showing normal karyotyping (Fig. 7D), and teratoma formation with the three germ layers (Fig. 7E).

Discussion

Discussion

Cellular plasticity is a key feature of the reprogramming process but considerable efforts are still needed to elucidate the combined cellular, molecular, and epigenetic mechanisms and to make iPSCs safe for use in regenerative medicine. Despite elegant investigations since 2006, the path involved in the establishment of the pluripotency network is still not fully understood.

In the present study we identified a biphasic role for the calcineurin/NFAT signaling pathway in reprogramming. During the early phase of reprogramming, calcineurin is required for the maintenance of proper cell cycle proliferation by suppressing negative cell cycle regulators, known barriers in the early phase of reprogramming [4, 13, 21]. Consistent with the findings of the present study, previous studies have reported that calcineurin phosphatase activity regulates mammalian cell proliferation[22, 23]. In addition, suppressing calcineurin results in delaying the MET, another event reported to occur during the initiation phase of reprogramming that is essential for cell dedifferentiation and transformation to epithelial-like colonies[5, 6]. Together, these findings highlight the positive role of calcineurin in the early phase of reprogramming (Fig. 8).

During the late phase of reprogramming, transitional expression of NFATc2 and its translocation to the nucleus in response to calcineurin play a negative role in reprogramming. The repressive effects of NFATc2 in our system emanated from its direct interaction with Suv39h1, Hdac3, and Ezh2. Suv39h1 is one of the major H3K9 methyltransferases and its knockdown has been reported to increase reprogramming efficiency by

enhancing the expression of core pluripotency genes, including *Sox2*[24, 25]. The repressive role of HDACs in reprogramming has been overcome using pharmacological inhibitors such as trichostatin A (TSA), butyrate, and valproic acid (VPA), which enabled replacement of c-Myc and Klf4 in reprogramming cocktails [26]. However, Hdac3 did not have any significant effect on reprogramming in the present study. Conversely, the H3K27 methyltransferase activity of Ezh2 is essential for reprogramming. Inhibition or knockdown of Ezh2 impairs the reprogramming process because of failure to repress the somatic cell program [7, 25]. To explain our data in this context, the physical interaction of NFATc2, Suv39h1, Ezh2, and Hdac3 induces compact heterochromatin formation mediated by H3K9 and H3K27 trimethylation over *Sox2* and *Klf2* loci and represses their expression. In agreement with these results, previous studies showed that overexpression of *ca-NFATc2* induces mouse ESC differentiation by downregulating pluripotency markers, and calcineurin/NFAT inhibition promotes the naïve condition whereby *Klf2* is highly expressed [12, 27].

Furthermore, the reprograming factors OSKM bind to promoters of *Gnaq*, *plcb1*, calcineurin subunits, and NFATc isoforms and regulate their expression at the onset of reprogramming. Activation of Gnaq/plcb1 triggers depletion of endoplasmic reticulum (ER) Ca2+ stores, with the increased intracellular Ca2+ activating calmodulin, which, in turn, binds and activates the calcineurin/NFAT signaling pathway[9]. Gnaq/calcineurin/NFATc2 form an inhibitory axis over *Sox2* and *Klf2* regulatory regions triggering their repression mediated with Suv39h1, and eventually decreasing reprogramming efficiency (Fig. 8), thus, inhibition of calcineurin *or* NFATc2 in the late phase of reprogramming could reactivate

endogenous *Sox2* to produce iPSCs, although at a lower efficiency compared with ectopic expression of *Sox2*.

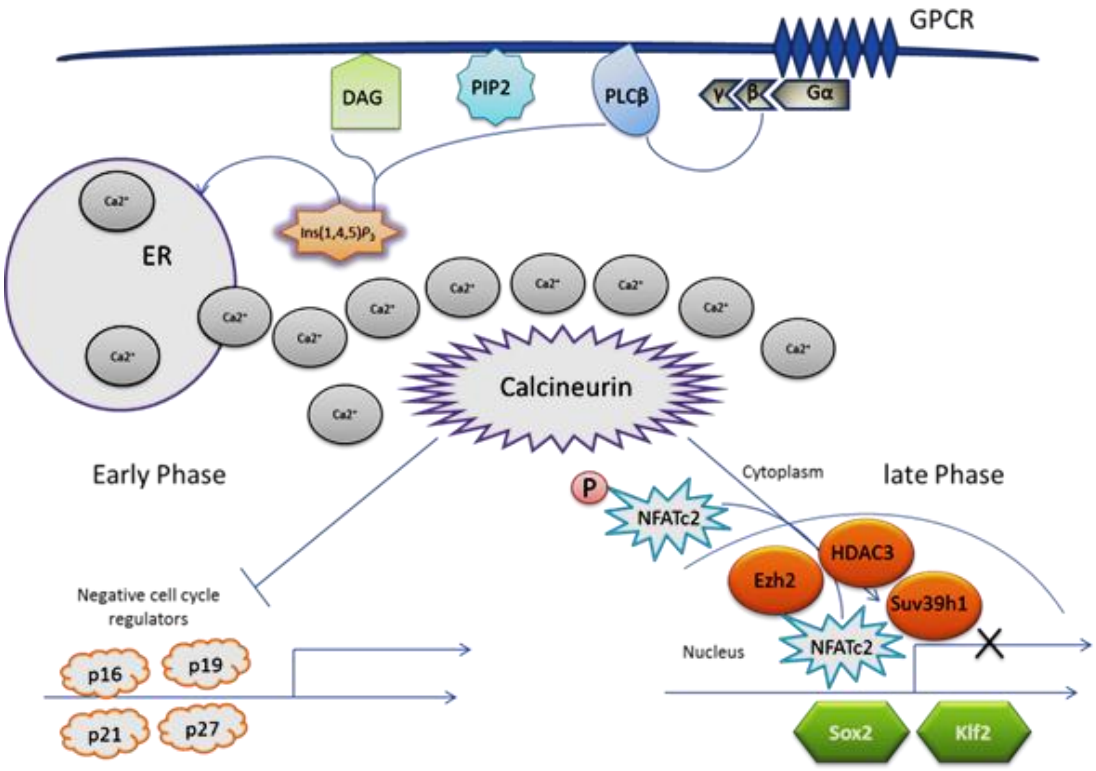

Figure 8. The reprogramming on the model represented in this study. The Gnaq level is upregulated during reprogramming process and triggers activation of calcineurin which possesses a positive role in early of reprogramming by maintaining proper cell cycle division, while the late phase, the inhibitory of calcineurin is mediated by NFATc2 which is transiently expressed and recruit Hdac3, ezh2, and Suv39h1 over the regulatory region of Sox2 and Klf2 regulatory loci and inhibits their expression and hence decreasing the reprograming efficiency.

On the road to iPS production, the endogenous *Sox2* expression represents the deterministic factor which enhances the reprogramming

model by activating different downstream pluripotency markers to produce iPS colonies [7]. Despite the importance of Sox2 in the reprogramming process, to the best of our knowledge, it remains the most replaced factor in the context of reprogramming. Replacement of Sox2 can be achieved by pharmacological inhibition of TGF-β signaling to induce Nanog expression [28], depletion of the tumor suppressor $p27^{Kip1}$ [29], ablation of Tcf3 a terminal downstream transcription factor of Wnt signal [30], and recently, knockdown tumor suppressor *Rb* [31]. Thus, various factors appear to regulate Sox2 during reprogramming, meaning that the exact molecular regulatory mechanism remains elusive. Consistent with the findings of the present study, the fact that sequential use of a combination of reprogramming factors followed by the later addition of Sox2 during reprogramming (at 4.5 days) enhances reprogramming efficiency explains how the need for Sox2 during the early phase of reprogramming can be bypassed [32].

On the other hand, Klf2 is superior to Klf1, Klf4, and Klf5 proteins to enhance somatic cell reprogramming [33, 34], despite the fact that it seems not to be essential for reprogramming because of functional redundancy with other Klfs [35] . Overexpression of *Klf2* with *Nanog* is required to ensure the naïve condition in human ESCs [36], however, in mouse ES cell the ground state can be established under 2i condition containing GSK3 and MEK inhibitors [37]. In a recent study, it was reported that maintenance of Klf2 protein by preventing its phospho-degradation mediated by MEK/extracellular signal-regulated kinase (ERK) sustained the self-renewal of mouse ESCs [38]. In addition, it has been reported that the combined effects of Klf2 and PR Domain Containing 14 known as *Prdm14* promote

the conversion of epiblast stem cells (EpiSCs) into mouse ESCs because of the ability of Klf2 to repress the primordial germ cell (PGC) network [39]. Thus, we conclude that enhancing *Klf2* expression by decreasing the repressive marks over its promoter (mediated by NFATc2 binding) could enhance reprogramming.

Klf2 is normally expressed in mature thymocytes, naïve T cells, and memory T cells [40]. NFAT isoforms are also expressed in T cells and the overlap between NFAT isoforms and Klf2 to regulate interleukin2 (IL2) expression in T cells, where calcineurin/NFAT is activated, provides us with the possibility of subsequent coordination among them to achieve proper functional aim [41].

Recent studies demonstrated the oncogenic activity of NFATc2 in melanoma and other cancers [42-45]. Interestingly, activation of the Gnaq q209l mutant has been linked to melanoma [46-48], and several studies have demonstrated the role of Sox2 in melanoma initiation [49-51]. Thus, we could predict that the Gnaq/calcineurin/NFATc2 axis would be a useful tool for studying the predisposition of iPSCs for malignancy or as a potential target for cancer therapy.

Conclusion

Conclusion

In our study we dissected the biphasic role for calcineurin/NFAT that could be useful for enhancing reprogramming efficiency. In the early phase the calcineurin seems to be necessary for proper cell cycle division, and knockdown of calcineurin causes cell cycle arrest at the G1 phase, and that is also accompanied with delaying in the rate of MET. While in the late phase, we found that NFATc2 is transiently expressed and localized mainly in the nuclear part due to upregulation of calcineurin activity.

The NFATc2 physically interacted with Hdac3, ezh2, and Suv39h1 and bound to the regulatory region of Klf2 and Sox2 and then the repressive marks H3K9me3 and H3K27me3 enrichments were enhanced, resulting in downregulating their expression.

Besides, we identified Gnaq as upstream regulator of calcineurin and Gnaq/Calcineurin/NFATc2 forms inhibitory axis over Klf2, and Sox2 regulatory loci. Then by inhibition the calcineurin activity chemically or genetically or by knockdown of NFATc2, we can replace Sox2 in reprogramming process with lower affinity.

Despite that, we cannot exclude the possibility of signaling cross-talk because, under specific pathophysiological conditions, cross-talk has been reported for calcineurin with different signaling pathways, including c-Jun N-terminal kinase (JNK), MEK, protein kinase C, p38 MAPK, Wnt, and Notch [52, 53]. Finally, it would be interesting to study calcineurin signaling in the reprogramming of T lymphocytes, as it is the one of the major signals.

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