A critical role for AID in the initiation of reprogramming to induced pluripotent stem cells

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ABSTRACT **Mechanistic insights into the reprogramming of fibroblasts to induced pluripotent stem cells (iPSCs) are limited, particularly for early acting molecular regulators. Here we use an acute loss of function approach to demonstrate that activation-induced deaminase (AID) activity is necessary for the initiation of reprogramming to iPSCs. While AID is well known for antibody diversification, it has also recently been shown to have a role in active DNA demethylation in reprogramming toward pluripotency and development. These findings suggested a potential role for AID in iPSC generation, yet, iPSC yield from AID-knockout mouse fibroblasts was similar to that of wild-type (WT) fibroblasts. We reasoned that an acute loss of AID function might reveal effects masked by compensatory mechanisms during development, as reported for other proteins. Accordingly, we induced an acute reduction (>50%) in AID levels using 4 different shRNAs and determined that reprogramming to iPSCs was significantly impaired by 79 ± 7%. The deaminase activity of AID was critical, as coexpression of WT but not a catalytic mutant AID rescued reprogramming. Notably, AID was required only during a 72-h time window at the onset of iPSC reprogramming. Our findings show a critical role for AID activity in the initiation of reprogramming to iPSCs.—Bhutani, N., Decker, M. N., Brady, J. J., Bussat, R. T., Burns, D. M., Corbel, S. Y., Blau, H. M. A critical role for AID in the initiation of reprogramming to induced pluripotent stem cells. *FASEB J.* 27, 1107–1113 (2013). www.fasebj.org**

Key Words: activation-induced deaminase · DNA demethylation · early events in iPSC generation

Reprogramming of somatic cells to generate induced pluripotent stem cells (iPSCs) has unprecedented potential for regenerative medicine, for cell-based therapies, for modeling human diseases in culture, and for drug discovery. Yet, despite years of effort, the frequency of reprogramming remains low, and the time course is slow and asynchronous (1–3). To reliably generate iPSCs with less heterogeneity, greater fidelity, and increased efficiency, an elucidation of the molecular regulators underlying the reprogramming process would be invaluable. Although much research has focused on iPSCs since their landmark discovery (2, 4, 5), mechanistic insights into the process of reprogramming remain somewhat limited. Several groups have reported that elimination of tumor suppressors, such as p53 and Ink4a/Arf, accelerates cell division and leads to higher iPSC frequencies (6–9); factors like Glis1, SUV39H1, DOT1L, and YYI alter the frequency of iPSCs (10, 11), and Essrb and Utf1 expression predicts successful reprogramming (12), but an understanding of the molecular regulators required at the onset of reprogramming is generally lacking. To meet this need, we previously employed a cell-fusion approach (heterokaryons) ideally suited to studying the initiation of reprogramming in general (13) and toward pluripotency (14), as reprogramming is initiated rapidly, synchronously, and at high efficiency within hours postfusion. We hypothesized that heterokaryons could serve as a discovery tool for mechanisms mediating reprogramming, such as DNA demethylation (3). In nondividing heterokaryons formed by fusing human fibroblasts with an excess of mouse embryonic stem cells, we demonstrated that replication-independent activation-induced deaminase (AID)-mediated active DNA demethylation is critical for initiating reprogramming toward pluripotency (14). This study was the initial report implicating the AID family of deaminases as part of the mammalian DNA demethylation machinery. Since then, many other studies have identified additional molecules and enzymes that mediate DNA repair in active DNA demethylation (15, 26, 17–19). These include AID, APOBEC family members, the hydroxymethylases, ten-eleven translocation (TET) proteins (17–19), and the base-excision repair DNA glycosylases, including thymine DNA glycosylase (TDG) (15, 16). However, to date there is no

Abbreviations: 4F, 4 transcription factors; AID, activation-induced deaminase; iPSC, induced pluripotent stem cell; KO, knockout; MEF, mouse embryonic fibroblast; Rb, retinoblastoma; TET, ten-eleven translocation; TTF, tail-tip fibroblast; WT, wild-type

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evidence that these regulators play a role in the reprogramming of fibroblasts to iPSCs.

We sought to test whether iPSC reprogramming requires the deaminase AID. Although AID is critical to heterokaryon reprogramming toward pluripotency (14), the fusion and iPSC approaches to reprogramming differ in their requirement for cellular proliferation. In heterokaryons, AID is crucial to the initiation of reprogramming and DNA demethylation during a 3-d period when cell division and DNA replication do not occur. DNA demethylation is also known to be required during reprogramming to iPSCs, as iPSCs that are only partially reprogrammed are incompletely demethylated at pluripotency gene promoters (20). However, since rapid and continuous cell division accompanies iPSC reprogramming over a period of weeks, DNA demethylation is generally considered to occur *via* a DNA (cytosine-5)-methyltransferase 1-dependent process whereby methylation is passively lost over the course of multiple cell divisions (20, 21).

MATERIALS AND METHODS

Lentiviral shRNA

The lentiviral nontarget shRNA and the shRNAs targeting AID (shAID3 and shAID4) were as described previously to target AID in B cells (22) and were from Sigma (St. Louis, MO, USA). shAID5 and shAID6 (V2LMM_44041, and V2LMM_42053, respectively) were from Thermo Scientific OpenBioSystems (Waltham, MA, USA).

Viral production, concentration, and titration

Lentiviral or retroviral constructs and the appropriate packaging plasmids were transfected into subconfluent HEK293T cells using Fugene (Roche, Indianapolis, IN, USA). Viral medium was harvested at 48 and 72 h following transfection and concentrated by ultracentrifugation. Virus was titered by p24 ELISA using the Lenti-X p24 Rapid Titer Kit (Clontech, Mountain View, CA, USA). Viral titers were matched for AID, AID-E58Q, and mock. Viral titer for shCon was always matched to the highest shAID titer.

Viral constructs

For the retroviral human AID (hAID) construct, the full-length hAID cDNA was amplified from pMSCV-AID-ires-Thy1.1 using the following primers: hAID *Bam*HI, 5′-CTAGGATCCATGGACAGCCTCTTGATGAAC-3′; and hAID *Eco*RI, 5′-CTAGAATTCTCAAAGTCCCAAAGTACGAAA-3′. The resulting fragment was cloned into the *Bam*HI/*Eco*RI site of the pBABE-puro vector. mAIDwt (wild-type) and mAID(E58Q) were a generous gift from M. Nussenzweig (Rockefeller University, New York, NY, USA).

Production and concentration of viral supernatants

HEK293T cells were plated at 5×10^6 cells/10-cm dish. The next day, the cells were transfected with 3.2 μg lentiviral vector, 4 μg Gag-Pol vector, 0.4 μg Tat vector, 0.4 μg Rev vector, and 0.4 μg VSVg vector using 24 μl Fugene in 160 μl Opti-MEM (Invitrogen, Grand Island, NY, USA). For retroviral production, 4 μg of retroviral vector was transfected with 4 μg pCL-ECO vector using 24 μl Fugene. Supernatant was collected 48 and 72 h post-transfection and filtered through 0.45-μm pore filters. For concentration, viral supernatant was centrifuged at 18,000 rpm for 1.5 h at 4°C, and the pellets were resuspended in mouse embryonic fibroblast (MEF) medium at 20–30× concentration. Concentrated lentivirus aliquots were stored at −80°C until use.

Six-well assays for iPSC generation

MEF or tail-tip fibroblast (TTF) cells (passage 2 to passage 3) were plated in 6-well dishes at 60,000 cells/well. The following day, the cells were infected with lentiviruses encoding 4 transcription factors (4F: Oct4, Klf4, Sox2, and c-Myc) and shRNA in the presence of 8 μg/ml polybrene. During rescue experiments, cells were infected with AID expression retroviruses 1 d prior to addition of 4F and shRNA lentiviral supernatant. In the staggered knockdown experiments, cells were infected with lentiviral shRNA at 3 or 5 d following 4F infection. Following the final round of viral infection, cells were cultured for up to 21 d in MEF medium. At the end of the culture period, the number of iPSC colonies in each well was scored by morphology and by Nanog staining.

Ninety-six-well assay

MEF or TTF cells (passage 2 to passage 3) were plated in 6-well dishes at 50,000 cells/well. After 24 h, the cells were infected with lentivirus in the presence of 8 μg/ml polybrene. The following day, the cells were trypsinized and split into 96-well plates at 100 cells/well, preplated with a feeder layer of irradiated MEFs at 1000 cells/well. Daily medium changes for secondary MEFs were supplemented with doxycycline (1 μg/ml). The cells were cultured for up to 21 d. At the end of the culture period, the number of iPSC-positive wells was scored. This stringent 96-well assay, in which a well containing one or more iPSC colonies was scored singly, corroborated the 6-well assay.

MEF isolation

MEFs were isolated from E13.5 BALB/c mouse embryos [either wild-type (WT) or AID-knockout (KO), generously provided by T. Honjo, Kyoto University, Kyoto, Japan], expanded in culture until passage 1 or 2, frozen, and stored in liquid nitrogen.

Generation of Nanog GFP 2° MEFs for iPSC reprogramming

Genetically identical 2° MEFs that harbor dox-inducible reprogramming factors were generated as described previously (1). Briefly, Nanog-GFP 1° MEFs expressing the reverse tetracycline transactivator (M2rtTA) were infected with dox-inducible lentiviruses encoding the 4F reprogramming factors. Doxycycline-induced reprogramming led to primary iPSC colonies that were propagated and injected into mouse blastocysts. MEFs were isolated at E13.5 of gestation from the resulting chimeric mice and selected for 3 d in puromycin, yielding a genetically homogeneous population of 2° MEFs that reprogrammed to iPSCs upon doxycycline addition.

Alkaline phosphatase staining

Alkaline phosphatase staining was performed using the Vector Red Alkaline Phosphatase Substrate Kit I (cat. no. SK-5100; Vector Laboratories, Burlingame, CA, USA) following the manufacturer's protocol. Images were taken using a Nikon D3100 SLR camera (Nikon, Tokyo, Japan).

Immunofluorescence

Selected wells were blocked (20% goat serum, 0.3% Triton X-100) and stained for Nanog (cat. no. A300-398A; 1:200 antibody dilution; Bethyl Laboratories, Montgomery, TX, USA). Images were acquired using an epifluorescent microscope (Axio Observer.A1; Carl Zeiss MicroImaging, Thornwood, NY, USA), Zeiss Neofluar ×10/0.3 or ×20/0.4 objective lens, and a microimaging camera (AxioCam MRC; Carl Zeiss MicroImaging). The software used for acquisition was AxioVision 4.8.1 (Carl Zeiss MicroImaging).

Real-time PCR

Real-time PCR for AID and GAPDH was performed using an ABI 7900HT Real time PCR system using the Sybr Green PCR mix (Applied Biosystems, Carlsbad, CA, USA). Samples were cycled at 94°C for 2 min, 40× (94°C for 20 s, 58°C for 45 s).

Statistical analyses

Data are presented as the mean ± SD. Comparisons between groups used Student's *t* test assuming 2-tailed distributions.

RESULTS

We designed experiments to determine whether AID plays a role in reprogramming fibroblasts to iPSCs. MEFs were transduced with a single lentivirus (Lenti 4F) encoding the 4 defined factors, Oct4, Sox2, c-Myc, and Klf4, as described previously (23), to reprogram the MEFs to iPSC colonies. To determine whether AID plays a role in reprogramming to iPSCs, we first performed gain of function experiments and assessed the frequency of Nanog-expressing colonies in each case. Since viral load greatly affects reprogramming efficiency, we carefully controlled for viral titers in each experiment (Supplemental Fig. S1). In initial gain of function experiments in which hAID was constitutively overexpressed together with the 4F virus, we observed a consistent 2-fold increase in iPSC colony frequency in 3 independent experiments. These experiments showed that AID alone augmented the reprogramming process (**Fig. 1*A*, *B***). We confirmed that AID addition increases the efficiency of reprogramming to iPSCs using a second scenario, secondary MEFs (2° MEFs) generated from chimeric mice in which induction of the 4 factors is under the control of a doxycycline-inducible promoter (1). A major advantage of the secondary system is that the MEFs are exposed to a reduced viral load as only a single infection of the AID or mock virus is required. In addition, the 2° MEFs harbored a Nanog-GFP knock-in reporter construct enabling a fluorescent readout for Nanog expression. Like 1° MEFs, AID overexpression in 2° MEFs led to a doubling in colony number (Fig. 1*C*, *D*). We characterized the iPSC colonies obtained on constitutive overexpression of AID and found no differences from WT iPSCs in their karyotype, global gene expression patterns, teratoma formation, and potential to contribute to all three germ layers (data not shown). AID presumably requires additional factors, such as components of DNA repair pathways, for full efficacy (15, 16); however, these data show that an increase in AID alone is capable of significantly increasing the frequency of normal iPSCs.

To further test the requirement for AID in reprogram-

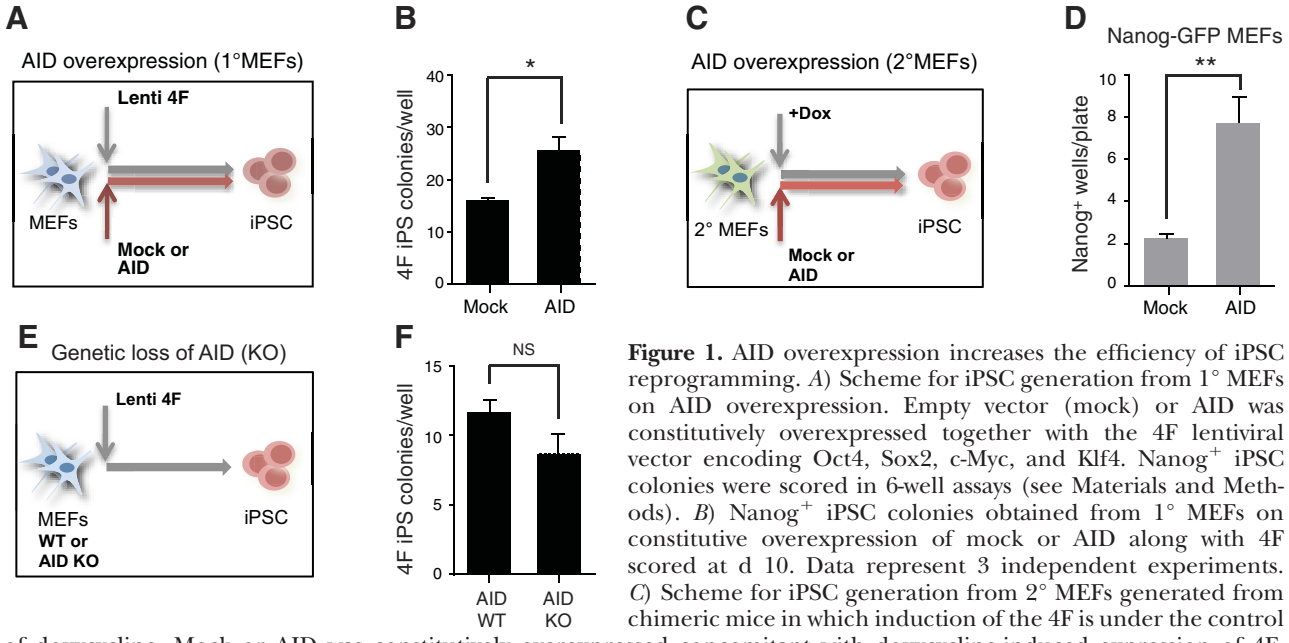

Figure 1. AID overexpression increases the efficiency of iPSC reprogramming. *A*) Scheme for iPSC generation from 1° MEFs on AID overexpression. Empty vector (mock) or AID was constitutively overexpressed together with the 4F lentiviral vector encoding Oct4, Sox2, c-Myc, and Klf4. Nanog$^+$ iPSC colonies were scored in 6-well assays (see Materials and Methods). *B*) Nanog$^+$ iPSC colonies obtained from 1° MEFs on constitutive overexpression of mock or AID along with 4F scored at d 10. Data represent 3 independent experiments. *C*) Scheme for iPSC generation from 2° MEFs generated from chimeric mice in which induction of the 4F is under the control of doxycycline. Mock or AID was constitutively overexpressed concomitant with doxycycline-induced expression of 4F. Nanog$^+$ iPSC wells were scored at d 21 in 96-well assays (see Materials and Methods). *D*) Nanog$^+$ (GFP$^+$) iPSC colonies derived from 2° MEFs following constitutive overexpression of AID or a mock vector. Data represent 8 replicates from 2 independent experiments. *E*) Scheme for iPSC generation from 1° MEFs derived from WT or AID-KO mice. Nanog$^+$ iPSC colonies from WT or AID-KO 1° MEFs transduced with the 4F lentivirus were scored in 6-well assays. *F*) Nanog$^+$ iPSC colonies obtained from WT or AID-KO 1° MEFs at d 16 after 4F transduction. Data represent 3 independent experiments. *$P < 0.05$, **$P < 0.01$; 2-tailed Student's *t* test.

ming, we performed loss of function experiments. We first compared reprogramming of fibroblasts genetically lacking AID to WT controls and did not observe any significant change in reprogramming (Fig. 1*E*). The frequency of iPSCs derived from fibroblasts isolated from AID-KO mice, which were created by homologous recombination and lack AID throughout development (24), to fibroblasts from strain-, age-, and sex-matched WT mice did not differ (Fig. 1*F*), when either embryonic fibroblasts (MEFs; Fig. 1*F* and Supplemental Fig. S2*A*) or adult dermal fibroblasts from mouse tail tips (TTFs) were used for reprogramming (Supplemental Fig. S2*B*). Although AID-KO mice are known to have impaired antibody generation and diversification in B cells (24) and exhibit somewhat reduced DNA demethylation in their germ cells (25), they are viable and fertile (24). We therefore hypothesized that in the course of development, AID function might be largely compensated for by one or more related family members or other components of the DNA demethylation machinery. Such genetic compensation has been previously reported for families of proteins including cyclin-dependent kinases (CDKs), forkhead homeobox type O (FOXO) transcription factors, and retinoblastoma (Rb) tumor suppressors (26–28).

We designed a strategy for inducing an acute loss of AID function, to avoid potential compensatory mechanisms (**Fig. 2*A***). We used lentivirus encoding shRNAs (shAID3 and shAID4) known to specifically and efficiently knock down AID in B cells, as described previously by Nussenzweig and colleagues (22), and two additional shRNAs (shAID5 and shAID6) that target the 3′-untranslated region of the AID transcript (Supplemental Fig. S3*A*). As a control, an shRNA was used that did not target AID (shCon). In WT MEFs, a knockdown of AID gene expression of ~85% was seen with shAID3 and shAID4 and expression of 50% with shAID5 and shAID6 (Fig. 2*B*). Each of these shRNAs was then introduced into MEFs simultaneously with Lenti-4F virus (Fig. 2*A*). We took great care to control for variables that influence iPSC reprogramming efficiency: cell density and rates of growth were similar (Supplemental Fig. S4); early passage MEFs were used to avoid senescence; and viral load was carefully titered and kept constant during infection. Instead of sequential introduction of the shRNAs and the iPSC factors (Lenti-4F), the viruses were delivered simultaneously in order to further minimize MEF doublings and senescence.

Notably, an acute loss of AID function using all four shRNAs targeting AID, shAID3, shAID4, shAID5, and shAID6, greatly diminished reprogramming of WT MEFs compared to the shCon (Fig. 2*C*.) iPSC colonies were infrequent in 3 independent experiments in which MEFs were treated with either of shAID3, shAID4, shAID5, or shAID6, assessed either by alkaline-phosphatase staining (Fig. 2*D*) or by the more stringent assay of staining for the pluripotency marker, Nanog, using immunofluorescence (Fig. 2*E*) as compared to shCon. To determine the specificity of the shRNA viruses, they were transduced along with 4F virus into AID-KO MEFs genetically lacking AID, in which they should have no effect. As expected, reprogramming efficiency was comparable to WT MEFs transduced with the shCon, ensuring that the phenotype observed is not due to off target effects of the 4 shRNAs. In contrast to

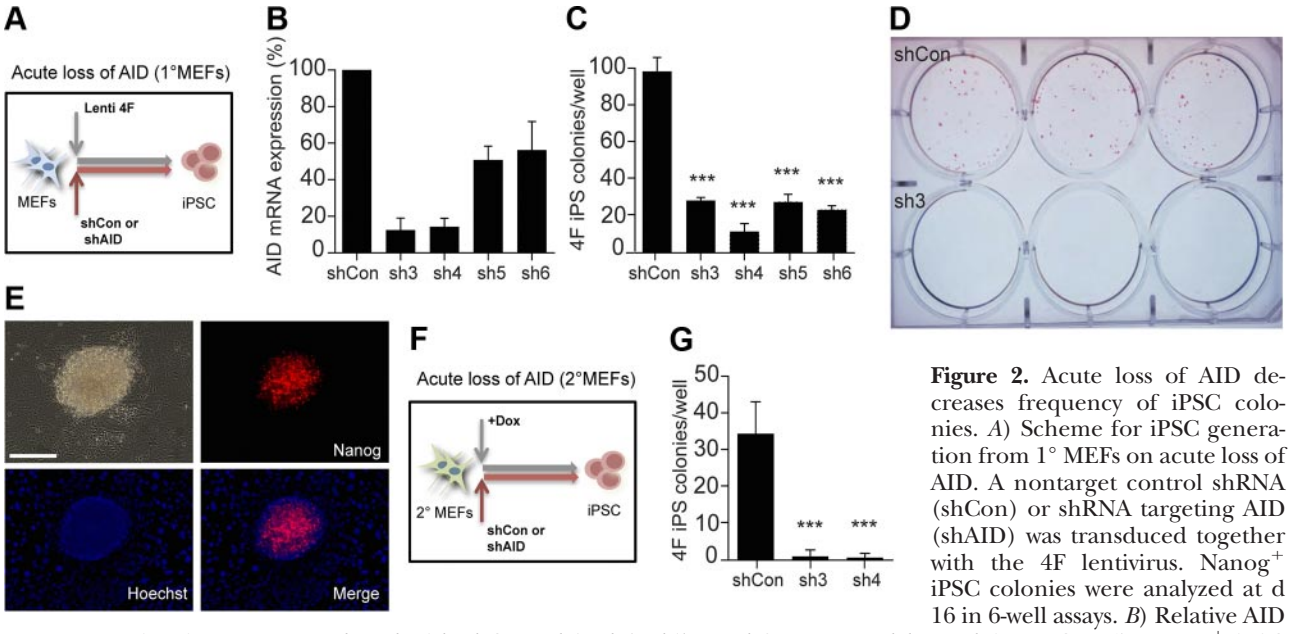

Figure 2. Acute loss of AID decreases frequency of iPSC colonies. *A*) Scheme for iPSC generation from 1° MEFs on acute loss of AID. A nontarget control shRNA (shCon) or shRNA targeting AID (shAID) was transduced together with the 4F lentivirus. Nanog$^+$ iPSC colonies were analyzed at d 16 in 6-well assays. *B*) Relative AID gene expression in MEFs transduced with shCon, sh3, sh4, sh5, or sh6 as assessed by real-time PCR. *C*) Nanog$^+$ iPSC colonies obtained from 1° MEFs transduced with shCon or shAID (3, 4, 5, or 6) in 6-well assays scored on d 16. Data represent 3 independent experiments. *D*) Representative iPSC colonies generated from 1° MEFs transduced with the 4F virus and shCon (top) or shAID3 (bottom) in 6-well assays, stained with alkaline phosphatase. *E*) Nanog staining of a representative iPSC colony generated by Lenti-4F virus in 1° MEFs in the presence of shCon; Nanog staining (red) and nuclear Hoechst staining (blue). Scale bar = 200 μm. *F*) Scheme for iPSC generation from 2° MEFs. shCon or shAID was transduced concomitant with doxycycline-induced expression of 4F and Nanog$^+$ iPSC colonies were analyzed in 6-well assays. *G*) Nanog$^+$ iPSC colonies obtained from 2° MEFs transduced with shCon or shAID (3 or 4) concomitant to doxycycline induction of 4F, scored on d 16. Data represent 3 independent experiments. ***$P < 0.001$; 2-tailed Student's t test.

AID-KO MEFs, shRNA-mediated acute loss of function in WT MEFs greatly diminished reprogramming efficiency (Supplemental Fig. S3*B*). These experiments provided the first evidence that AID is required for the reprogramming of fibroblasts to iPSCs.

The requirement for AID in reprogramming to iPSCs was further validated using a second reprogramming scenario, the 2° MEFs (1). The secondary system is advantageous, as the stress of viral load on the MEFs is reduced, since the 4F virus is omitted, and only a single lentiviral infection of shCon or shAID is required. In addition, the secondary system rules out any adverse effects of the simultaneous transduction with the shRNA and 4F virus. Knockdown largely abrogated reprogramming in the 2° MEFs, and iPSC colonies were extremely rare in 3 independent experiments (Fig. 2*F*, *G*). These experiments confirm the findings in 1° MEFs (Fig. 2*C*) and further demonstrate a critical role for AID in the reprogramming of fibroblasts to iPSCs.

To ensure that the block in reprogramming was specific to loss of AID function, we performed rescue experiments. For this purpose, initially the WT functional AID was overexpressed. To ensure that AID was expressed prior to shRNA knockdown of endogenous AID, we transduced MEFs with AID (or mock) 1 d before transducing them with shRNAs (shAID3 or shCon) and the 4F virus (**Fig. 3*A***). A representative Nanog$^+$ iPSC colony obtained in the rescue experiments is shown (Fig. 3*B*). Despite the increased viral load, which was similar for all samples, these experiments clearly showed that in the presence of functional AID, reprogramming efficiency was restored in 2 replicate experiments, showing a statistically significant rescue of the AID loss of function (Fig. 3*C*).

To determine whether the deaminase function of AID was critical to its effects in reprogramming to iPSCs, we introduced a catalytic mutant, AID(E58Q) (Fig. 3*A*). This mutant was previously used in B lymphocytes and shown to abrogate the function of AID in class switch recombination, which requires its deaminase activity (29). AIDwt led to a 100 ± 10-fold increase, and AID(E58Q) led to a similar 105 ± 16-fold increase in AID gene expression levels, assayed by real-time PCR. Despite similar expression levels, in contrast to WT AID, no rescue of reprogramming was observed when the deaminase mutant of AID, AID(E58Q), was expressed (Fig. 3*C*), showing that the deaminase activity is essential to its function in reprogramming to iPSCs (Fig. 3*C*).

We hypothesized that AID is required only during a brief time window at the onset of reprogramming to iPSCs. Accordingly, we designed experiments to identify the time window of AID requirement (**Fig. 4*A***). The shRNAs targeting AID were delivered to MEFs at different times after initiating the reprogramming process. As shown in Fig. 4*A*, the 4F virus was introduced into the MEFs, and an AID-targeting shRNA (shAID3), or shCon was introduced either simultaneously with the 4F virus on d 0 (as in prior experiments) or on d 3 or 5 after transduction with the 4F virus. Notably, AID was required only during the earliest interval, immediately following transduction with the 4F virus. Indeed, when AID was knocked down 3 or 5 d after the initiation of reprogramming, there was no discernible effect on iPSC frequency (Fig. 4*B*). These data show that AID is required only at the initiation of reprogramming to iPSCs.

DISCUSSION

Our studies provide novel insights into an early regulatory step required for iPSC reprogramming. Specifically we show that AID activity is necessary for reprogramming to iPSCs, as acute loss of function at the onset of 4F delivery significantly impairs reprogramming (Fig. 4*C*). Two different systems for inducing pluripotency, 1° and 2° MEFs, show a marked reduction in colony formation in the absence of AID. Moreover, the loss of function was mediated by 4 shRNAs to different AID sequences. The block in reprogramming was specific, as it could be rescued by expression of WT but not mutant AID lacking the catalytic residue essential to its

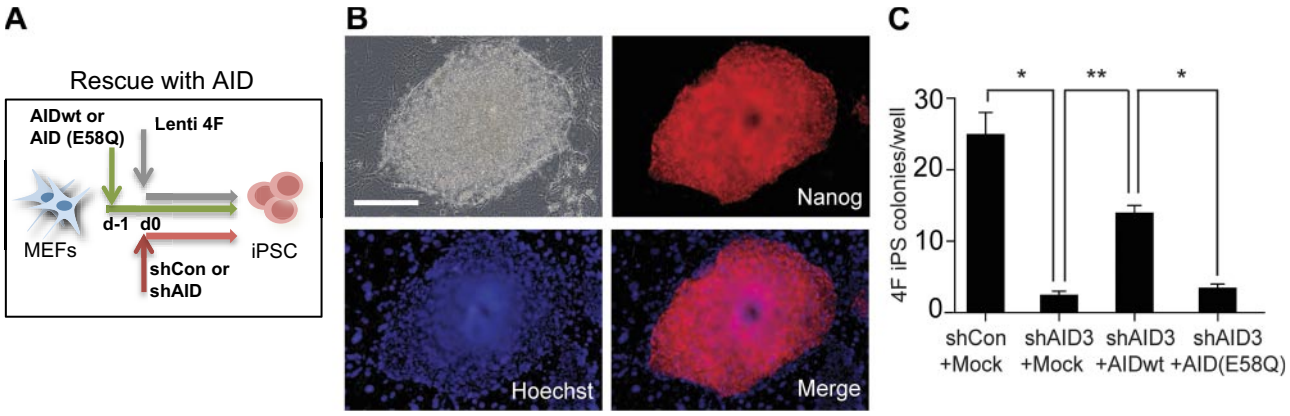

Figure 3. AID loss of function is rescued by overexpression of WT but not deaminase mutant AID. *A*) Scheme for rescue of acute loss of AID by overexpression of AIDwt or a deaminase mutant, AID(E58Q). Primary MEFs were infected with mock (empty vector control) or AIDwt or AID(E58Q) 1 d before transduction with shRNA (shCon or shAID3) lentivirus and the Lenti-4F virus. Nanog$^+$ iPSC colonies were analyzed in 6-well assays. *B*) A representative Nanog-stained iPSC colony generated by the rescue of the effect of shAID3 by AID overexpression (d 16); Nanog staining (red) and nuclear Hoechst staining (blue). Scale bar = 200 μm. *C*) Nanog$^+$ iPSC colonies obtained from 1° MEFs transduced with mock or AIDwt or AID(E58Q) scored at d 16. Data represent 2 independent experiments. $^*P < 0.05$, $^{**}P < 0.01$; 2-tailed Student's *t* test.

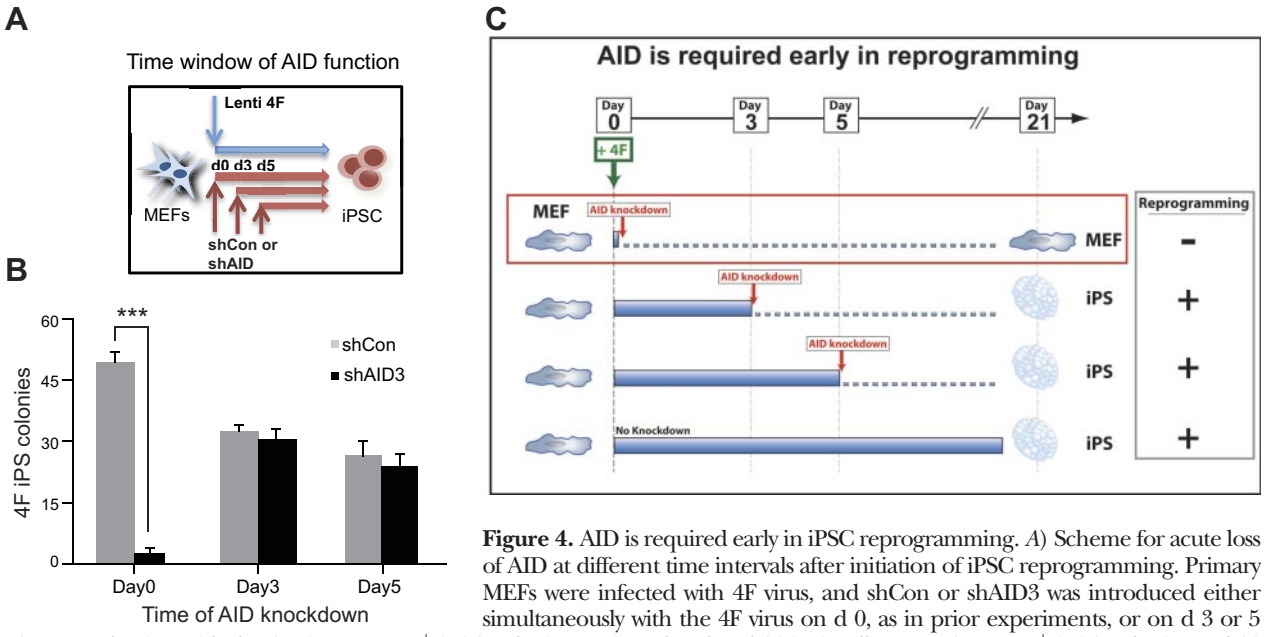

Figure 4. AID is required early in iPSC reprogramming. *A*) Scheme for acute loss of AID at different time intervals after initiation of iPSC reprogramming. Primary MEFs were infected with 4F virus, and shCon or shAID3 was introduced either simultaneously with the 4F virus on d 0, as in prior experiments, or on d 3 or 5 after transduction with the 4F virus. Nanog$^+$ iPSC colonies were analyzed at d 16 in 6-well assays. *B*) Nanog$^+$ iPSC colonies at d 16 obtained from 1° MEFs transduced with 4F at d 0, concomitant with shCon or shAID3 introduced at d 0, 3, or 5. Data represent 4 independent experiments. ***$P < 0.001$; 2-tailed Student's t test. *C*) Model for AID action in iPSC reprogramming. Acute loss by shRNA to AID inhibits iPSC reprogramming revealing a critical role for AID. AID function is required at the onset of reprogramming, as acute loss at 3–5 d after initiation has no effect.

function as a deaminase (29). In addition, the loss of reprogramming potential was not observed in AID-KO MEFs, suggesting that compensation by other molecules and enzymes may have occurred during development. In support of this hypothesis, a lack of phenotype has also been reported for genetic elimination of single members of other protein families (26–28). For example, an acute loss of the Rb protein was required to reveal a phenotype that was not apparent in genetically null Rb cells due to compensation by the family members p107 and p130 (28).

Recently, insights into the mechanisms of mammalian DNA demethylation have increased substantially. Our initial study in nondividing heterokaryons identified AID as critical for demethylation of Oct4 and Nanog promoters (14). Later studies by others suggested that AID may act in concert with the TET enzymes, such that 5hmC is converted to 5hmU by deamination and then excised by TDG in neuronal cells (15, 16). Recent biochemical studies have revealed a 10-fold higher deamination activity of AID for 5mC relative to 5hmC, suggesting that AID may act independently of TET (30). A potential caveat of these *in vitro* biochemical studies is that they may not fully reflect the action of AID *in vivo*. *In vitro* studies lack the context of the intact cell that may provide essential cellular cofactors, post-translational modifications, and compartmentalization not included when using short DNA substrates and purified enzymatic components. Concrete evidence for DNA demethylation is particularly difficult to establish in iPSCs, since unlike nondividing heterokaryons (14), the time course of reprogramming is asynchronous, and the frequency of reprogramming is very low (1, 2, 4, 5). Therefore promoter demethylation analyses using techniques such as bisulfite sequencing or chromatin immunoprecipitation would largely reflect the unsuccessfully reprogramming population (>95%) and be uninformative. As a result, we cannot exclude alternative consequences of deamination by the AID/APOBEC family, such as RNA editing or deamination of 5mC prior to hydroxylation by the TET family (31). Thus, although AID deaminase activity is required at the onset of reprogramming to iPSCs, whether AID has a role in DNA demethylation in iPSCs remains to be determined.

The cumulative data presented here demonstrate that in the absence of AID, the initiation of reprogramming of fibroblasts to iPSCs is inhibited and the frequency greatly diminished. Since a role for AID in reprogramming toward pluripotency was first shown in heterokaryons, this finding underscores the possibility that molecular regulators of iPSC reprogramming can be elucidated by utilizing this alternative reprogramming approach (3). Our study shows that AID is a common early regulator not only in cell fusion-based reprogramming (14) but also in reprogramming to iPSCs, suggesting that the different approaches to reprogramming are at least in some respects mechanistically similar. We postulate that an identification of such early regulators will not only increase our understanding of the mechanisms underlying reprogramming but also increase the efficiency of iPSC generation. The relatively modest 2-fold increase in reprogramming efficiency on AID overexpression suggests that AID is not a rate-limiting factor, but rather acts in conjunction with other molecules, such as factors that have been implicated in the mammalian DNA demethylation pathway like TDG (32, 33) or as yet unidentified factors. Elucidation of these additional enzymes or regulators, their mechanistic interactions, and their role in nuclear reprogramming to pluripotency will be of great interest. FJ

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