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NAMPT/SIRT2–mediated inhibition of the p53-p21 signaling pathway is indispensable for maintenance and hematopoietic differentiation of human iPS cells

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List of abbreviations

Akt	Protein kinase B
ANKLE2	Ankyrin repeat and LEM domain containing 2
ASK1	Apoptosis signal-regulating kinase 1
BrdU	Bromodeoxyuridine / 5-bromo-2'-deoxyuridine
CDK2	Cyclin dependent kinase2
CDK4/6	Cyclin dependent kinase 4/6
CK1	Casein kinase 1
CRISPR	Clustered regularly interspaced short palindromic repeats
ECCs	Embryonal carcinoma cells
ESCs	Embryonic stem cells
EB	Embryoid body
FOXO1	Forkhead box O1
FOXO3a	Forkhead box O3
G-CSF	Granulocyte-colony stimulating factor
H4K16ac	Histone H4 acetylation at lysine 16
H3K27me3	Trimethylation of lysine residue 27 at histone 3
HSP90	Heat shock protein 90
iPS cells	Induced pluripotent stem cells
MEFs	Murine embryo fibroblasts
MAPK	Mitogen-activated protein kinases
NA	Nicotinic acid
NAD^+	Nicotinamide adenine dinucleotide
NAM	Nicotinamide
NAMPT	Nicotinamide phosphoribosyl transferase
NBT/BCIP	5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium
NK	Natural killer
NMN	Nicotinamide mononucleotide
NMNAT	Nicotinamide mononucleotide adenylyl transferase

NFκB	Nuclear Factor NF-Kappa-B
OXPHOS	Oxidative phosphorylation
p53AIP1	p53-regulated apoptosis-inducing protein 1
PEPCK1	Phosphoenolpyruvate carboxy kinase
pRB	Retinoblastoma protein
PSCs	Pluripotent stem cells
REFs	Rat embryo fibroblasts
ROCK	Rho kinase
ROS	Reactive oxygen species
STAT	Signal transducer and activator of transcription
SIR2	Silent information regulator 2
SIRT1	Sirtuin 1
SIRT2	Sirtuin 2
SIRT6	Sirtuin 6
SSEA-4	Stage-specific embryonic antigen-4
Trp	Tryptophan
2D	Two-dimensional
3D	Three-dimensional

1. Introduction

Inhibitors of nicotinamide phosphoribosyl transferase (NAMPT) have been confirmed as therapeutic targets in leukemia. The main practical problem confronting us in this domain is the leukemogenic transformation mechanism downstream of NAMPT. Our lab has identified that NAMPT is a crucial enzyme regulating granulocyte-colony stimulating factor (G-CSF)-dependent granulopoiesis via an NAD^+ - sirtuin1(SIRT1) - dependent pathway [1]. Subsequent studies demonstrated that the upregulation of NAMPT and sirtuin 2 (SIRT2) was related to the abnormal proliferation and survival of leukemic cells by further activating of the AKT/ glycogen synthase kinase-3β/β-catenin pathway [2]. However, this remains an open question of whether the NAMPT/SIRT2 pathway is involved in mediating of human induced pluripotent stem (hiPS) cells maintenance and hematopoietic differentiation.

1.1. NAMPT-NAD^+-SIRTUIN pathway

It is well known that nicotinamide adenine dinucleotide (NAD^+) plays a vital role in modulating cellular metabolism and homeostatic control of energy balance. There are two pathways for NAD^+ biosynthesis: the *de novo* pathway and salvage pathway (**Fig. 1**) [3]. Typtophan (Trp) convert to NAD^+ through the *de novo* pathway. In the classical salvage pathway, NAM is converted into nicotinamide mononucleotide (NMN) by NAMPT, which is then turned into NAD^+ by nicotinamide mononucleotide adenylyl transferase (NMNAT)[4, 5].

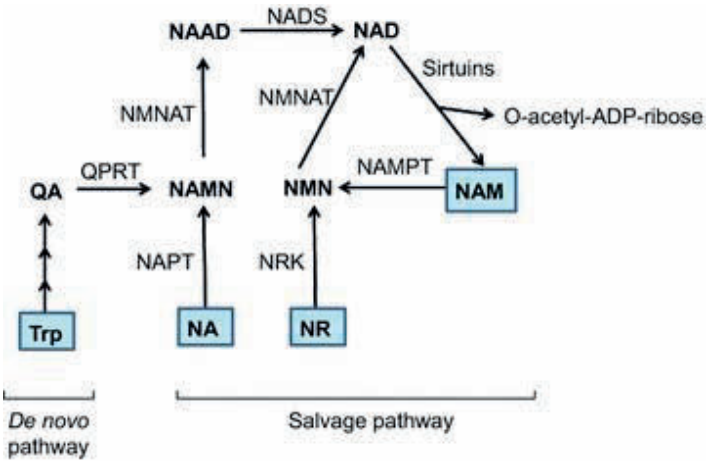

Figure 1. Schematic diagram of the de novo and salvage pathways of NAD+ biosynthesis. (adopted from

Srivastava, S., Clin Transl Med, 2016)

NAMPT is a rate-limiting enzyme in NAD^+ synthesis, and provides NAD^+ to sirtuins for functional activation of protein deacetylase and further transcriptional regulation (**Fig.2**) [2]. Sirtuins belong to the Silent information regulator 2 (SIR2)-like family of NAD^+ dependent protein lysine deacetylases. Their enzymatic activity plays an essential role in cell survival, aging and apoptosis. There are seven members, SIRT1–SIRT7, which display diversity in subcellular localization and functions, they are involved in life-span prolongation, obesity, cardiovascular disease, neurological functions and cancer [6].

Of the seven sirtuins encoded in the mammalian genome, the SIRT1 protein has been most extensively studied due to its impact on metabolism and related diseases, while the biological and therapeutic roles of other sirtuins are still unclear in many circumstances. Accumulating evidence has uncovered the SIRT2crucial functions in a broad range of biological procedures, such as in the nervous system, mitosis modulation, genome integrity, cell differentiation, cell homeostasis, and oxidative stress [7]. The first function of SIRT2 was found in moderating mitotic. Throughout the G2/M transition of the cell cycle, SIRT2 transiently shuttles from the cytoplasm to the nucleus where SIRT2 acetylate histone H4 lysine 16 (H4K16ac) and subsequently modulating chromosomal condensation during mitosis [8]. SIRT2 is as well plays a critical role in metabolic homeostasis. For instance, SIRT2 protects against insulin resistance by deacetylating Forkhead Box O1 (FOXO1) and activating Protein kinase B (Akt) as well as downstream targets [9, 10]. SIRT2 also regulates mitochondrial biogenesis through deacetylating Forkhead Box O3 (FOXO3a) and, thus, diminishes Reactive oxygen species (ROS) levels [11]. Most importantly, some evidence indicates a role for SIRT2 in tumorigenesis. SIRT2-specific inhibitors exhibit broad anticancer activity [2, 12].

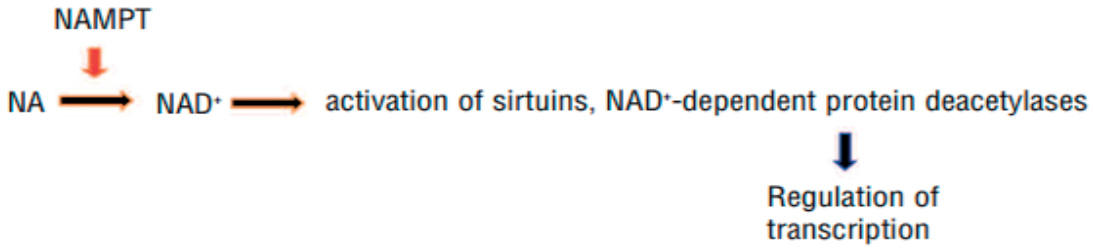

Figure 2. Model of the NAMPT action. (adopted from Dan, L., et al., Haematologica, 2012)

1.2. Experimental technologies and clinical applications of iPS cells

iPS cells were first generated by Takahashi and Yamanaka et al. in 2006. They reprogrammed mouse fibroblasts into embryonic stem (ES)-like cells via the retrovirus-regulated transfection of four transcription factors, Sox2,Oct3/4, Klf4 and c-Myc [13]. The iPS cells exhibit features of the embryonic stem cells (ESCs), including cell morphology, growth properties *in vitro*, and retention of the developmental potential to differentiate into any cell type. The generation of iPS cells also solved the ethical controversy that scientists had with the human ESCs application, because iPS cells are easily generated from somatic cells [14, 15]. Given the feasible production of almost infinite of patient-specific stem cells, iPS cells have a significant prospect in clinical and translational applications, such as disease modeling, drug screening toxicity testing, and transplantation therapies (**Fig. 3**) [16].

Nowadays, iPS cells are commonly used to study different processes of hematopoiesis *in vitro.* Immunotherapy using T lymphocytes, natural killer (NK) cells, and macrophages; blood transfusion therapy with platelets and erythrocytes are being promoted using iPS cells as a source. Experimental iPS cells-based models of the monogenic congenital disorders or multifactorial hematopoietic malignant diseases can also be used to investigate novel treatment options, including CRISPR/Cas9 based gene therapy and identification of novel drugs using high throughput drug screening. Many improvements have been made in the methods for generating high quality clinical-grade human iPS cells with unlimited self-renewal potential, though without chromosomal aberrations.

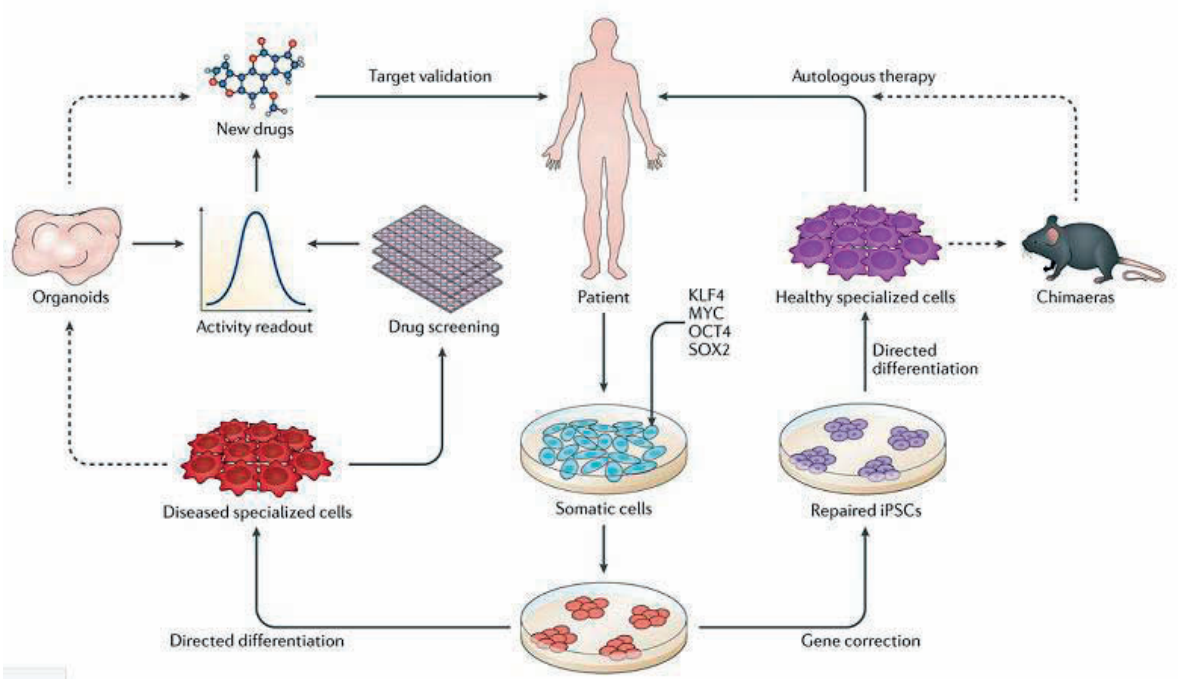

Figure 3. Current progress in the experimental and translational applications of iPS cells (adopted from Rowe, R.G., et al., Nat Rev Genet, 2019)

1.3. The role of NAMPT -NAD^+-Sirtuins signaling in iPS cells homeostasis

Over recent years, an essential connection between cell fate decisions and metabolic activity has been involved in mechanisms strengthening reprogramming, embryogenesis and disease pathogenesis [17]. For example, Nicotinamide was reported to promote iPS cells survival and differentiation through the regulation of Rho kinase (ROCK)-and Casein Kinase 1 (CK1) pathway [4]. A recent study by Son et al. has provided evidence that NAD^+ deficiency with FK866 (a specific noncompetitive inhibitor of the NAD^+ biosynthesis) was fatal for iPS cells, especially during reprogramming of somatic cells to pluripotent cells and in maintaining pluripotency of iPS cells [18].

It has been demonstrated that SIRT1 plays a crucial role in proficient telomere elongation, which is imperative for unlimited self-renewal, pluripotency, and chromosomal stability of iPS cells [19]. Our laboratory has demonstrated that SIRT1 is also crucial for granulocytic differentiation and leukemogenicity of hematopoietic cells. It's shown that the binding between CCAAT/enhancer and proteins C/EBPα and C/EBPß is regulated by SIRT1, which is

implicated in the G-CSF synthesis. Another report indicates that GADD45A, which involves in the G-CSF triggered myeloid differentiation, is mediated by NAMPT-SIRT1 via fork head box transcription factor FOXO3[20, 21].

Ryall et al. showed that knock-out of the SIRT1 deacetylase domain gives rise to premature differentiation of skeletal muscle stem cells *in vitro* and *in vivo* [22]. Peng Xu et al. concluded that the loss of function of Sirtuin 6 (SIRT6) could significantly decrease the reprogramming efficiency of iPS cells. Furthermore, they showed that $SIRT6^{-/-}$ iPS-like cell line has intrinsic differentiation defects, simultaneously retaining normal self-renewal [23].

SIRT2 is also reported to be associated with the regulation of the pluripotency of iPS cells. During reprogramming of iPS cells from somatic cells, SIRT2 downregulation increases the activity of glycolytic enzymes, subsequently promoting the conversion of oxidative phosphorylation (OXPHOS) to glycolysis [24]. However, the ultimate molecular mechanisms of SIRT2 involved during iPS cells culture and differentiation are not fully elucidated yet.

1.4. The link between cell cycle and cell fate decision in iPS cells homeostasis

The combination of the cell cycle and pluripotency regulating networks has been displayed a mechanistic association to cell fate decisions (**Fig. 4**) [25]. As mentioned above, iPS cells are characterized by two features: capacity for self-renewal and pluripotency, which means that iPS cells have an infinite proliferation capacity, simultaneously preserving the ability to develop the three embryonic germ layers. There are five different fates a cell may select in the process of cell cycle, a cell may adopt one of the: proliferation, differentiation, quiescence, senescent or apoptosis. The cell may choose an early checkpoint that decides the cell fate selection upon detecting DNA damage or stress stimuli throughout the G1 phase of the cell cycle. Therefore, the fate selection is generally made in the G1 phase [26].

Cultured human pluripotent stem cells (PSCs) including ESCs, iPS cells, and embryonal carcinoma cells (ECCs) show unusual cell-cycle features. The doubling time of PSCs is about 15-18 hours, compare to 24-32 hours in lineage committed cells. Furthermore, PSCs have remarkably shorter G1 phases than somatic cells: G1 length is 2-3hours in PSCs while it lasts

around 8-12hours in lineage committed cells [27-29].

The association between the length of cell cycle and the process of differentiation has been well characterized in cancer cells, somatic cell lines and adult stem cells. For example, neural progenitor cells can be induced to form neurons when the cell cycle was prolonged by cyclin-dependent kinases inhibitor. [30] On the other hand, the differentiation may be deterred when the cell cycle of neural progenitor cells was cut down [31]. These investigations implied that G1 phase correlated to a window of elevated sensitivity of stem cells to differentiation signals.

Filipczyk et al. has suggested that p21 inhibits cyclin dependent kinase2 (CDK2) and cyclin dependent kinase 4/6 (CDK4/6), which phosphorylate the retinoblastoma protein (pRb) required for the induction of G1-S delay in human ESCs. Subsequently, the upregulated dephosphorylated pRb promotes the absence of Oct4 in human ESCs cultures. Oct4 is a stem cell marker indispensable for preserving of pluripotency, whose expression is markedly downregulated with the start of differentiation in mouse and human ESCs [32, 33]. This observation clearly demonstrated that p21 is critical not only for the short G1 cell cycle phase and rapid proliferation of ESCs and iPS cells, but also for the inhibition of differentiation.

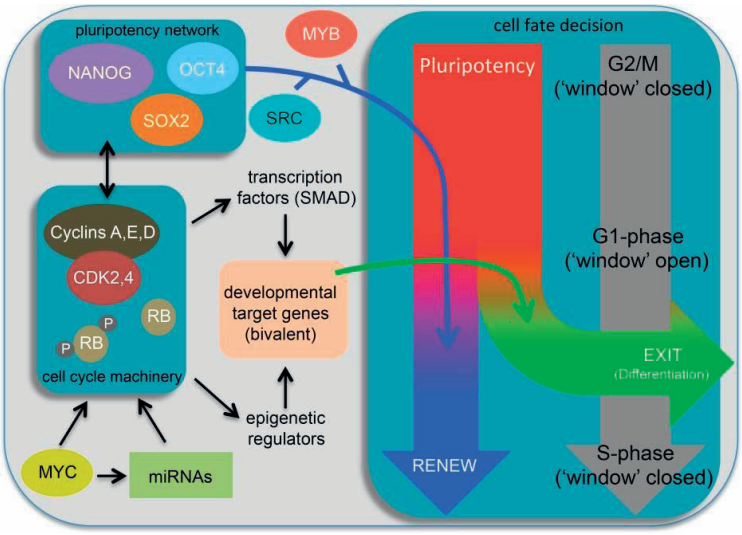

Figure 4. Signaling networks regulating proliferation and differentiation decisions in iPS cells (adopted from Boward, B., et al., Stem Cells, 2016)

1.5. The role of p53-p21 pathway in the regulation of the cell cycle and apoptosis in stem cells

It is well known that p53 is involved in an vital way in regulating cell cycle, apoptosis, and genomic stability [34, 35]. p53 transactivates downstream target genes, such as MDM2, p21, BAX, NOXA and PUMA [36].

Already in 1991, Martinez et al. confirmed that wild-type p53 acts at the G1 phase of the cell cycle by blocking cells from entering the S phase in transformed primary rat embryonic fibroblasts [37]. Furthermore, p53 was proved to control proliferation and cell death in ESCs. The truncation of p53 causes to accelerated proliferation and decreased spontaneous apoptosis in mouse ESCs. Treatment of human ESCs with Nutlin, a selective inhibitor of p53 , led to a rapid accumulation of p21 and cell cycle arrest at the boundary between the G1 and S phases [38, 39].

For decades, p53 was known as a well-established regulator of p21 expression. Stabilized p53 leads to a prolongation of the G1 phase of the cell cycle and increased spontaneous differentiation. p53 stabilization mechanism was shown to be involved in a complex formation with p21. The expression of p21 in stem cells is remarkably low or even no detectable. Neganova et al. found that p21 is epigenetic silencing through H3K27me3 in human ESCs. This observation stresses the significance of having an effective mechanism to suppress p21 expression in ESCs [40, 41]. The low expression of p21 owing to the high activity of CDK2 during the cell cycle was found as a primary motivation for the rapid proliferation of ESCs [42, 43].

1.6. Aims of the study

Based on the above information, the aims of the thesis were the following:

i. To explore the role of NAMPT/SIRT2 signaling in the proliferation, apoptosis and maintenance of pluripotency of iPS cells.

ii. To investigate the interference of the NAMPT/SIRT2 signaling on the hematopoietic differentiation of iPS cells.

iii. To study the interplay between NAMPT/SIRT2 and p53-p21 signaling pathways in the iPS cells integrity.

2. Publication

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NAMPT/SIRT2-mediated inhibition of the p53-p21 signaling pathway is indispensable for maintenance and hematopoietic differentiation of human iPS cells

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Abstract

Background: Nicotinamide phosphoribosyltransferase (NAMPT) regulates cellular functions through the protein deacetylation activity of nicotinamide adenine dinucleotide (NAD^+)-dependent sirtuins (SIRTs). SIRTs regulate functions of histones and none-histone proteins. The role of NAMPT/SIRT pathway in the regulation of maintenance and differentiation of human-induced pluripotent stem (iPS) cells is not fully elucidated.

Methods: We evaluated the effects of specific inhibitors of NAMPT or SIRT2 on the pluripotency, proliferation, survival, and hematopoietic differentiation of human iPS cells. We also studied the molecular mechanism downstream of NAMPT/SIRTs in iPS cells.

Results: We demonstrated that NAMPT is indispensable for the maintenance, survival, and hematopoietic differentiation of iPS cells. We found that inhibition of NAMPT or SIRT2 in iPS cells induces p53 protein by promoting its lysine acetylation. This leads to activation of the p53 target, p21, with subsequent cell cycle arrest and induction of apoptosis in iPS cells. NAMPT and SIRT2 inhibition also affect hematopoietic differentiation of iPS cells in an embryoid body (EB)-based cell culture system.

Conclusions: Our data demonstrate the essential role of the NAMPT/SIRT2/p53/p21 signaling axis in the maintenance and hematopoietic differentiation of iPS cells.

Keywords: iPSC maintenance, Hematopoietic differentiation of iPSCs, NAMPT/SIRT2 pathway, p53 deacetylation, p21 activation

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Highlights

- NAMPT regulates proliferation and survival of iPS cells via SIRT2
- SIRT2 deacetylates p53 leading to inhibition of p21 in iPS cells
- NAMPT and SIRT2 are important for the EB-based hematopoietic differentiation of iPS cells

Introduction

Understanding the mechanisms underlying the maintenance and hematopoietic development of induced pluripotent stem (iPS) cells is essential for the establishment of efficient protocols for iPS cell generation, ex vivo blood cell formation, and the identification of new treatment options for benign and oncogenic hematological disorders.

Although protocols for iPS cell generation are well established, there is still room for improvement in terms of the efficient generation of high-quality iPS cells. Understanding the mechanisms that maintain pluripotency and proliferation of iPS cells will be helpful in screening for highly pluripotent, high-quality iPS cells as well as improving protocols for the large-scale generation and maintenance of high-quality iPS cells.

Establishment of protocols for in vitro hematopoietic differentiation of embryonic stem (ES) cells and iPS cells has enabled the identification of a plethora of extrinsic and intrinsic factors essential for the regulation of blood cell differentiation at different developmental stages, starting from very early stages of mesodermal specification and the generation of early hemogenic progenitors [1, 2]. Diverse, crucial hematopoietic transcription factors are deregulated in human bone marrow failure syndromes and leukemia [3–5]. Regulation of these key factors can take place at the transcriptional or translational level, but there is also growing evidence for post-translational modifications, such as phosphorylation or de-/acetylation, in the regulation of protein functions [6, 7]. For instance, protein deacetylation triggered by NAMPT (nicotinamide phosphoribosyltransferase) and downstream NAD^+-dependent sirtuins (SIRT) is important for myeloid differentiation and leukemogenic transformation of hematopoietic cells through regulation of the CCAAT/enhancer-binding proteins C/EBPα and C/EBPß, the serine/threonine kinase AKT, the tumor-suppressor p53, and the forkhead box transcription factor FOXO3 [8–12]. In these studies, SIRT1 and SIRT2, members of the SIRT family of NAD^+-dependent class III histone deacetylases [13], were found to activate target proteins in hematopoietic cells upon NAMPT activation. It has been demonstrated that a SIRT1 deficiency compromises hematopoietic differentiation of mouse ES cells and embryonic and adult hematopoiesis in the mouse [14]. However, the role of NAMPT and SIRT2 during maintenance and myeloid differentiation of iPS cells is largely unknown. Identification of specific selective inhibitors of NAMPT and SIRT2 [15, 16] has made it possible to evaluate the specific roles of each of these factors in different physiological and pathological processes. We recently identified important roles of NAMPT and SIRT2 during early stages of hematopoietic differentiation of iPS cells using a feeder-free, serum-free monolayer-based differentiation protocol [17]. However, we did not study the granulocytic differentiation of iPS cells using this method.

In the current study, we evaluated the roles of NAMPT and SIRT2 in the maintenance and granulocytic differentiation of iPS cells. We found that NAMPT/SIRT2-mediated deacetylation of p53 is important for iPS cell maintenance through deactivation of p53 and subsequent suppression of p21.

Material and methods

Cell culture

Healthy donor-derived human iPS cells (hiPSCs, hCD34-iPSC16) [18] were provided by Dr. Nico Lachmann and Dr. Thomas Moritz (Hannover Medical School, Hannover, Germany). This hiPSC line was maintained on Geltrex LDEV-Free Reduced Growth Factor Basement Membrane Matrix (Cat Nr. A1413302, Thermo Fisher Scientific)-coated cell culture plates in Stemflex medium with 10% Stemflex Supplement (Cat Nr. A3349401, Thermo Fisher Scientific) and 1% penicillin/streptomycin. The medium was changed every day or every second day. Cells were passaged every 5 or 6 days in 1:10 or 1:15 ratios depending on their density.

Treatment of iPS cells with FK866 or AC93253

5×10^4 hiPSCs/well were seeded into one well of a 6-well plate, were kept in maintenance for 48 h, and were then treated with different doses of FK866 (Cat Nr. F8557-25MG, Sigma-Aldrich) or AC93253 (Cat Nr. A9605-10MG, Sigma-Aldrich). The corresponding concentration of dimethylsulfoxide (DMSO; Sigma-Aldrich) was used as vehicle control. After 48 h cells were collected for further analysis.

Flow cytometry analysis

To assess the pluripotency of iPS cells, the antibodies TRA1-60-PE (Cat Nr. MA1-023-PE, eBioscience) and SSEA4-FITC (Cat Nr. 560126, BD biosciences, BD) were used. Dead cells were excluded from the analysis by 4′, 6-diamidino-2-phenylindole (DAPI; 1μg/ml) (Cat Nr. D3571, Thermo Fisher Scientific) staining. For detection of hematopoietic progenitor cells, the antibodies CD33-BV421 (Cat Nr. 366622, BioLegend, BL), CD34-PE-Cy7 (Cat Nr. 343615, BL), KDR-AF647 (Cat Nr. 359909, BL), CD43-PE (Cat Nr. 343204, BL), CD41a-FITC (Cat Nr. 303703, BL), CD235a-FITC (Cat Nr. 349103, BL), CD45-

BV510 (Cat Nr. 103138, BL) and 7-AAD (Cat Nr. 420404, BL) were used as an "early-stage" multicolor hematopoietic cell panel. For the detection of mature myeloid cells, the antibodies CD15-PE (Cat Nr. 301905, BL), CD16-FITC (Cat Nr. 302005, BL), CD14-APC-H7 (Cat Nr. 367117, BL), CD45-BV510 (Cat Nr. 103138, BL), CD33 BV-421 (Cat Nr. 366622, BL) and 7-AAD (Cat Nr. 420404, BL) were used as a "late-stage" multicolor myeloid differentiation panel. Anti-mouse IgGk beads were used for compensation. Antibodies and beads for flow cytometry were purchased from BD Biosciences unless otherwise indicated. Samples were analyzed using a FACS Canto II flow cytometer (Becton-Dickinson) and FlowJo software (FLOWJO, LLC, Ashland, OR).

RNA isolation and qRT-PCR

RNA was isolated using the RNeasy mini kit (Qiagen), and cDNA was prepared from 500 ng RNA by oligo primer using the Omniscript-RT kit (Qiagen). All procedures were performed following the manufacturers' instructions. Quantitative polymerase chain reaction (qPCR) was performed using LightCycler® 480 SYBR Green I Master (Roche Applied Science). Real-time PCR detection was performed using a LightCycler 480 Real-Time PCR System (Roche Applied Science). Quantification of target gene expression was conducted in comparison to the reference GAPDH gene expression and depicted as ΔΔCt relative to GAPDH. Primer sequences are shown in Table S1.

Lentivirus-mediated gene knockdown in hiPSCs

HEK293T cells were used for lentivirus production. On the day before transfection, 8×10^6 HEK293T cells were plated in each T75 flask. Cells were co-transfected with target shRNA expression vector (NAMPT shRNA in pRRL.PPT.SF.i2RFP, SIRT2 shRNA in pRRL.PPT.SF.i2GFP, p53 shRNA in pRRL.PPT.SF.i2YFP) or control vector (pRRL.PPT.SF.i2GFP, pRRL.PPT.SF.i2RFP, or pRRL.PPT.SF.i2YFP), psPAX2 packaging vector (#12260, Addgene), and pMD2.G envelope vector (#12259, Addgene) using TransIT®-LT1 Transfection Reagent (#MIR2305, Mirus Bio LLC). Oligonucleotide sequences for shRNA are available upon request. Lentivirus-containing supernatants were harvested at 48 h after transfection, passed through a 0.22-μm filter, and incubated with Lenti-X™ Concentrator (#631232, Takara Clonetech) overnight at 4 °C. After centrifugation, the virus pellet was resuspended with complete DMEM medium and titrated by FACS.

For knockdown of NAMPT, SIRT2, or p53, hiPS cells were seeded in a 6-well plate (1×10^5 cells/well) 24 h before transduction. Cells were transduced by incubation and centrifugation with lentiviral supernatant at a multiplicity of infection (MOI) 40. After 72 h incubation at 37 °C, transduction efficiency was quantified by qPCR and western blot.

Western blot analysis

Whole-cell lysates were obtained by lysing equal numbers of cells with 3× laemmli buffer (30% glycerol, 6% SDS, 7.5% β-Mercaptoethanol, 0.75% Bromphenol blue in 200 nM Tris-HCL [pH 6.8]), which were subsequently heated at 95 °C for 5 min and spun down. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (GE healthcare life sciences). The WB membranes were blocked with 5% non-fat dry milk-TBST (10 mM Tris-HCL [pH 8.0], 150 mM NaCl, 0.1% Tween 20) for 1 h at room temperature. Primary antibodies were incubated overnight at 4 °C. After washing 4 times for 5 min with TBST, membranes were incubated with secondary antibodies for 1 h at room temperature. The protein bands were detected using Pierce ECL Western Blotting substrate (Thermo Fisher Scientific) or Luminata Forte Western HRP substrate (Millipore, Billerica, MA) and visualized by exposure to X-ray film (GE healthcare life sciences). The following antibodies were used: rabbit monoclonal antibody to GAPDH (Cat Nr. 2118, Cell Signaling Technology), rabbit monoclonal antibody to p21 (Cat Nr. 2947s, Cell Signaling Technology), mouse monoclonal antibody to p53 (Cat Nr. sc-126, Santa Cruz Biotechnology), rabbit monoclonal antibody to acetyl-p53 (Lys382) (Cat Nr. 2525, Cell Signaling Technology), rabbit polyclonal antibody to NAMPT (Cat Nr. PAB17046, Abnova).

Assessment of cell proliferation with Incucyte® S3 live-cell analysis system

2×10^4 hiPSCs were seeded in Geltrex-coated cell culture plates in Stemflex medium with 10% Stemflex Supplement (Cat Nr. A3349401, Thermo Fisher Scientific). The cells were incubated in the medium supplemented with FK866 1 nM/2 nM, AC93253 50 nM/100 nM or DMSO at the corresponding concentrations in IncuCyte Live Cell Analysis System (Essen Bio) at 37 °C, 5% CO_2. The cell growth images were recorded every 6 h and analyzed by IncuCyte S3 Software (Essen Bio).

Cell cycle analysis

For cell cycle analysis, iPS cells were incubated with 1 mM of BrdU for 30 min and BrdU uptake was quantified using APC BrdU Flow Kit (Cat Nr. 557892, Becton-Dickinson, Franklin Lakes, NJ, USA). Samples were analyzed using a FACS Canto II flow cytometer (Becton-Dickinson) and FlowJo software (FLOWJO, LLC, USA).

Assessment of apoptosis

Apoptosis was analyzed using FITC Annexin V Apoptosis Detection Kit (Cat Nr. 556547, Becton-Dickinson)

following the manufacturer's instructions. Samples were analyzed using a FACS Canto II flow cytometer (Becton-Dickinson) and FlowJo software (FLOWJO, LLC).

Embryoid body (EB)-based hematopoietic differentiation of hiPSCs

hiPS cells were kept in maintenance on Geltrex-coated plates for 5 days until confluency. iPS cells were dissociated by PBS/EDTA (0.02%) for 5–7 min. EB induction was achieved via Spin EBs (20.000 cells/EB) in 96-well plates using APEL serum-free differentiation medium (Stemcell Technologies) supplemented with bFGF (20 ng/μl) and ROCK Inhibitor (R&D). After 24 h, BMP4 (40 ng/μl) was added to the culture to induce mesodermal differentiation. After 2 days, EBs were plated on Geltrex-coated 6-well plates (10 EBs/well) in hematopoietic stem cell differentiation medium (APEL medium supplemented with 40 ng/μl VEGF, 50 ng/μlSCF, and 50 ng/μl IL-3). After 3 days, medium was changed to the neutrophil differentiation medium (APEL medium supplemented with 50 ng/μl IL3 and 50 ng/μl G-CSF). DMSO, FK866 (1 nM and 2 nM), or AC93253 (50 nM and 100 nM) were added to the culture medium starting at day 3 of culture. Medium with DMSO, FK866, or AC93253 was exchanged every 3 days. Hematopoietic cells appeared on days 12–14 of culture. They were harvested for various analyses on days 18 and 25. All cytokines were purchased from R&D System unless otherwise indicated. Cell morphology was evaluated on cytospin preparations of suspension hematopoietic cells generated on day 25 of culture. For this, 2×10^4 cells were centrifuged on the cytospin centrifuge at 400 rpm for 4 min. Cytospin slides were stained with Wright-Giemsa stain using the Hema-Tek slide stainer (Ames).

Three-germ-layer differentiation assay

The three-germ-layer differentiation of hiPS cells was performed using STEMdiff™ Trilineage Differentiation Kit following the manufacturer's instructions (Cat Nr. 05230, Stemcell Technologies). In some experiments, cell culture medium was supplemented with FK866 1 nM, AC93253 50 nM, or DMSO. For ectoderm lineage, 4×10^5 hiPS cells per well were plated in 24-well plate on day 0. After 24 h, the culture medium was changed from Stemflex medium to STEMdiff™ Trilineage Ectoderm Medium (Cat Nr. 05231, Stemcell Technologies). The medium was changed daily until day 7. 1×10^5 hiPS cells per well for mesoderm lineage and 4×10^5 hiPS cells per well for endoderm lineage were plated in 24-well plates on day 2. The cells were supplemented daily with STEMdiff™ Trilineage Mesoderm Medium (Cat Nr. 05232, Stemcell Technologies) and STEMdiff™ Trilineage Endoderm Medium (Cat Nr. 05233, Stemcell Technologies) with inhibitors or DMSO starting from day 3 till day 7.

Cell differentiation was analyzed on day 7 using the Human Three Germ Layer 3-color Immunocytochemistry Kit according to the manufacturer's instructions (Cat Nr. SC022, R&D Systems). Cells were fixed in PBS containing 4% paraformaldehyde (Cat Nr. 158127, Sigma-Aldrich) and blocked with PBS containing 10% normal donkey serum (Cat Nr. D9663, Sigma-Aldrich), 0.3% Triton™ X-100 (Cat Nr. 93443, Sigma-Aldrich), and 1% BSA (Cat Nr. A2058, Sigma-Aldrich). After that, cells were incubated with conjugated antibodies corresponding to the cell lineage of interest: Otx-2 for ectoderm, Brachyury for mesoderm, and GATA-4 for endoderm. After 3 h incubation at room temperature in the dark, cells were washed and kept with PBS containing 1% BSA and DAPI (1:1000 dilution). Images were taken on ZEISS Apotome microscope.

Statistical analyses

Statistical analyses were conducted using Student's t test or Boost Ratio [19] Statistical significance was taken to be $p < 0.05$.

Results

Inhibition of NAMPT or SIRT2 suppresses growth and induces cell cycle arrest and apoptosis in human iPS cells

We tested the in vitro effect of FK866 (a specific inhibitor of NAMPT) and AC93253 (a highly selective SIRT2 inhibitor) on the growth of human iPS cells (Fig. 1a, b). We found that treatment with FK866 or AC93253 caused a concentration-dependent decrease in the absolute number of iPS cells (Fig. 1c). These results are in line with reduced NAD^+ levels in FK866-treated cells (Fig. S1A). Morphologically, iPS cells treated with FK866 or AC93253 failed to form compact colonies, compared with control cells treated with DMSO (Fig. 1d). To explore the mechanism underlying the defect in iPS cell proliferation, we measured apoptosis and assessed cell cycle distribution using Annexin V and BrdU assays after treating cells with different concentrations of FK866 (1 and 2 nM), AC93253 (50 and 100 nM), or DMSO (vehicle control). We found that, compared with control cells, treatment with FK866 (2 nM) or AC93253 (50 and 100 nM) induced early and late apoptosis in iPS cells (Fig. 1e, Fig. S2A). Additionally, inhibition of NAMPT or SIRT2 caused cell cycle arrest in G0/G1 phase in iPS cells (Fig. 1f, Fig. S2B).

Similar results were observed by inhibiting NAMPT or SIRT2 by means of lentivirus-based transduction of iPS cells with shRNAs specifically targeting human NAMPT or SIRT2, as compared to control shRNA-transduced samples (Fig. 2a–d, Fig. S5A).

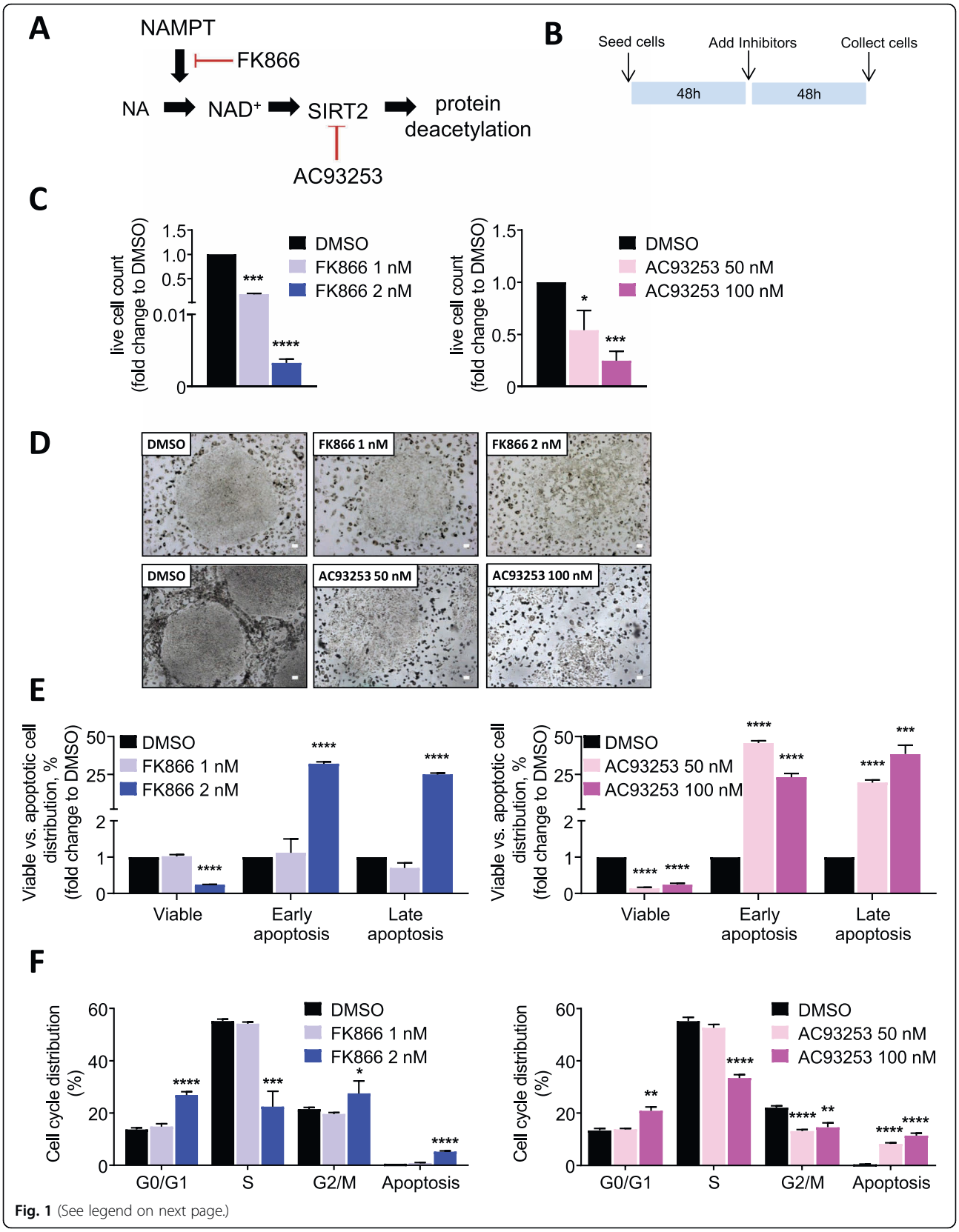

Fig. 1 (See legend on next page.)

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Fig. 1 Inhibition of NAMPT or SIRT2 suppresses the proliferation of human iPS cells by enhanced apoptosis and cell cycle arrest. **a** Schematic of the NAMPT-NAD^+-SIRT2 pathway. NAMPT is the rate-limiting enzyme that converts nicotinamide (NA) into NAD^+ that subsequently activates the NAD^+ dependent protein deacetylase, SIRT2. Specific small molecule inhibitors for NAMPT (FK866) and SIRT2 (AC93253) are depicted in red. **b** 5×10^4 human iPS cells were seeded on a Geltrex-coated 6-well plates, as described in MM. After 48 h of culture, different doses of FK866 or AC93253 were added to the culture medium. DMSO was used as vehicle control. Cell numbers were counted after 48 h of culture. **c** Numbers of viable iPS cells treated with FK866 (left) or AC93253 (right) were quantified using trypan blue dead cell exclusion. DMSO was used as vehicle control. Fold change differences of live cells relative to DMSO-treated cells are shown. Data represent means ± SD from two independent experiments, each in triplicates (*$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$). **d** Representative images of human iPS cells treated with DMSO, FK866, or AC93253 for 48 h. Scale bars: 50 μm. **e** Analysis of apoptosis of FK866 (left) or AC93253 (right)-treated human iPS cells using Annexin V assay. DMSO was used as vehicle control. Diagrams show the fold change differences in the percentage of each cell fraction (early apoptosis, late apoptosis, viable cells) relative to DMSO-treated cells. Data represent means ± SD from two independent experiments, each in triplicates (***$p < 0.001$, ****$p < 0.0001$, compared to DMSO-treated cells). **f** Analysis of cell cycle of FK866 (left) or AC93253 (right)-treated human iPS cells using BrdU assay. DMSO was used as vehicle control. Data represent means ± SD from two independent experiments, each in triplicates, (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$)

Inhibition of NAMPT or SIRT2 affects the pluripotency of human iPS cells

We further evaluated the effects of NAMPT or SIRT2 inhibition on the pluripotency of human iPS cells. We found that neither SSEA4 and Tra-1-60 protein expression nor alkaline phosphatase staining were not affected by treatment of iPS cells with FK866 or AC93253 compared with DMSO-treated control group (Fig. 3a, Figs. S3A-B, S4A). At the same time, SOX2 mRNA expression in iPS cells was inhibited by FK866 treatment compared with DMSO controls, whereas Oct4 and NANOG mRNA levels were increased by FK866 or AC93253 treatment (Fig. 3b). An analysis of mRNA expression of genes specific for each of three germ layers, ectoderm (Pax6, TUB3), mesoderm (MYH6, BRACH), and endoderm (FOXA2, AFP), revealed that TUB3, BRACH, and AFP were upregulated by treatment with FK866 (2 nM), whereas incubation with AC93253 induced Pax6, BRACH, and FOXA2 expression compared to the vehicle control (Fig. 3c). At the same time, protein expression of endoderm-, mesoderm-, and ectoderm-specific proteins was not affected (Fig. 3d and data not shown).

Interestingly, differentiation of iPSCs into ectoderm and endoderm in the presence of NAMPT or SIRT2 inhibitors resulted in a strong suppression of the endodermal differentiation, but no effects on the ectodermal differentiation (Fig. 3e and data not shown). At the same time, pre-culture of iPSCs with NAMPT or SIRT2 inhibitors for 48 h and subsequent differentiation into endoderm and ectoderm in the absence of inhibitors revealed no differences between studied groups (Fig. 3f and data not shown).

These results indicate that inhibition of NAMPT or SIRT2 in human iPS cells deregulates controlled expression of pluripotency and germ layer genes affecting endodermal, but not ectodermal differentiation. This process is reversible, since endodermal differentiation was not affected upon removal of the inhibitors.

Inhibition of NAMPT or SIRT2 attenuates mesodermal and neutrophil differentiation of human iPS cells

We further evaluated the effects of NAMPT and SIRT2 inhibitors on the mesodermal and hematopoietic/granulocytic differentiation of iPS cells. We recently described a novel mechanism for the granulocytic differentiation of hematopoietic stem cells by NAMPT-mediated protein deacetylation [8]. We therefore evaluated the role of NAMPT and SIRT2 in the neutrophilic differentiation of iPS cells using an embryoid body (EB)-based culture system (Fig. 4a). We found that cells treated with 2 nM FK866 produced significantly fewer hematopoietic cells compared with control cells. Treatment with AC93253 (50 or 100 nM) completely suppressed hematopoietic differentiation. In contrast, treatment with 1 nM of FK866 had almost no effect on differentiation (Fig. 4b, c). We also analyzed these differentiated cells by flow cytometry and found a decrease in erythro-megakaryocytic progenitors ($CD43^+CD41a^+CD235a^+CD45^-$) [20] and myeloid-committed multilineage progenitors ($CD43^+CD41a^-CD235a^-CD45^+$) [20] in cells treated with 2 nM FK866 compared with cells treated with DMSO or 1 nM FK866, whereas multilineage progenitors with lymphoid potential ($CD43^+CD41a^-CD235a^-CD45^-$) [20] were unchanged. We also observed a decrease in monocytes ($CD45^+CD11b^+CD14^+$), immature neutrophils ($CD45^+CD11b^+CD15^+$), and mature neutrophils ($CD45^+CD16^+CD15^+$) in response to treatment with 2 nM FK866, but not 1 nM FK866 or DMSO (Fig. 4d, Figs. S6A, S7A). In addition, an evaluation of cytospin slides showed an increased number of immature myeloid cells as a consequence of 2 nM FK866 treatment (Fig. 4e). These findings are in line with reduced levels of intracellular NAD^+ in suspension EB-derived hematopoietic cells and in adherent EB cells assessed on day 29 of culture (Fig. S8A). Collectively, these data suggest an important role for NAMPT, SIRT2, and NAD^+ in the regulation of mesodermal and granulocytic differentiation of iPS cells.

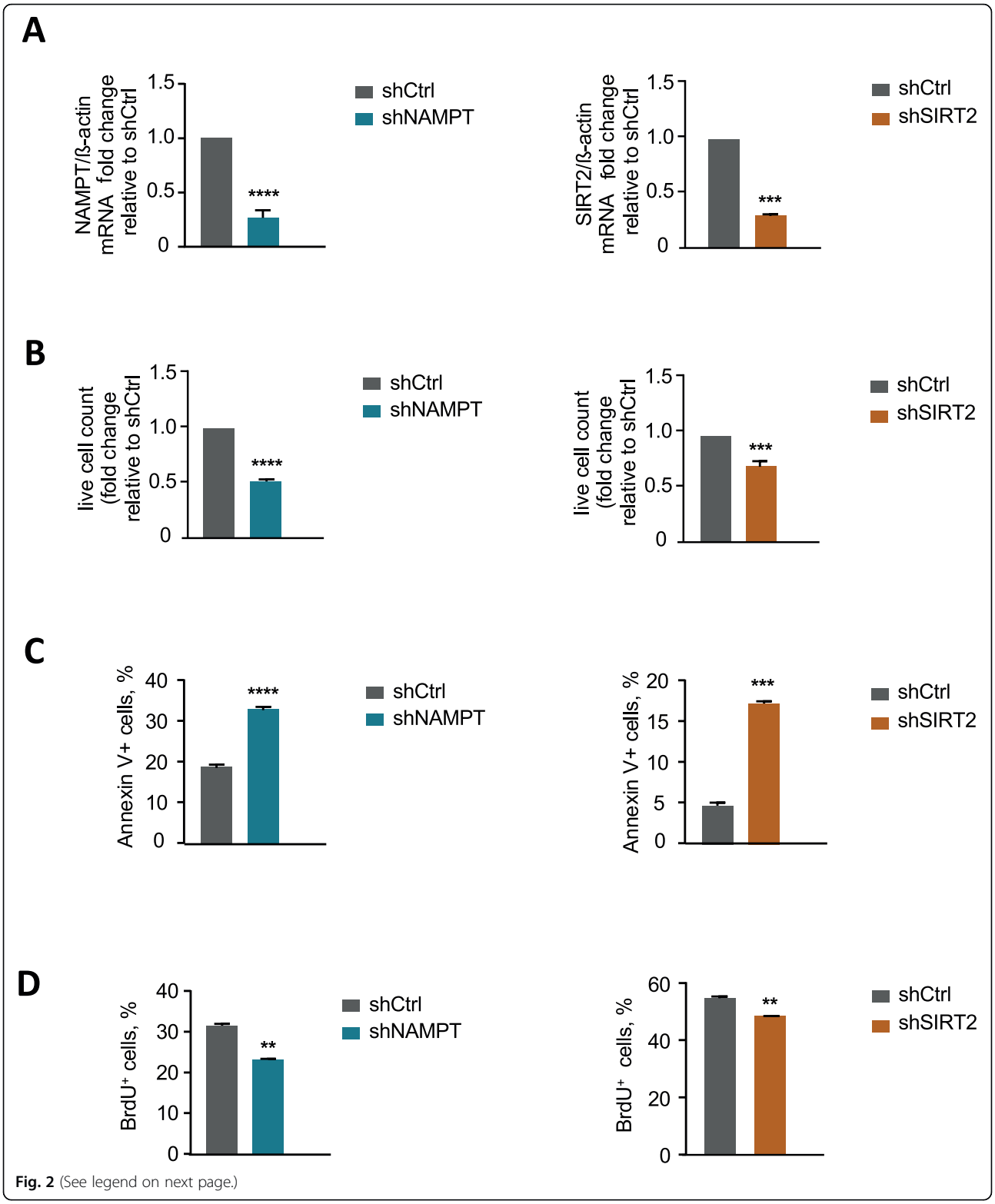

Fig. 2 (See legend on next page.)

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Fig. 2 Effects of NAMPT or SIRT2 knockdown on the proliferation and survival of human iPS cells. **a** mRNA expressions of NAMPT (left) and SIRT2 (right) in iPS cells transduced with shNAMPT (left) or shSIRT2 (right) LV were evaluated. iPS cells transduced with shCtrl LV was used as control. Fold changes relative to control are shown (***$p < 0.001$, ****$p < 0.0001$). **b** Cell growth of iPS cells transduced with shNAMPT (left) or shSIRT2 (right) lentivirus (LV) were quantified using trypan blue dead cell exclusion. iPS cells transduced with shCtrl LV was used as control. Fold changes relative to control are shown (***$p < 0.001$, ****$p < 0.0001$). **c** Apoptosis was analyzed in shNAMPT (left) or shSIRT2 (right) LV-transduced iPS cells using Annexin V assay. iPS cells transduced with shCtrl LV were used as control. Diagrams show the percentage of Annexin V-positive apoptotic cells (**$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$). **d** Cell cycle was analyzed in shNAMPT (left) or shSIRT2 (right) LV-transduced iPS cells using BrdU assay. iPS cells transduced with shCtrl LV was used as control. Diagrams show the percentage of cells in S phase (**$p < 0.01$)

Inhibition of NAMPT or SIRT2 activates the p53-p21 pathway through lysine acetylation of p53 in human iPS cells

We next sought to determine the signaling pathways regulated by NAMPT/SIRT2 in iPS cells. We previously demonstrated the essential role of NAMPT in the regulation of p53 activity in AML cells through lysine-382 deacetylation [11, 21]. p53 plays an important role during the maintenance of iPS cells [22]. In line with these observations, we detected dramatically elevated levels of acetylated and total p53 protein in lysates of FK866- or AC93253-treated iPS cells compared with DMSO-treated samples (Fig. 5a). p53 directly activates p21 [23, 24], which functions as a regulator of cell cycle progression at G1/S phase [25, 26]. Interestingly, p21 mRNA and protein expression were strongly upregulated in response to all tested concentrations of FK866 and AC93253 compared with the DMSO-treated group (Fig. 5b, c).

Inhibition of NAMPT or SIRT2 using shRNA also resulted in the elevated p21 expression due to the activation of p53 by acetylation (Fig. 5d).

Moreover, inhibition of p53 using transduction of iPS cells with p53-specific shRNA (Fig. 6a, b) resulted in a markedly diminished reduction of cell proliferation in the presence of NAMPT or SIRT2 inhibitors, as compared to control cells and analyzed using as evaluated using an IncuCyte cell proliferation assay (Fig. 6c, d).

These results suggest that the p53-p21 pathway plays an essential role in NAMPT/SIRT2-mediated proliferation of human iPS cells.

Discussion

In the present study, we demonstrated for the first time that NAMPT and SIRT2 are indispensable for the maintenance of iPS cells. In doing so, we have opened a new field for further investigation of the role of NAMPT-mediated protein deacetylation in developmental biology. It would be interesting to investigate whether NAMPT-triggered protein deacetylation governs the development of tissues and organs of mesoderm, endoderm or ectoderm origin, and whether SIRT2 or other SIRTs are involved. We recently demonstrated that NAMPT and SIRT2 are essential for early blood cell formation [17]. The results of the present study complemented these findings. Using an EB-based approach, we identified an essential role for NAMPT and SIRT2 in the maintenance of iPS cells and in their hematopoietic differentiation. Until now, nothing was known about the role of NAMPT signaling in endodermal or ectodermal cell specification. Here, we found deregulated expression of genes specific for the three germ layers. We also observed no effects of NAMPT or SIRT2 inhibition on the ectodermal differentiation, but strong effect on the endodermal and mesodermal differentiation. Our observations may help to identify improved culture conditions through pharmacological modulation of NAMPT/SIRT signaling, extending existing novel strategies for in vitro growth of different tissues as organoid cultures for translational and even therapeutic use. Our observations suggest that addition of NAMPT, NA, or NAD^+ may be useful for the maintenance or generation of iPS cells. Moreover, knowledge about the essential role of NAMPT/SIRT2 signaling for the maintenance of pluripotency in iPS cells may suggest better screening strategies for distinguishing pluripotent, high-quality iPS cells from differentiated, low-quality iPS cells based on an analysis of NAMPT/SIRT2 pathway activity. In addition, by modulating NAMPT/SIRT2 signaling in iPS cell culture, we may improve the quality of generated iPS cells. Our findings also suggest that NAMPT/SIRT2 signaling regulates iPS cell proliferation, an observation that may help in generating iPS cells more efficiently for large-scale culture.

The effects of NAMPT or SIRTs on the specification and differentiation of tissues and organs may be cell type-, concentration-, and differentiation stage-dependent. Dose- and cell type-dependent functions of NAMPT are known: although NAMPT is required for proper myeloid cell formation, hyper-activated NAMPT induces proliferation of hematopoietic stem cells and causes acute myeloid leukemia [9]. In the present study, we demonstrated that inhibition of NAMPT in iPS cells abrogated early-stage hematopoietic differentiation, arguing for a pro-differentiation role of NAMPT in this cell type. It is known that a SIRT1 deficiency causes increased apoptosis and diminished hematopoietic differentiation of mouse ES cells [14, 27]. We found here that SIRT2 also plays a role in

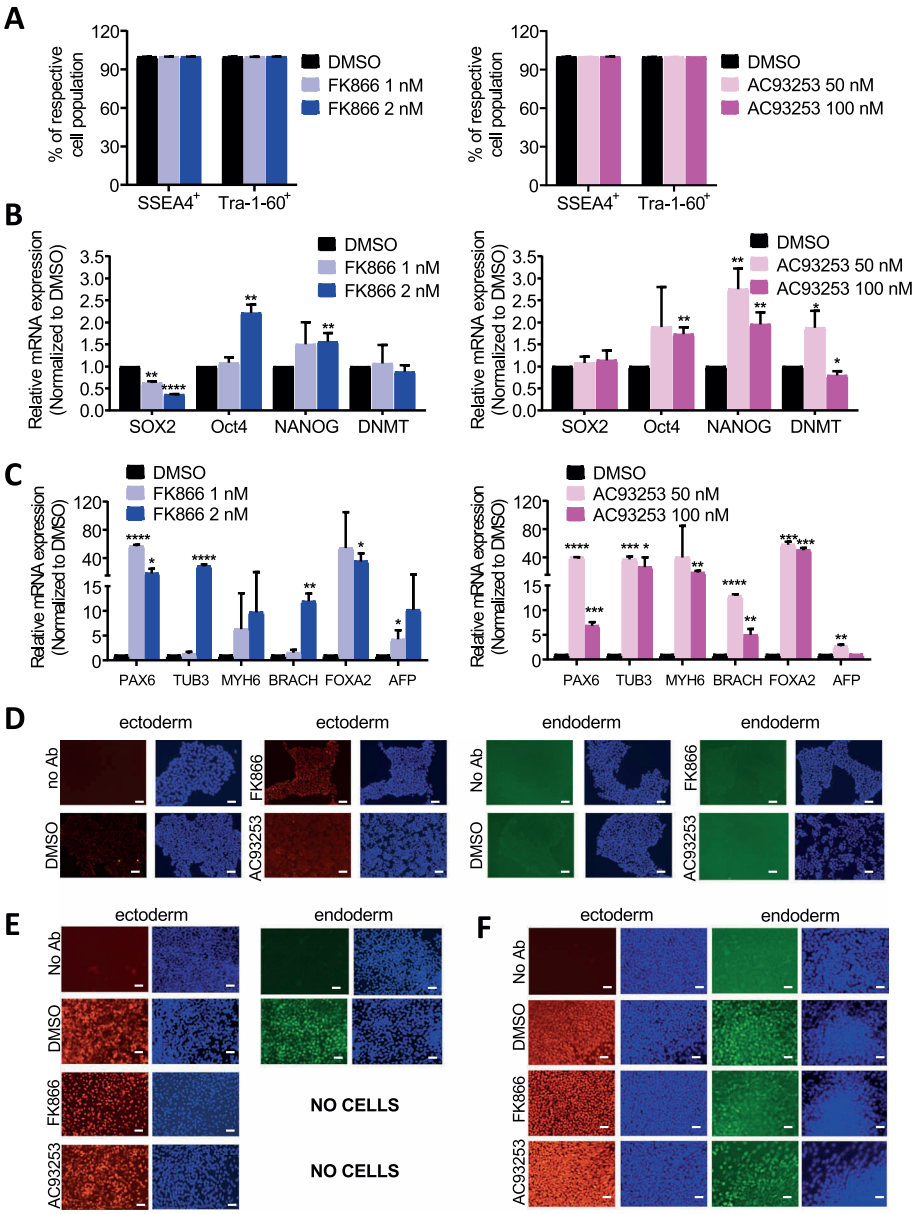

Fig. 3 Effect of NAMPT or SIRT2 inhibition on the iPS cell pluripotency. **a** Analysis of the expression of the pluripotent stem cell surface markers, SSEA4 and Tral-1-60, on FK866 (left) or AC93253 (right)-treated human iPS cells using FACS. DMSO was used as vehicle control. Data represent means ± SD from two independent experiments, each in triplicates. **b** mRNA expression of pluripotency genes in human iPS cells treated with FK866, AC93253, or DMSO for 48 h. Fold changes of pluripotency markers relative to DMSO-treated cells are shown. Data represent means ± SD from two independent experiments, each in triplicates (*$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$). **c** mRNA expression of genes specific for the three germ layers in human iPS cells treated with FK866, AC93253, or DMSO for 48 h. Fold changes of markers of differentiation relative to DMSO-treated cells are shown. Data represent means ± SD from two independent experiments, each in triplicates (*$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$). **d** iPS cells were treated with DMSO, 1 nM of FK866, or 50 nM of AC93253 for 48 h with subsequent ICH analysis of the ectodermal and endodermal differentiation, as described in "Material and methods". Representative images are depicted, no Ab: control staining w/o antibody. **e** Ectodermal and endodermal differentiation of iPS cells in the presence of DMSO, 1 nM FK866, or 50 nM AC93253 was evaluated, as described in "Material and methods". Representative images are depicted, no Ab: control staining w/o antibody. **f** iPS cells were treated with DMSO, 1 nM FK866, or 50 nM AC93253 for 48 h, after that ectodermal and endodermal differentiation was performed, as described in "Material and methods", without addition of inhibitors. Representative images are depicted, no Ab: control staining w/o antibody

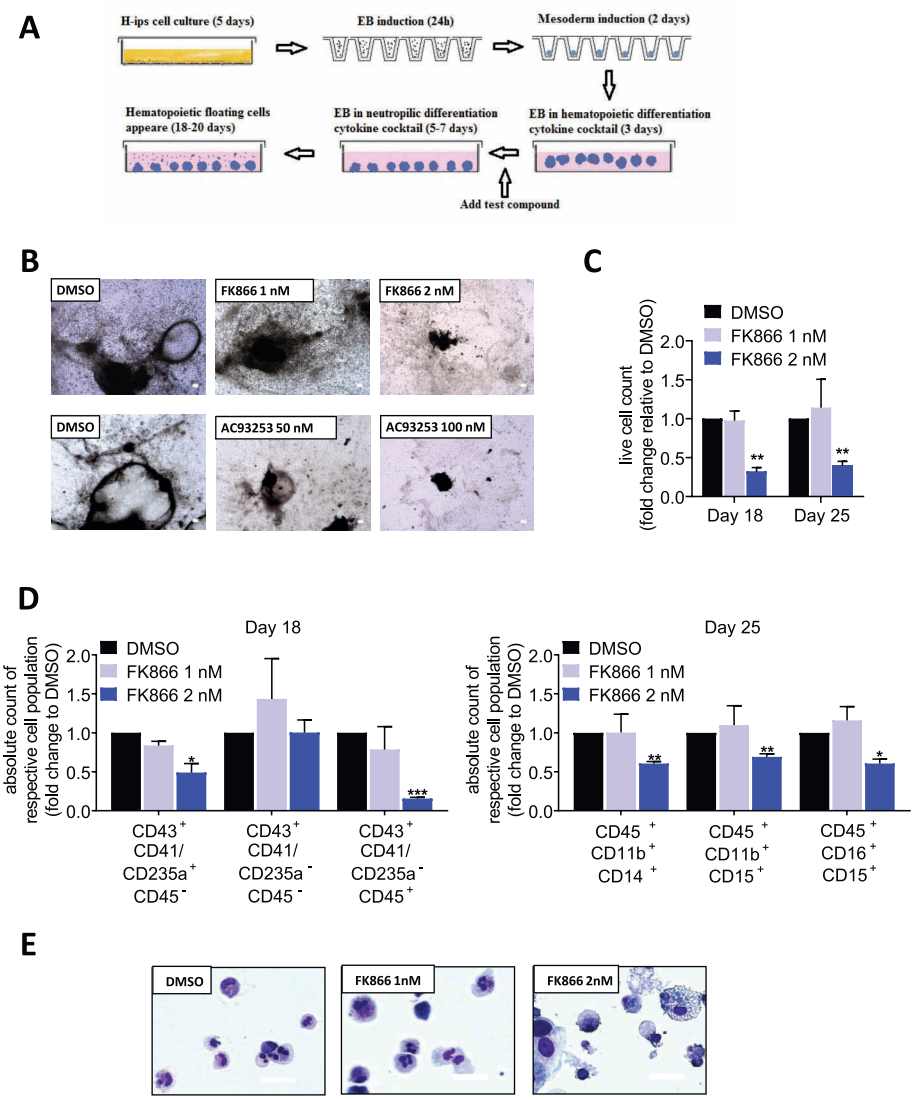

Fig. 4 NAMPT or SIRT2 inhibition suppresses hematopoietic differentiation of iPS cells. **a** Work flow of EB-based hematopoietic differentiation of iPS cells in the presence of DMSO, FK866, or AC93253. **b** Representative images of differentiated iPS cells treated with indicated drugs for 25 days. Images were taken using a Nikon Eclipse TS 100 microscope. Scale bars: 50 μm. **c** Number of viable hematopoietic cells generated in the presence of FK866 or DMSO using EB-based hematopoietic differentiation protocol at indicated time points, as assessed using trypan blue dead cell exclusion assay. Fold changes relative to DMSO-treated cells are shown. Data represent means ± SD from two independent experiments, each in duplicates (**$p < 0.01$). **d** Flow cytometry analysis of suspension cells harvested from EBs culture on day 18 and day 25 of differentiation for iPS cells treated with DMSO or FK866. Fold change difference of the absolute numbers of each cell fraction relative to DMSO are shown. Data represent means ± SD from two independent experiments, each in duplicates (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). **e** Representative cytospin images of suspension cells derived from differentiated human iPS cells treated with DMSO or FK866 on day 25 of culture. Scale bars: 400 μm

apoptosis induction and/or hematopoietic differentiation of iPS cells. We previously reported that SIRT2 inhibition resulted in apoptosis and diminished proliferation of acute myeloid leukemia cells through deacetylation/activation of Akt and subsequent activation of ß-catenin [9]. Both Akt and ß-catenin are important regulators of the proliferation of iPS cells and hematopoietic stem cells. But whether SIRT2 is connected to ß-catenin and Akt activation in the maintenance of the pluripotent state of iPS cells or induction of the mesodermal stages of hematopoiesis remains to be investigated.

A search for downstream targets of NAMPT/SIRT2 revealed that p53 is deacetylated by NAMPT/SIRT2 in iPS cells, leading to rapid and robust activation of p21 with subsequent cell cycle arrest and apoptosis of iPS cells. We recently reported that NAMPT deacetylates p53 in myeloid leukemia cells [11]. Our findings in iPS cells further confirm the important role of NAMPT/

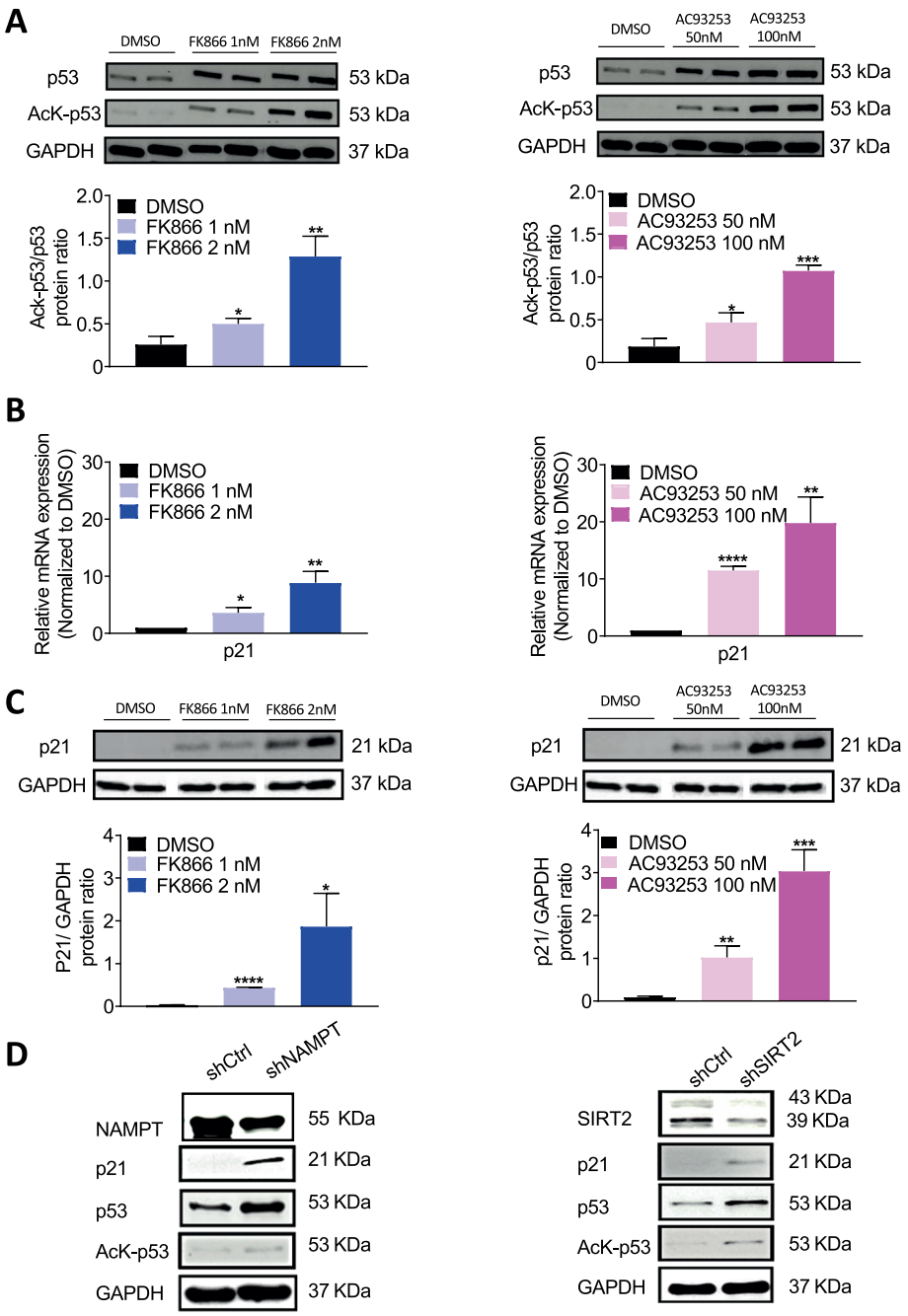

Fig. 5 NAMPT or SIRT2 inhibition led to diminished deacetylation of p53 with subsequent upregulation of p21 expression in human iPS cells. **a** Western blot analysis of total p53 and acetyl-K382 p53 protein expression in human iPS cells treated with FK866, AC93253, or DMSO for 48 h. GAPDH was used as loading control. Representative WB images are depicted. Diagrams show acetylated p53 to total p53 protein ratio in arbitrary units (AU). Data represent means ± SD from two independent experiments, each in duplicates (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). **b** mRNA expression of p21 in human iPS cells treated with FK866, AC93253, or DMSO for 48 h. Fold changes relative to DMSO-treated cells are shown. Data represent means ± SD from two independent experiments, each in triplicates (*$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$). **c** Western blot analysis of p21 protein expression in human iPS cells treated with FK866, AC93253, or DMSO for 48 h. GAPDH was used as loading control. Representative WB images are depicted. Diagrams show p21 to GAPDH protein ratio in arbitrary units (AU). Data represent means ± SD from two independent experiments, each in duplicates (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$). **d** Representative WB images of corresponding protein expression in human iPS cells transduced with the indicated shRNA constructs

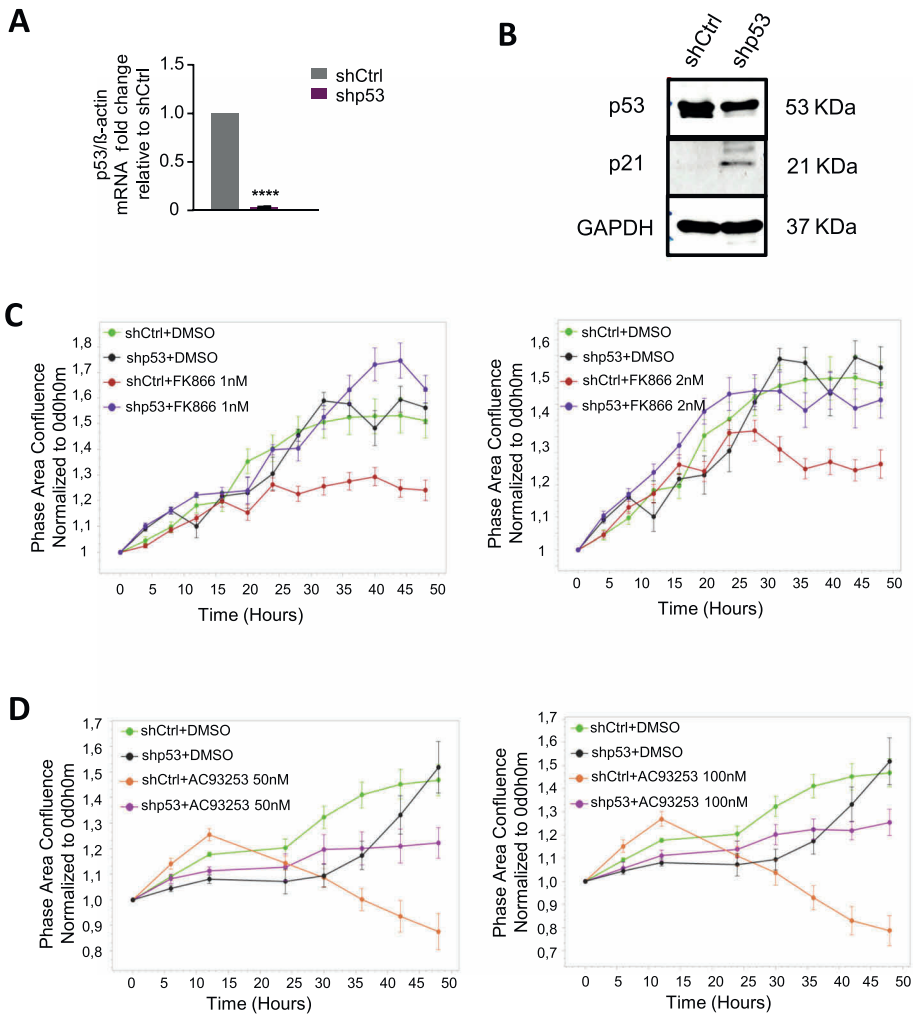

Fig. 6 Knockdown of p53 attenuates the growth inhibition in FK866- or AC93253-treated iPSCs. **a** mRNA expression of p53 in iPS cells transduced with shp53 LV was evaluated. iPS cells transduced with shCtrl LV was used as control. Fold change relative to control is shown (****$p < 0.0001$). **b** Western blot analysis of p53 protein expression in shp53 LV-transduced iPS cells. iPS cells transduced with shCtrl LV was used as control. GAPDH was used as loading control. Representative WB images are depicted. **c**, **d** Growth of iPS cells transduced with control shRNA or p53-specific shRNA and subsequently treated with DMSO, FK866 (1 nM or 2 nM), or AC93253 (50 nM or 100 nM) was evaluated over time with the IncuCyte S3 Live-Cell Analysis System

SIRT2 in p53 deactivation through lysine deacetylation. It has been reported that the p53-p21 pathway functions as a barrier that inhibits iPS cell generation and that p53 deletion improves the efficiency of iPS cell generation [28]. In this context, suppression of p53 activity through pharmacological modulation of the NAMPT/SIRT2 pathway may improve the efficiency of iPS cell generation. It would be interesting to investigate whether the p53-p21 pathway is connected to the ß-catenin-Akt pathway in iPS cells. The ultimate mechanism by which NAMPT and SIRT2 contribute to the regulation of hematopoietic differentiation also needs to be studied.

NAD^+ may not only regulate NAMPT/SIRT-triggered protein deacetylation, it may also affect intracellular metabolic processes. Metabolic regulation of iPS cells during reprogramming and self-renewal has recently been described [29, 30]. In line with these observations, it would be interesting to investigate the effect of NAMPT inhibition on the metabolic processes operating during iPS cell maintenance or hematopoietic differentiation. This may lead to the identification of novel small molecules connected to NAD^+ that are capable of inducing pluripotency or hematopoietic differentiation of iPS cells.

Taken together, our findings describe a novel mechanism of iPS cell maintenance by NAMPT/SIRT2 through post-translational regulation of p53. This mechanism is of central importance for developmental biologists, cell biologists, and clinical scientists.

Conclusions

Identification of the novel mechanisms essential for the proper proliferation and hematopoietic differentiation of iPS cells might lead to an establishment of novel improved protocols for the iPS cell maintenance and high-scale production for clinical use. We described here the novel role of the NAMPT/SIRT2 pathway in the regulation of p53 activation by deacetylation. We demonstrated that NAMPT or SIRT2 inhibition resulted in the strong induction of p21 expression that ultimately led to the cell cycle arrest, apoptosis, and disturbed differentiation of iPS cells.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13287-021-02144-9.

Additional file 1.

Additional file 2.

Additional file 3.

Abbreviations

NAMPT: Nicotinamide phosphoribosyltransferase; iPS: Induced pluripotent stem; EB: Embryoid body; ES: Embryonic stem; NAD: Nicotinamide adenine dinucleotide; DMSO: Dimethylsulfoxide; DAPI: 4′,6-Diamidino-2-phenylindole; FACS: Fluorescence-activated cell sorting; qPCR: Quantitative polymerase chain reaction; PBS: Phosphate-buffered saline; TBST: Tris buffered saline with Tween-20; NBT/BCIP: 5-Bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium; PFA: Paraformaldehyde; HRP: Horseradish peroxidase; ECL: Enhanced chemiluminescence; BrdU: Bromodeoxyuridine / 5-bromo-2'-deoxyuridine; EDTA: Ethylenediaminetetraacetic acid; G-CSF: Granulocyte-colony stimulating factor; CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats; PAX6: Paired Box 6; TUB3: Alpha-tubulin; MYH6: Myosin Heavy Chain 6; FOXA2: Forkhead Box A2; AFP: Alpha-fetoprotein

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Authors' contributions

Y.X., J.S., and T.M. made initial observations, designed the experiments, supervised experimentation, and wrote the manuscript; Y.X. performed all experiments and analyzed the data; B.D. and A.Z. assisted with culture of human iPS cells; M.N. assisted with the plasmid cloning; K.W., P.M., and M.N. assisted with the interpretation of the data and provided insightful comments. The author(s) read and approved the final manuscript.

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Availability of data and materials

Supporting data are available online.

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors agree with the publication of the herein presented manuscript.

Competing interests

The authors declare no competing financial interests and no competing non-financial interests.

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References

1. Park C, et al. A hierarchical order of factors in the generation of FLK1- and SCL-expressing hematopoietic and endothelial progenitors from embryonic stem cells. Development. 2004;131:2749–62.
2. Nostro MC, Cheng X, Keller GM, Gadue P. Wnt, activin, and BMP signaling regulate distinct stages in the developmental pathway from embryonic stem cells to blood. Cell Stem Cell. 2008;2:60–71.
3. Skokowa J, et al. LEF-1 is crucial for neutrophil granulocytopoiesis and its expression is severely reduced in congenital neutropenia. Nat Med. 2006;12:1191–7.
4. Skokowa J, et al. Cooperativity of RUNX1 and CSF3R mutations in severe congenital neutropenia: a unique pathway in myeloid leukemogenesis. Blood. 2014;123:2229–37.
5. Marcucci G, Haferlach T, Dohner H. Molecular genetics of adult acute myeloid leukemia: prognostic and therapeutic implications. J Clin Oncol. 2011;29:475–86.
6. Mowen KA, David M. Unconventional post-translational modifications in immunological signaling. Nat Immunol. 2014;15:512–20.
7. Wang YC, Peterson SE, Loring JF. Protein post-translational modifications and regulation of pluripotency in human stem cells. Cell Res. 2014;24:143–60.
8. Skokowa J, et al. NAMPT is essential for the G-CSF-induced myeloid differentiation via a NAD(+)-sirtuin-1-dependent pathway. Nat Med. 2009;15:151–8.
9. Dan L, et al. The role of sirtuin 2 activation by nicotinamide phosphoribosyltransferase in the aberrant proliferation and survival of myeloid leukemia cells. Haematologica. 2012;97:551–9.
10. Thakur BK, et al. NAMPT pathway is involved in the FOXO3a-mediated regulation of GADD45A expression. Biochem Biophys Res Commun. 2012;420:714–20.
11. Thakur BK, et al. Involvement of p53 in the cytotoxic activity of the NAMPT inhibitor FK866 in myeloid leukemic cells. Int J Cancer. 2013;132:766–74.
12. Koch C, et al. GM-CSF treatment is not effective in congenital neutropenia patients due to its inability to activate NAMPT signaling. Ann Hematol. 2017;96:345–53.
13. Nakagawa T, Guarente L. Sirtuins at a glance. J Cell Sci. 2011;124:833–8.
14. Ou X, et al. SIRT1 deficiency compromises mouse embryonic stem cell hematopoietic differentiation, and embryonic and adult hematopoiesis in the mouse. Blood. 2011;117:440–50.
15. Hasmann M, Schemainda I. FK866, a highly specific noncompetitive inhibitor of nicotinamide phosphoribosyltransferase, represents a novel mechanism for induction of tumor cell apoptosis. Cancer Res. 2003;63:7436–42.
16. Zhang Y, et al. Identification of a small molecule SIRT2 inhibitor with selective tumor cytotoxicity. Biochem Biophys Res Commun. 2009;386:729–33.
17. Morishima T, et al. LMO2 activation by deacetylation is indispensable for hematopoiesis and T-ALL leukemogenesis. Blood. 2019;134:1159–75.
18. Lachmann N, et al. Gene correction of human induced pluripotent stem cells repairs the cellular phenotype in pulmonary alveolar proteinosis. Am J Respir Crit Care Med. 2014;189:167–82.
19. Cleries R, et al. BootstRatio: a web-based statistical analysis of fold-change in qPCR and RT-qPCR data using resampling methods. Comput Biol Med. 2012;42:438–45.
20. Vodyanik MA, Thomson JA, Slukvin II. Leukosialin (CD43) defines hematopoietic progenitors in human embryonic stem cell differentiation cultures. Blood. 2006;108:2095–105.
21. Thakur BK, et al. Inhibition of NAMPT pathway by FK866 activates the function of p53 in HEK293T cells. Biochem Biophys Res Commun. 2012;424:371–7.

22. Solozobova V, Blattner C. p53 in stem cells. World J Biol Chem. 2011;2:202–14.
23. Macleod KF, et al. p53-dependent and independent expression of p21 during cell growth, differentiation, and DNA damage. Genes Dev. 1995;9:935–44.
24. el-Deiry WS, et al. WAF1, a potential mediator of p53 tumor suppression. Cell. 1993;75:817–25.
25. El-Deiry WS. p21(WAF1) mediates cell-cycle inhibition, Relevant to Cancer Suppression and Therapy. Cancer Res. 2016;76:5189–91.
26. Gartel AL, Tyner AL. The role of the cyclin-dependent kinase inhibitor p21 in apoptosis. Mol Cancer Ther. 2002;1:639–49.
27. Han MK, et al. SIRT1 regulates apoptosis and Nanog expression in mouse embryonic stem cells by controlling p53 subcellular localization. Cell Stem Cell. 2008;2:241–51.
28. Hong H, et al. Suppression of induced pluripotent stem cell generation by the p53-p21 pathway. Nature. 2009;460:1132–5.
29. Zhang J, Nuebel E, Daley GQ, Koehler CM, Teitell MA. Metabolic regulation in pluripotent stem cells during reprogramming and self-renewal. Cell Stem Cell. 2012;11:589–95.
30. Shyh-Chang N, Ng HH. The metabolic programming of stem cells. Genes Dev. 2017;31:336–46.

Publisher's Note

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CORRECTION Open Access

Correction: NAMPT/SIRT2-mediated inhibition of the p53-p21 signaling pathway is indispensable for maintenance and hematopoietic differentiation of human iPS cells

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1. In the original Fig. 5A, C the order of the samples was reorganized by cutting the bands from the original blot and putting them in the sequence DMSO, FK866 1nM, and FK866 2nM from left to right, but the vertical lines indicating the cutting positions of the blot were missing.

We have now replaced Fig. 5A, C with the original uncut Western Blot images. Note that the bands marked with the asterisk were excluded from the densitometry analysis of presented Western Blot images due to the poor protein quality of the sample, as indicated by the weak GAPDH signal. For the statistical analysis of the FK866 2nM datasets, we accidentally calculated the *p*-value of two datasets only instead of the required three, as was calculated for the DMSO and FK866 1nM datasets, respectively. Therefore, we removed the stars indicating the significance of the FK866 2nM datasets from the corresponding densitometry graphs, which doesn't change the conclusion, because the difference between DMSO and also FK866 2nM treated samples is obvious. We also corrected legends for Fig. 5A, C regarding the analyzed datasets of the presented WB images, for which statistics were performed.

†Tatsuya Morishima and Julia Skokowa contributed equally to this work

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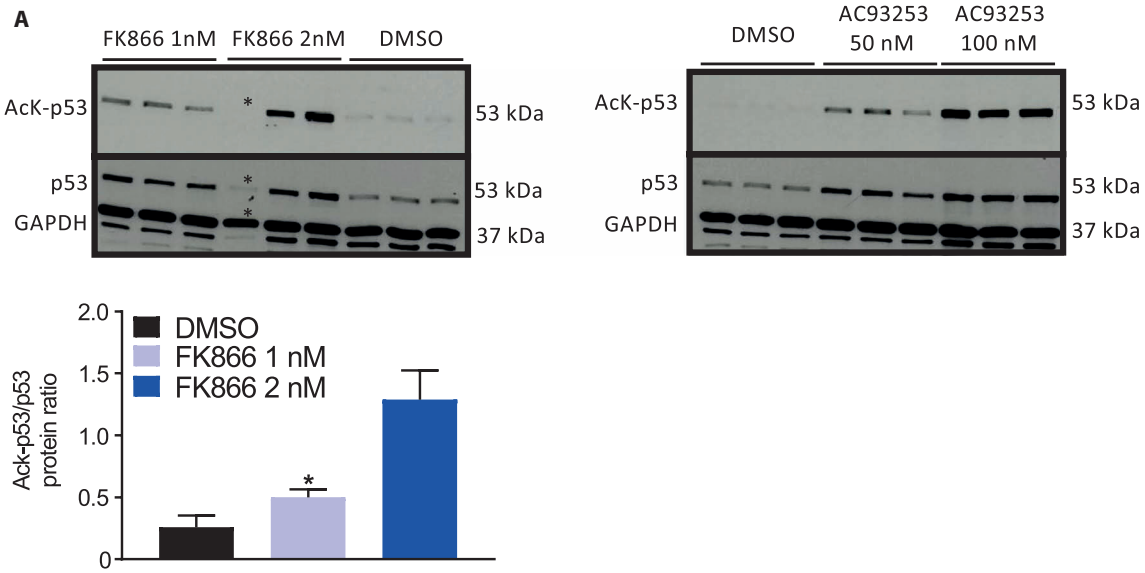
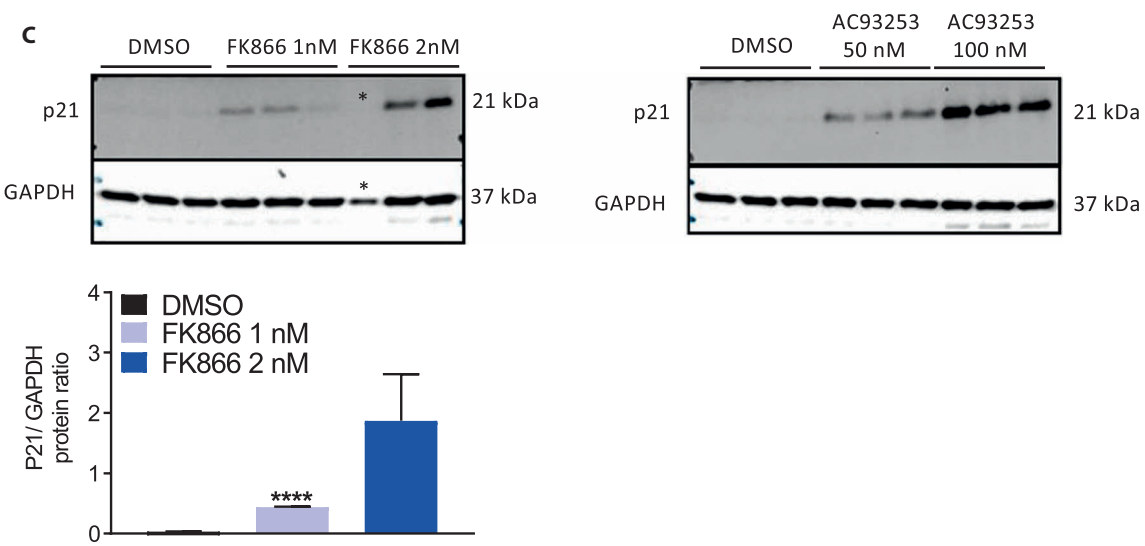
Corrected figure legend

Figure 5

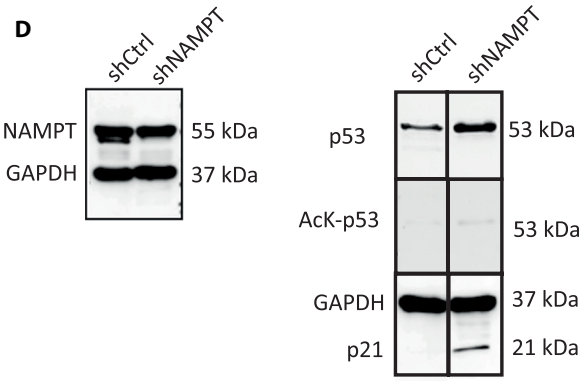


a Western blot analysis of total p53 and acetyl-K382 p53 protein expression in human iPS cells treated with FK866, AC93253, or DMSO for 48 h. GAPDH was used as a loading control. Representative WB images are depicted. The bands marked with the asterisk were excluded from the densitometry analysis due to the poor protein quality of the sample. Diagrams show the acetylated p53 to total p53 protein ratio in arbitrary units (AU). Data represent means ± SD (n = 2–3) (*$p < 0.05$, ***$p < 0.001$).



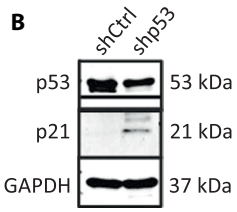
c Western blot analysis of p21 protein expression in human iPS cells treated with FK866, AC93253, or DMSO for 48 h. GAPDH was used as a loading control. Representative WB images are depicted. The bands marked with the asterisk were excluded from the analysis due to the bad protein quality of the sample. Diagrams show p21 to GAPDH protein ratio in arbitrary units (AU). Data represent means ± SD (n = 2–3) (**$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$)

2. **D**



In the original Fig. 5D, the GAPDH bands as loading controls for immune detection of p53, AcK-p53, and p21 were missing. We have now included the missing GAPDH bands.

3. **B**



In the original Fig. 6B, the line indicating the cutting position of the p53 blot was missing. We have now included this line.

None of the aforementioned corrections affect the conclusions of the respective experiments.

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3. Discussion of the results

In the present study, we used human iPS cells to understand the role of NAMPT/SIRT2 signaling during the initial stages of hematopoietic differentiation. Previously, our laboratory demonstrated a critical role of NAMPT/sirtuins in the hematopoietic differentiation and leukemogenicity of hematopoietic stem cells [1]. However, the role of NAMPT/sirtuins during the initial stages of hematopoiesis remained elusive. iPS cells are a well suitable experimental tool to study the initial steps of blood cell formation. Also, the number of hematopoietic stem cells that may be isolated from the bone marrow of healthy donors or patients is limited. Moreover, the capacity of primary hematopoietic stem cells to proliferate and to stay undifferentiated during *in vitro* culture is quite restricted. Thereby the hematopoietic cells derived from iPS cells are considered as an alternative source for translational research and therapeutic purposes. iPS cells are widely used for disease modeling, drug discovery, regenerative medicine, and the development of cell therapies [44]. A better understanding of the mechanisms underlying the self-renewal and proliferation of iPS cells is essential for the establishment of efficient protocols for iPS cells generation, maintenance, and differentiation.

3.1. Technical challenges of iPS cells maintenance and differentiation

Diverse extrinsic and intrinsic factors are indispensable for the modulation of iPS cells maintenance and blood cell formation.

Some studies claimed that co-culture on feeder cells has advantages over the extracellular matrix-coated dish approach. For instance, Matoba et al. indicated that for efficient hepatic differentiation, the maintenance of human iPS cells on mouse embryo fibroblasts (MEFs) is better than the feeder-free methods [45]. However, there are several limitations in the co-culture methods of iPS cells with feeder cells. First, for clinical grade transplantation of iPSC-derived cells, culturing iPS cells in feeder-free condition is considered a more suitable approach. iPS cells culture media should be well determined, xeno-free, and serum-free, these factors may heighten stem cell differentiation capacity [46]. Second, by testing small molecule compounds on their effects on iPS cells behavior, as in our case NAMPT and SIRT2 inhibitors, it is difficult

to exclude the effects of drugs on feeder cells, if using co-culture methods. Third, it is hard to clearly distinguish the cell morphology between fibroblasts and iPS cells in a co-culture setting, either under the microscope or using live-cell analysis systems, e.g., IncuCyte® system as in our case. Therefore, in our study we decided to keep iPS cells in maintenance using the feeder free protocol. Indeed, it performs well, giving good results for cell counting, apoptosis, cell cycle assay, and differentiation.

For the hematopoietic differentiation of iPS cells, compared to the standard two-dimensional (2D) flat-plate protocols, the three-dimensional (3D) spin-embryoid body (EB)-based iPS cells-differentiation system contribute to the stem cell-to-cell and cell-to-matrix interactions that promote the productivity of cell proliferation and functionality of iPS cells-derived granulocytes [47]. We induced the granulocytic differentiation of iPS cells applying the optimal EB-based protocol, which is well established in our lab [48]. These established protocols may be applied by other scientists for the efficient large-scale iPS cells maintenance and differentiation in the future.

Nowadays, the use of iPS cells in 3D spheroid and organoid culture is fast evolving. This method displays the complex tissue architecture more advance. It is reported that 3D cell culture of human iPS cells can model retinal cell, intestinal enteric nervous system, and myocardial cell differentiation [49]. Montel-Hagen's team successfully developed an efficient organoid system to induce hematopoietic specification and to generate conventional naive T cells from iPS cells [50]. Based on our study result, regulation of NAMPT or SIRT2 may promote the efficiency of 3D and organoid cell culture, thereby making iPSC culture systems more advanced in disease modeling and drug discovery.

3.2. NAMPT/SIRT2 pathway regulates iPSC proliferation via the p53-p21 signaling axis

In this study, we described an aberrant morphology and proliferation of iPS cells in which NAMPT or SIRT2 were inhibited using the specific inhibitors, FK866 or AC93253, respectively. These findings are in accordance with previous studies wherein it was demonstrated that

NAMPT promotes iPS cells survival [51] and that the depletion of SIRT6 hampers the iPS cells's maintenance [23].

SIRT2 was first identified to preferentially deacetylase Lys-40 residue of tubulin and Lys-16 residue of histone H4 proteins [8, 52]. Further studies indicate that there are much more proteins that are targets of this multi-faceted deacetylase, including FOXO, p300, phosphoenolpyruvate carboxy kinase 1(PEPCK1), ankyrin repeat and LEM domain containing 2 (ANKLE2), heat shock protein 90 (HSP90), nuclear factor NF-kappa-B (NFkB), and tumor suppressor protein p53 [12, 53-56]. Jin et al. demonstrated that the interaction between SIRT2 and 14-3-3 ß γ proteins could potentiate the reduction of acetylation and diminish the transcriptional activity of p53 [57]. Hoffmann et al. found that apoptosis was intensified in the existence of a SIRT2 inhibitor, AEM2, in non-small cell lung cancer cells, and this enrichment was accompanied by elevated levels of acetylated p53 [58]. Our results align with the previously published findings, showing that NAMPT/SIRT2 plays an important role in facilitating cell proliferation, suppressing p53 by deacetylation.

To identify the cell cycle stage that is adjusted by NAMPT/SIRT2 in iPS cells, we performed a BrdU assay. Here, we noticed that cell cycle arrested at G1 phase in FK866- or AC93253-treated iPS cells, compared to DMSO treated cells. Investigations using yeast and 293T cells have described the ability of SIRT2 to shuttle from cytoplasm to the nucleus in a cell cycle-dependent manner [8]. However, it was shown that SIRT2 inhibition induced cell cycle arrest at the G2/M stage, rather than G1 phase, as in our case. Inoue et al. demonstrated that SIRT2 could conduct in a mitotic checkpoint during the G2/M transition by blocking chromosome condensation under the glioma cell lines' mitotic stress [59]. Dan et al. observed that the inhibition of SIRT2 led to diminished growth of leukemia cells due to the G2/M phase cell cycle arrest [4]. The difference between the arrested cell cycle phases in response to SIRT2 inhibition might be attributable to iPS cells' unique cell cycle regulation features in our study and tumor cells investigated by other laboratories.

Because of the known role of p21 in the prolongation of the G1 phase of the cell cycle and inhibition of iPS cells proliferation, we decided to check p21 levels. As expected, we found that

p21 mRNA and protein levels were almost indistinguishable in iPS cells and markedly elevated after treatment with NAMPT or SIRT2 inhibitors.

p21 expression can be regulated in a p53-dependent manner, but also independently of p53. To address whether p53 is involved in the p21 regulation in iPS cells, we compared p53 expression levels in FK866- or AC93253-treated iPS cells with control DMSO treated cells. The enhanced expression and acetylation of p53 provided significant evidence for the p53-dependent up-regulation of p21. The presence of functional p53 is mainly ascribed to its acetylation by NAMPT-NAD^+/SIRT2 pathway. This finding was further confirmed by additional experiments in which we compared the effect of FK866 or AC93253 treatment on iPS cells transduced with non-target shRNA or p53-specific shRNA. As expected, we found that knockdown of p53 protects iPS cells from growth inhibition triggered by FK866 or AC93253 treatments. A similar conclusion was reached by Thakur et al. in 293T cells; they performed knock-down of p53 in 293T cells and observed elevated cell proliferation that was accompanied by a declined cell apoptosis and an accumulative S-phase arrest. This effect was more prominent in the FK866 treated group than the untreated control, and the authors indicated a direct involvement of p53 in the cytotoxic effect mediated by NAMPT inhibition [61].

We further verified our finding obtained using NAMPT and SIRT2 inhibitors through the genetic modification of NAMPT and SIRT2. Even though the study of CRISPR/Cas9-based gene-editing of iPS cells might be applied to answer our question, we previously found that NAMPT is essential for the cell survival and homozygous NAMPT knockout using CRISPR/Cas9 was lethal for iPS cells [60]. Hence, we alternatively knocked down the expression of NAMPT by applying lentiviral-based transduction to iPS cells with NAMPT-specific shRNA. For the purpose of consistency, SIRT2 expression was also inhibited in iPS cells using shRNA-based method. Similar to the observations using NAMPT or SIRT2 inhibitors, we found that NAMPT or SIRT2 knockdown hampered the outgrowth and survival of human iPS cells leading to reduced deacetylation of p53 following the upregulation of p21 expression.

Collectively, we observed diminished proliferation in iPS cells with reduced NAMPT/SIRT2 expression. Our investigations of the downstream targets of NAMPT/SIRT2 pathway evidenced that in iPS cells p53 is deacetylated by NAMPT/SIRT2, and NAMPT or SIRT2 blockage resulted in a fast and sturdy activation of p21 with consequential cell cycle arrest and apoptosis of iPS cells (**Fig. 5**). Standard techniques for iPS cells generation, maintenance and differentiation exhibit some limitations, such as low proliferation rate and loss of pluripotency of iPS cells, and inefficient differentiation with a high degree of variation from experiment to experiment. We provide an efficient approach to improve the methods of iPS cells maintenance and hematopoietic differentiation through modulating NAMPT/SIRT2/p53/p21 signaling pathway. The implementation of the above singnaling axis's pharmacological modulators may promote the widespread use of iPS cells in basic and translational research in the future.

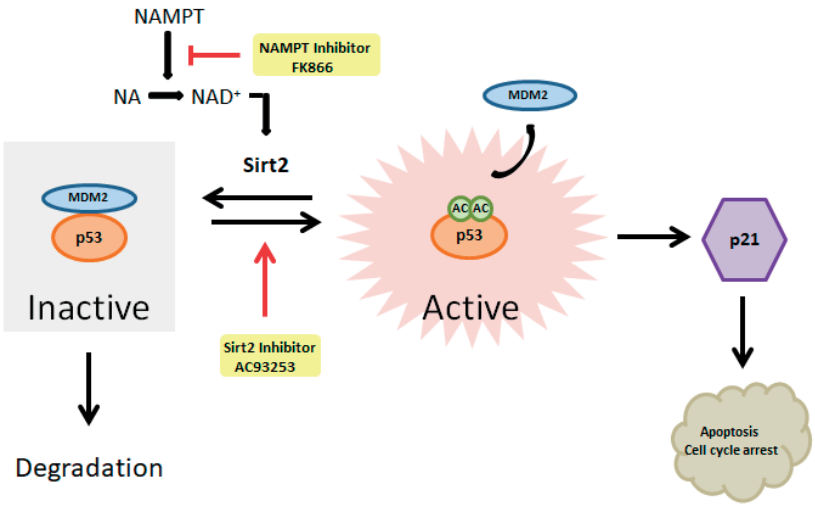

Figure 5. Schematic representation of the NAMPT/SIRT2/p53/p21 signaling axis.

3.3. The role of p53 and p21 in the coordination of iPS cells pluripotency

It was reported that p53 accumulation declines the mRNA expression of pluripotency coordinators like OCT4, SOX2 and NANOG, the abundance of the pluripotent cell surface marker SSEA-4 (stage-specific embryonic antigen-4), and spontaneous differentiation of human ESCs [62]. Qin et al. also observed that p53 activates miR-34a and miR-145, which negatively regulated the expression of pluripotency related genes OCT4, SOX2, KLF4 and LIN28A [63]. Besides, it was shown that the ectopic expression of p21 remarkably diminished

the expression of pluripotency markers NANOG, LIN28 andOCT4, increasing the expression of differentiation markers GATA4, CDX2and GATA6 in human ESCs[41]. However, the expression of pluripotency markers SOX2, OCT4, NANOG, DNMT, and stem cells markers SSEA-4 and Tra-1-60 were almost not affected in our study. This discrepancy is most probably attributable to the differences between iPS cells and ESCs. It is also possible, that a critical threshold of expression levels of crucial factors is required to affect pluripotency, which was not the case in iPS cells than ESCs. Another explanation might be that the duration of NAMPT or SIRT2 inhibitors treatment used in our study was too short to induce the characteristic changes in iPS cells, as it was demonstrated for ESCs.

3.4. NAMPT/SIRT2 regulates granulocytic differentiation of iPS cells

Our next novel finding is that the EB-based granulocytic differentiation of iPS cells was strongly diminished after inhibition of NAMPT with a complete loss of mature neutrophils after SIRT2 inhibition. These observations are in line with previous findings from our lab when studying more committed hematopoietic progenitors [1]. It was demonstrated, that NAMPT coordinates G-CSF and G-CSFR expression in hematopoietic cells via SIRT1-dependent activation of C/EBPα and C/EBPß. The present data of this thesis goes beyond the previous report showing that granulopoiesis is also dependent on SIRT2, and both NAMPT and SIRT2 are essential for the myeloid differentiation starting from immature cells in the iPSC settings [1]. Thus, after FK866 treatment, the percentages of erythro-megakaryocytic ($CD43^+CD41a^+CD235a^+CD45^-$cells) and myeloid-committed ($CD43^+CD41a^-CD235a^-CD45^+$cells) progenitors were decreased at early stage of iPS cells differentiation, but NAMPT inhibition did not affect the lymphoid differentiation potential (appearance of $CD43^+CD41a^-CD235a^-CD45^-$ cells). At the late stage of the differentiation, all myeloid cell subtypes, including monocytes ($CD45^+CD11b^+CD14^+$cells), immature neutrophils ($CD45^+CD11b^+CD15^+$cells), and mature neutrophils ($CD45^+CD16^+CD15^+$cells) were influenced with the FK866 treatment, as compared to control DMSO treated samples. Most probably, NAMPT or SIRT2 inhibitor caused a shift in the hematopoietic lineage cell fate

decision at the very early stage of stem cell specification, which may severely affect further differentiation stages.

It remains to be investigated whether the diminished hematopoietic differentiation upon NAMPT or SIRT2 inhibition is due to the cell cycle arrest in the S phase, or caused by the direct "regulatory" defects in the differentiation capacity of iPS cells, in addition to the cell cycle defects. In order to answer this question, we treated iPS cells with FK866 or AC93253 for 72 hours and evaluated their three-germ layer differentiation in subsequent culture. Interestingly, we found that both, FK866 and AC93253, treatments led to a strong abolishment of the endodermal differentiation without any influence on the ectodermal differentiation. However, comparing the results of the mRNA expression levels of differentiation markers, specifically expressed in one of the three embryonic germ layers, we found that not only *PAX6* and *TUB3* (ectoderm - specific factors), but also *MYH6* and *BRACH* (endoderm - specific factors) as well as *FOXA2* and *AFP* (mesoderm - specific factors) were up-regulated in FK866 or AC93253 treated iPS cells compared to DMSO treated iPS cells. This difference between mRNA expression levels and cell differentiation potential may be explained by different expression kinetics of mRNA and proteins - we need to evaluate protein expression of the above mentioned factors to be able to confirm this [64].

3.5. The role of p21 in regulating apoptosis

Contradictory data are describing the role of p21 in the regulation of apoptosis. Several studies have pointed out that p21is a positive regulator of apoptosis in many systems [65]. One study revealed that p21 interrupts the squamous carcinoma cells's apoptotic process of by inducing cycle arrest in G1 phase upstream of the caspase-3 activation [66] . Consistent with this observation, interleukin 6-typecytokines were shown to induce the p21 gene promoter by a signal transducer and activator of transcription (STAT)-dependent mechanism in human osteoblastic cells, protecting these cells from apoptosis [67]. However, we detected a pro-apoptotic effect of elevated p21 in iPS cells. The ultimate mechanism of p53-p21 dependent regulation of apoptosis in iPS cells remains to be investigated. Numerous other pro-apoptotic targets of p53 have been described, such as Bcl2 and p53-regulated apoptosis-inducing protein

1 (p53AIP1) [68, 69]. It would be important to investigate if p53 regulates other factors and signaling pathways besides of p21 in iPS cells.

3.6. Limitations of the study

First of all, the stabilization and activation of p53 might be regulated by acetylation and phosphorylation [70]. It is known that different kinases mediate p53 phosphorylation on Thr18 and/or Ser20 in response to distinct stress signals in somatic cells [71]. The role of p53 phosphorylation on the regulation of proliferation, survival and pluripotency of iPS cells was not studied yet. Thakur et al. detected no differences in the phosphorylation status of p53, but a markedly increased p53 acetylation in FK866 treated HEK293T WT cells, compared to untreated control cells [61]. However, it is unknown whether p53 phosphorylation is involved in the mediation of iPS cells cell proliferation or pluripotency. It remains to be investigated if acetylation of p53 affects its phosphorylation, or vice versa in iPS cells.

Additionally, the effect of SIRT2 inhibitors seems to be complicated and firmly dependent on the cell type and the genetic connection. The variable expression of different cell cycle coordinator such as c-MYC, CYCLIN D1, CDK2, in addition to p21 regulation, might be obligated to mediate the balance between self-renewal and differentiation of iPS cells. Therefore, not only p21 but also these additional cell cycle regulatory factors should be investigated in detail in iPS cells, in connection with NAMPT/SIRT2 signaling activation [72].

4. Summary

NAMPT modulates cellular functions by means of the protein deacetylation activity of NAD^+-dependent sirtuins which determines functions of histones and none-histone proteins via deacetylation. The role of NAMPT and SIRT2 in maintenance and hematopoietic differentiation of iPS cells is poorly investigated. We assessed the effect of NAMPT and SIRT2 on the pluripotency, proliferation, survival, and granulocytic differentiation of human iPS cells. We applied two approaches to inhibit NAMPT or SIRT2, the addition of specific chemical inhibitors of NAMPT or SIRT2 (FK866 and AC93253, respectively) to the culture medium, shRNA mediated NAMPT/SIRT2 knockdown in iPS cells. The molecular mechanism downstream of NAMPT/SIRT2 functions in iPS cells is also investigated. We provide that evidence that NAMPT is essential for the maintenance, survival and myeloid differentiation of iPS cells. We found that of the NAMPT or SIRT2 blockage in iPS cells induces p53 upregulation by enhancing its lysine acetylation. This results in activation of p21, the target of p53, consequently, the cell cycle arrested in G1 phase and apoptosis was induced in iPS cells. NAMPT and SIRT2 inhibition also abolished iPS cells' granulocytic differentiation in an embryoid body (EB)-based differentiation. Taken together, our data shows that the NAMPT/SIRT2/p53/p21 signaling axis plays an essential role in the iPS cells maintenance and hematopoietic differentiation.

5. Zusammenfassung

NAMPT moduliert zelluläre Funktionen mittels der Protein-Deazetylase-Aktivität von NAD+-abhängigen Sirtuinen (SIRTs), zum Beispiel der Deazetylierung von Histonen und anderer Proteine. Die Rolle von NAMPT in der Regulierung von SIRT2 bei der Kultivierung und hämatopoetischer Differenzierung von induzierten pluripotenten Stammzellen (iPS-Zellen) ist unklar. Wir haben die Effekte von NAMPT und davon abhängig SIRT2 auf die Pluripotenz, die Proliferation, das Überleben und auf die hämatopoetische Differenzierung von humanen iPS-Zellen untersucht. Wir haben zwei experimentelle Ansätze verwendet. In dem ersten wurden iPS-Zellen in Anwesenheit von spezifischen chemischen Inhibitoren von NAMPT oder SIRT2 (FK866 für NAMPT und AC93253 für SIRT2) kultiviert. In dem zweiten Ansatz, wurden NAMPT oder SIRT2 mittels lentiviraler Transduktion mit spezifischen shRNA Konstrukten in iPS-Zellen inhibiert. Wir haben auch den molekuleren Mechanismus von NAMPT/SIRT2 in iPS-Zellen untersucht. Wir konnten zeigen, dass NAMPT unabdingbar für die Kultivierung, das Überleben und für die hämatopoetische Differenzierung von iPS-Zellen ist. Wir haben auch herausgefunden, dass die Hemmung von NAMPT oder SIRT2 in iPS-Zellen die Expression von p53 Protein induziert und das p53 Protein azetyliert. Dadurch wird das p53 Zielgen p21 aktiviert, was zum Stop des Zellzyklus und der Induktion von Apoptose in iPS-Zellen führt. Zusätzlich hemmt die NAMPT oder SIRT2 Inhibition die hämatopoetische Differenzierung von iPS-Zellen. Zusammenfassend konnten wir also die wichtige Rolle des NAMPT/SIRT2/p53/p21 Signalweges in der Kultivierung und hämatopoetischer Differenzierung von iPS-Zellen aufzeigen.

6. Outlook

Overall, this work shows a new mechanism of iPSC maintenance through post-translational regulation of p53 by NAMPT/SIRT2 signaling. Implementation of the knowledge of this study may contribute to the establishment of improved protocols of high-scale generation of iPS cells and hematopoietic cells for clinical applications in the future.

In the ongoing work, it would be interesting to perform CRISPR/Cas9-mediated knockout of SIRT2 and NAMPT in iPS cells to verify further our conclusion, that were mostly based on the implementation of small molecule chemical inhibitors. We previously described a NAMPT/SIRT2-dependent activation of the ß-catenin-Akt pathway in acute myeloid leukemia. Therefore, it would be compelling to investigate if the NAMPT/SIRT2-regulated p53-p21 signaling is associated with the ß-catenin-Akt pathway in the maintenance of iPS cells. The terminal mechanism by which NAMPT and SIRT2 regulate the hematopoietic differentiation is a crucial component attempts to overcome. Investigating 3D cell printing iPS cells and organoid-induced hematopoietic differentiation might also prove vital.

7. References

1. Skokowa, J., et al., NAMPT is essential for the G-CSF-induced myeloid differentiation via a NAD(+)-sirtuin-1-dependent pathway. Nat Med, 2009. **15**(2): p. 151-8.
2. Dan, L., et al., The role of sirtuin 2 activation by nicotinamide phosphoribosyltransferase in the aberrant proliferation and survival of myeloid leukemia cells. Haematologica, 2012. **97**(4): p. 551-9.
3. Srivastava, S., Emerging therapeutic roles for NAD(+) metabolism in mitochondrial and age-related disorders. Clin Transl Med, 2016. **5**(1): p. 25.
4. Meng, Y., et al., Nicotinamide Promotes Cell Survival and Differentiation as Kinase Inhibitor in Human Pluripotent Stem Cells. Stem Cell Reports, 2018. **11**(Dec 11): p. 9.
5. Maiese, K., et al., The vitamin nicotinamide: translating nutrition into clinical care. Molecules, 2009. **14**(9): p. 3446-85.
6. Blander, G. and Guarente, L., The Sir2 family of protein deacetylases. Annu Rev Biochem, 2004. **73**: p. 417-35.
7. Wang, Y., et al., SIRT2: Controversy and multiple roles in disease and physiology. Ageing Res Rev, 2019. **55**: p. 100961.
8. Vaquero, A., et al., SirT2 is a histone deacetylase with preference for histone H4 Lys 16 during mitosis. Genes Dev, 2006. **20**(10): p. 1256-61.
9. Jing E., G.S., Kahn C.R. , SIRT2 regulates adipocyte differentiation through FoxO1 acetylation/deacetylation. Cell Metab. 2007;6(2):105-114.
10. Szego, E.M., E. Gerhardt, and T.F. Outeiro, Sirtuin 2 enhances dopaminergic differentiation via the AKT/GSK-3beta/beta-catenin pathway. Neurobiol Aging, 2017. **56**: p. 7-16.
11. Wang, F., et al., SIRT2 deacetylates FOXO3a in response to oxidative stress and caloric restriction. Aging Cell, 2007. **6**(4): p. 505-14.
12. Peck, B., et al., SIRT inhibitors induce cell death and p53 acetylation through targeting both SIRT1 and SIRT2. Mol Cancer Ther, 2010. **9**(4): p. 844-55.
13. Takahashi, K. and S. Yamanaka, A decade of transcription factor-mediated reprogramming to pluripotency. Nat Rev Mol Cell Biol, 2016. **17**(3): p. 183-93.
14. Halevy, T. and Urbach, A., Comparing ESC and iPSC-Based Models for Human Genetic Disorders. J Clin Med. 2014 Dec; 3(4): p. 1146–1162.
15. Moradi, S., et al. Research and therapy with induced pluripotent stem cells (iPSCs): social, legal, and ethical considerations. Stem Cell Res Ther. 2019; p.10: 341.
16. Rowe, R.G. and G.Q. Daley, Induced pluripotent stem cells in disease modelling and drug discovery. Nat Rev Genet, 2019. **20**(7): p. 377-388.
17. Cliff, T.S. and S. Dalton, Metabolic switching and cell fate decisions: implications for pluripotency, reprogramming and development. Curr Opin Genet Dev, 2017. **46**: p. 44-49.
18. Son, M.J., et al., Nicotinamide overcomes pluripotency deficits and reprogramming barriers. Stem Cells, 2013. **31**(6): p. 1121-35.
19. De Bonis, M. L., Ortega, S., and Blasco, M. A., SIRT1 is necessary for proficient telomere elongation and genomic stability of induced pluripotent stem cells. Stem Cell Reports, 2014. 2: p. 690–706.

20. Thakur, B.K., et al., NAMPT pathway is involved in the FOXO3a-mediated regulation of GADD45A expression. Biochem Biophys Res Commun, 2012. **420**(4): p. 714-20.
21. Skokowa, J., et al., LEF-1 is crucial for neutrophil granulocytopoiesis and its expression is severely reduced in congenital neutropenia. Nat Med, 2006. **12**(10): p. 1191-7.
22. Ryall, J.G., et al., The NAD(+)-dependent SIRT1 deacetylase translates a metabolic switch into regulatory epigenetics in skeletal muscle stem cells. Cell Stem Cell, 2015. **16**(2): p. 171-83.
23. Xu, P., et al., Sirt6 regulates efficiency of mouse somatic reprogramming and maintenance of pluripotency. Stem Cell Res Ther, 2019. **10**(1): p. 9.
24. Liu, T.M. and N. Shyh-Chang, SIRT2 and glycolytic enzyme acetylation in pluripotent stem cells. Nat Cell Biol, 2017. **19**(5): p. 412-414.
25. Boward, B., T. Wu, and S. Dalton, Concise Review: Control of Cell Fate Through Cell Cycle and Pluripotency Networks. Stem Cells, 2016. **34**(6): p. 1427-36.
26. Blomen and Boonstra. Cell fate determination during G1 phase progression. Cell Mol Life Sci. 2007 Dec;64(23): p. 3084-104.
27. Chen, X., A. Hartman, and S. Guo, Choosing Cell Fate Through a Dynamic Cell Cycle.Curr Stem Cell Rep, 2015. **1**(3): p. 129-138.
28. Kapinas, K., et al., The abbreviated pluripotent cell cycle. J Cell Physiol, 2013. **228**(1): p. 9-20.
29. White, J. and Dalton, S., Cell cycle control of embryonic stem cells. Stem Cell Rev. 2005;1: p. 131–8.
30. Calegari, F. and Huttner, W.B., An inhibition of cyclin-dependent kinases that lengthens, but does not arrest, neuroepithelial cell cycle induces premature neurogenesis. J Cell Sci. 2003 Dec 15;116(Pt 24): p. 4947-55.
31. Calegari F., Selective lengthening of the cell cycle in the neurogenic subpopulation of neural progenitor cells during mouse brain development. J Neurosci. 2005 Jul 13;25(28): p. 6533-8.
32. Filipczyk, A.A., et al., Differentiation is coupled to changes in the cell cycle regulatory apparatus of human embryonic stem cells. Stem Cell Res, 2007. **1**(1): p. 45-60.
33. Neganova, I., et al., Expression and functional analysis of G1 to S regulatory components reveals an important role for CDK2 in cell cycle regulation in human embryonic stem cells. Oncogene, 2009. **28**(1): p. 20-30.
34. Gottlieb TM., p53 and apoptosis. Semin Cancer Biol.1998;8(5): p. 359-68.
35. Ko LJ., p53: puzzle and paradigm. Genes Dev.1996 May 1;10(9): p.1054-72.
36. Johnstone, R.W., et al. Apoptosis: A Link between Cancer Genetics and Chemotherapy. Cell.2002 Jan 25;108(2):p.153-64.
37. Martinez J., Cellular localization and cell cycle regulation by a temperature-sensitive p53 protein. Genes Dev. 1991 Feb;5(2): p.151-9.
38. Sabapathy K., et al., Regulation of ES cell differentiation by functional and conformational modulation of p53. EMBO J 1997; 16: p.6217-6229.
39. Vassilev LT., Small-molecule antagonists of p53-MDM2 binding: research tools and potential therapeutics. Cell Cycle. 2004; 3: p. 419-421.
40. Maimets T., et al., Activation of p53 by nutlin leads to rapid differentiation of human embryonic stem cells. Oncogene 2008; 27: p.5277-5287.

41. Itahana, Y., et al., Histone modifications and p53 binding poise the p21 promoter for activation in human embryonic stem cells. Sci Rep, 2016. **6**: p. 28112.
42. Stead E., Pluripotent cell division cycles are driven by ectopic Cdk2, cyclin A/E and E2F activities. Oncogene. 2002 Nov 28;21(54): p.8320-33.
43. Neganova, I. and M. Lako, G1 to S phase cell cycle transition in somatic and embryonic stem cells. J Anat, 2008. **213**(1): p. 30-44.
44. Amabile, G. and Meissner, A., Induced pluripotent stem cells: current progress and potential for regenerative medicine. Trends Mol Med, 200915: p.59–68.
45. Pettinato, G., X. Wen, and N. Zhang, Formation of well-defined embryoid bodies from dissociated human induced pluripotent stem cells using microfabricated cell-repellent microwell arrays. Sci Rep, 2014. **4**: p. 7402.
46. Liu, G., et al. Advances in Pluripotent Stem Cells: History, Mechanisms, Technologies, and Applications. Stem Cell Rev and Rep, 2020; (16) p. 3–32.
47. Dannenmann B., et al., CRISPR/Cas9 Genome Editing of Human-Induced Pluripotent Stem Cells Followed by Granulocytic Differentiation. In: Sioud M. (eds) RNA Interference and CRISPR Technologies. Methods in Molecular Biology,2020; vol 2115. Humana, New York, NY.
48. Dannenmann, B., et al., CRISPR/Cas9 Genome Editing of Human-Induced Pluripotent Stem Cells Followed by Granulocytic Differentiation. Methods Mol Biol, 2020. **2115**: p. 471-483.
49. Eglen, R.M. and T. Reisine, Human iPS Cell-Derived Patient Tissues and 3D Cell Culture Part 2: Spheroids, Organoids, and Disease Modeling. SLAS Technol, 2019. **24**(1): p. 18-27.
50. Montel-Hagen A., et al., Organoid-Induced Differentiation of Conventional T Cells from Human Pluripotent Stem Cells. Cell Stem Cell. 2019 Mar 7;24(3): p. 376-389.
51. Meng, Y., et al., Nicotinamide Promotes Cell Survival and Differentiation as Kinase Inhibitor in Human Pluripotent Stem Cells. Stem Cell Reports, 2018. **11**(6): p. 1347-1356.
52. North BJ., The human Sir2 ortholog, SIRT2, is an NAD+-dependent tubulin deacetylase. Mol Cell.2003Feb;11(2): p.437-44.
53. Sayd, S., M.P. Junier, and H. Chneiweiss, SIRT2, a multi-talented deacetylase. Med Sci (Paris), 2014. **30**(5): p. 532-6.
54. Kaufmann T., SIRT2 regulates nuclear envelope reassembly through ANKLE2 deacetylation. J Cell Sci. 2016 Dec 15;129(24): p.4607-4621.
55. Peck, B. et al., SIRT inhibitors induce cell death and p53 acetylation through targeting both SIRT1 and SIRT2. Mol. Cancer Ther. 9: p. 844–855.
56. Min JS., SIRT2 reduces actin polymerization and cell migration Biochim Biophys Acta Mol Cell Res. 2018 Sep;1865(9): p.1230-1238.
57. Jin, Y.H., et al., Sirt2 interacts with 14-3-3 beta/gamma and down-regulates the activity of p53. Biochem Biophys Res Commun, 2008. **368**(3): p. 690-5.
58. Hoffmann, G., et al., A novel sirtuin 2 (SIRT2) inhibitor with p53-dependent pro-apoptotic activity in non-small cell lung cancer. J Biol Chem, 2014. **289**(8): p. 5208-16.
59. Inoue, T., et al., SIRT2, a tubulin deacetylase, acts to block the entry to chromosome condensation in response to mitotic stress. Oncogene, 2007. **26**(7): p. 945-57.

60. Zhang, L.Q., et al., Metabolic and molecular insights into an essential role of nicotinamide phosphoribosyltransferase. Cell Death Dis, 2017. **8**(3): p. e2705.
61. Thakur, B.K., et al., Inhibition of NAMPT pathway by FK866 activates the function of p53 in HEK293T cells. Biochem Biophys Res Commun, 2012. **424**(3): p. 371-7.
62. Qin, H., et al., Regulation of apoptosis and differentiation by p53 in human embryonic stem cells. J Biol Chem, 2007. **282**(8): p. 5842-52.
63. Abhinav K., et al., p53 Regulates Cell Cycle and MicroRNAs to Promote Differentiation of Human Embryonic Stem Cells. PLoS Biol. 2012 Feb; 10(2): e1001268.
64. Fortelny, N., et al. Can we predict protein from mRNA levels?. Nature 2017; 547, E19–E20.
65. Gartel AL et al., The role of the cyclin-dependent kinase inhibitor p21 in apoptosis. Mol Cancer Ther. 2002 Jun;1(8): p.639-49.
66. Bellido, et al., Transcriptional activation of the p21(WAF1, CIP1, SDI1) gene by interleukin-6 type cytokines. A prerequisite for their pro-differentiating and antiapoptotic effects on human osteoblastic cells. J. Biol. Chem., 273: p.21137–21144, 1998.
67. Bellido, T., et al., Transcriptional activation of the p21(WAF1,CIP1,SDI1) gene by interleukin-6 type cytokines. A prerequisite for their pro-differentiating and anti-apoptotic effects on human osteoblastic cells. J Biol Chem, 1998. **273**(33): p. 21137-44.
68. Vousden KH., p53: Death Star. Cell. 2000 Nov 22;103(5): p.691-4.
69. Oda K., p53AIP1, a potential mediator of p53-dependent apoptosis, and its regulation by Ser-46-phosphorylated p53. Cell. 2000 Sep 15;102(6): p.849-62.
70. Nicola J. Maclaine., The regulation of p53 by phosphorylation: a model for how distinct signals integrate into the p53 pathway. Aging (Albany NY). 2009 May; 1(5): p.490–502.
71. MacLaine, N.J. and T.R. Hupp, How phosphorylation controls p53. Cell Cycle, 2011. **10**(6): p. 916-21.
72. Enver, T., et al., Cellular differentiation hierarchies in normal and culture-adapted human embryonic stem cells. Hum Mol Genet, 2005. **14**(21): p. 3129-40.

8. Declaration

Y.X., J.S. and T.M. made initial observations, designed the experiments, and wrote the manuscript.; Y.X. planned and performed all the experiments, analyzed the data and performed trouble-shooting; B.D., A.Z., assisted with culture of human iPS cells; M.N. assisted with the plasmid cloning; K.W., P.M. and M.N. assisted with the interpretation of the data and provided insightful comments.

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