Gadd45a is a heterochromatin relaxer that enhances iPS cell generation

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Abstract

Reprogramming of somatic cells to induced pluripotent stem cells rewrites the code of cell fate at the chromatin level. Yet, little is known about this process physically. Here, we describe a fluorescence recovery after photobleaching method to assess the dynamics of heterochromatin/euchromatin and show significant heterochromatin loosening at the initial stage of reprogramming. We identify growth arrest and DNA damage-inducible protein a (Gadd45a) as a chromatin relaxer in mouse embryonic fibroblasts, which also enhances somatic cell reprogramming efficiency. We show that residue glycine 39 (G39) in Gadd45a is essential for interacting with core histones, opening chromatin and enhancing reprogramming. We further demonstrate that Gadd45a destabilizes histone–DNA interactions and facilitates the binding of Yamanaka factors to their targets for activation. Our study provides a method to screen factors that impact on chromatin structure in live cells, and identifies Gadd45a as a chromatin relaxer.

Keywords chromatin relaxer; FRAP; Gadd45a; heterochromatin relaxation; reprogramming

Subject Categories Chromatin, Epigenetics, Genomics & Functional Genomics; Stem Cells

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Introduction

Reprogramming somatic cells to induced pluripotent stem cells (iPSCs) by defined factors opened up many interesting avenues for biology and medicine. For example, it is feasible to generate patient-specific iPSCs that can be used to model diseases for drug

developments or generate functional cells to treat illness through cell transplantation [1,2]. Yet, recent reports have suggested that iPSCs generated with current technologies may have potential problems in clinical applications due to genome integration and immunogenicity [3,4]. Thus, further refinement and improvement of current reprogramming methods may mitigate some of those concerns.

Reprogramming is an epigenetic process. For example, DNA demethylation enzymes such as the Tet family have been shown to play a critical role in the reestablishment of pluripotency [5–7]. Likewise, histone modification enzymes or small molecule inhibitors/activators for these enzymes have been shown to regulate reprogramming efficiency [8–11]. At the chromatin level, remodeling factors such as Brg1 and Baf155 have been shown to promote reprogramming [12], whereas other modifying enzymes such as Dot1l or Mbd3 have been shown to inhibit reprogramming [13,14]. These insights not only enhanced our understanding of reprogramming, but also provided means to improve the reprogramming process. At the cellular level, mouse embryonic fibroblasts (MEFs) appear to undergo a mesenchymal-to-epithelial transition (MET) at the early phase of reprogramming [15,16]. Beyond the MET, there is an intermediate state called pre-iPSCs [17,18], which can be further converted to fully reprogrammed iPSCs by vitamin C (Vc) apparently through histone demethylases specific for H3K9me3 [19]. Therefore, factors capable of modulating the epigenetic state may be beneficial to reprogramming.

iPSCs, like ESCs, have less condensed heterochromatin foci and hyperdynamic chromatin proteins compared to MEFs [20,21]. Therefore, reprogramming of MEFs into iPSCs must undergo a gradual loosening or relaxation of the tightly packed heterochromatin in MEFs. It has been reported that fluorescence recovery after photobleaching (FRAP) can be used to analyze the dynamic interaction among chromatin proteins [22,23]. Here in this report, we describe a FRAP-based method to analyze heterochromatin and euchromatin

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dynamics during reprogramming and the identification of Gadd45a as a powerful chromatin relaxer that also enhances reprogramming robustly.

Results

FRAP reveals heterochromatin dynamics in the early phase of somatic cell reprogramming

Heterochromatin has been shown to be the main difference between somatic and pluripotent stem cells and also one of the major barriers for reprogramming [24–26]. To understand the dynamic changes in heterochromatin during reprogramming, we took advantage of a previously reported live cell imaging method to detect chromatin dynamics called FRAP [22,23] and made several critical modifications such that we can monitor and quantify euchromatin and heterochromatin remodeling dynamics. First, we labeled heterochromatin protein 1a (HP1a) with mCherry and chromatin-associated protein Histone1 (H1) with green fluorescent protein (GFP) (Fig 1A). HP1a-mCherry should allow us to distinguish between heterochromatin and euchromatin by selecting the region of interest (ROI) within (heterochromatin) or outside (euchromatin) HP1a foci; meanwhile, H1-GFP has been widely used in FRAP analysis of chromatin [27]. We also developed FRAP of H1-GFP in these ROIs to analyze the dynamics of heterochromatic and euchromatic H1, respectively (Fig 1A). We recorded FRAP curves for a period of 120 s after bleaching and analyzed the mobile fraction (MF), which refers to the recovery ratio in 120 s post-bleaching. This method allows a concise determination of heterochromatin and euchromatin dynamics by sequential HP1a region selection and H1 FRAP.

We then performed FRAP analysis and found that only heterochromatin, not euchromatin, exhibits greater MFs in cells undergoing reprogramming (SKO-D3) than in control MEFs (Flag-D3) (Fig 1B and C), suggesting that heterochromatin undergoes a relaxation process at the early phase of reprogramming. We then quantified heterochromatin with HP1a stain and showed that it overlaps with 4', 6-diamidino-2-phenylindole (DAPI) staining (Fig EV1A and B). Interestingly, in MEFs undergoing SKO or Sox2, Klf4, Oct4, c-Myc (SKOM) reprogramming, we observed a dramatic decrease in heterochromatin at the very early phase of reprogramming (day 3) (Fig EV1A–D), a known feature characteristic of pluripotent stem cells [28,29]. We also performed immunofluorescence *in situ* hybridization (immuno-FISH) to map the endogenous *Oct4* locus and HP1a foci in MEFs infected with SKO or SKOM. While the *Oct4* loci overlapped with HP1a foci in control MEFs, no such association was found between them in MEFs undergoing SKO or SKOM reprogramming (Figs 1D and E, and EV1E and F). Taken together, our results demonstrate that heterochromatin undergoes significant relaxation during early stages of reprogramming.

FRAP as a tool to identify heterochromatin relaxers

As heterochromatin relaxes during the conversion of somatic cells into iPSCs, we wish to employ FRAP to screen for factors that can relax heterochromatin and perhaps enhance reprogramming also. We firstly tested six chemicals or protein factors with FRAP in MEFs: valproic acid (VPA) as positive control [27], protein factors

(p53, p21 and Gadd45a) in cell cycle regulation [30–32], and Jumonji family proteins (Jhdmlb and Utx) with the ability to enhance reprogramming [9,10,33] (Fig EV2A). VPA is a histone deacetylase inhibitor known to enhance heterochromatin dynamics and somatic cell reprogramming [27,34], and thus serves as a positive control. Interestingly, we discovered that Gadd45a also increases heterochromatin dynamics (Figs 2A and EV2A). In contrast, p53 and p21, which have been reported to inhibit reprogramming [35–37], suppress heterochromatin dynamics (Fig EV2A and B). On the other hand, Jhdmlb and Utx, which have been reported to facilitate reprogramming [9,10,33], surprisingly, have no effect on heterochromatin dynamics (Fig EV2A and B).

We then showed that the H1 dynamics in MEFs gradually decreases during culture from day 3 to day 10 perhaps due to cell senescence and can be reversed by Gadd45a (Fig EV2C and D). It appears that both heterochromatin and euchromatin are affected by Gadd45a under the same experimental settings (Fig EV2E). To further understand the role of Gadd45a on chromatin density status, we performed chromatin immunoprecipitation (ChIP) assays with antibodies targeting H3K9Ac, H3K9me2, H3K9me3, H3K27Ac, H3K27me2, and H3K27me3 at the promoters of the pluripotency genes *Oct4*, *Nanog*, and *Sox2* in MEFs infected with Gadd45a. Overexpression of Gadd45a could increase the H3K9Ac and H3K27Ac levels and reduce the H3K9me2/3 and H3K27me2/3 levels (Appendix Fig S1). Methylation of H3K9 and H3K27 is thought to be a marker for heterochromatin [24], and H3K27 methylation could be established by dense chromatin [38]. Histone acetylation is always associated with active gene promoters and transcription [39]. Taken together, our results suggest that Gadd45a regulates chromatin density and can relax it significantly.

Gadd45a relaxes heterochromatin during reprogramming

Based on the ability of Gadd45a to relax both heterochromatin and euchromatin in MEFs (Figs 2A and EV2A and E), we next tested whether Gadd45a could further relax chromatin during reprogramming. We co-infected MEF cells with Gadd45a and the reprogramming factors SKO, and found that Gadd45a increases heterochromatin H1 dynamics by FRAP, though it has no further effect on euchromatin dynamics (Figs 2B and EV2F). ChIP assays with antibodies targeting H3K9Ac, H3K9me2, H3K9me3, H3K27Ac, H3K27me2, and H3K27me3 during reprogramming with SKO or SKO plus Gadd45a on day 3 of reprogramming were performed to show an enhancement of the H3K9Ac and H3K27Ac levels and a reduction of the H3K9me2, H3K9me3, H3K27me2, and H3K27me3 levels at the promoters of the pluripotency genes *Oct4*, *Nanog*, and *Sox2* in the presence of Gadd45a (Fig 2C). These results suggest that Gadd45a dynamically regulates modifications of H3K9 and H3K27, that is, acetylation enhancement and methylation reduction, during reprogramming. Based on these observations, we further conclude that Gadd45a relaxes heterochromatin in reprogramming cells.

Gadd45 proteins enhance reprogramming

We next ask whether Gadd45a enhances reprogramming. To this end, we co-infected MEFs containing a transgenic *Oct4* promoter driving GFP expression (OG2 MEFs), with Gadd45a and the

Figure 1. Heterochromatin loosening in the early phase of somatic cell reprogramming.

A Scheme of H1-GFP FRAP in heterochromatin foci (with HP1a-mCherry) and euchromatin (without HP1a-mCherry). The relative FRAP curves of euchromatin (Eu) and heterochromatin (Het) are shown divided into two parts—the mobile fraction (MF) and the immobile fraction (IF) after bleaching recovery. ROI, region of interest.

B FRAP curves of H1-GFP in reprogramming with SKO. The mean values of relative fluorescence recovery are shown in the curves. For heterochromatin, the recovery ratio is higher in cells transfected with reprogramming factors than that in the control on day 3. Changes are significantly different. More than 16 cells were analyzed for each group. * $P \leq 0.05$.

C The ratio of MF at 120 s post-bleaching in FRAP was compared in the reprogramming stages. For heterochromatin, cells transfected with SKO showed much more rapid recovery than control cells (Flag) on day 3. More than 16 cells were analyzed for each group. * $P \leq 0.05$.

D DNA FISH images showing the localizations of endogenous *Oct4* locus and HP1a foci in MEFs infected with SKO or Flag control. More than 72 cells were analyzed for each group. Scale bar: 5 μ m.

E Summary of percentage of co-localization between the *Oct4* locus and HP1a foci in SKO-mediated reprogramming. More than 72 cells were analyzed for each group.

Data information: In (B), data are presented as mean value; in (C), data are presented as mean $\pm$ SEM. P -values were calculated using an unpaired two-tailed Student's t -test.

Figure 2. Gadd45a as a heterochromatin relaxer that enhances reprogramming.

A The recovery kinetics of heterochromatin in MEFs infected with Flag or Gadd45a on day 3. The ratio of MF at 120 s post-bleaching is shown at the right panel. More than 20 cells were analyzed for each group. *** $P \leq 0.001$.

B The recovery kinetics of heterochromatin in MEFs infected with Flag alone or SKO plus Flag or Gadd45a on day 3. The ratio of MF of heterochromatin is shown at the right panel. More than 19 cells were analyzed for each group. * $P \leq 0.05$.

C ChIP-PCR analysis of H3K9Ac, H3K9me2, H3K9me3, H3K27Ac, H3K27me2, or H3K27me3 modification levels in Oct4 binding sites of MEFs infected with SKO plus Flag or Gadd45a on day 3. ** $P \leq 0.01$; $n = 3$.

Data information: In (A and B), data are presented as mean $\pm$ SEM; in (C), data are presented as mean $\pm$ SD. P -values were calculated using an unpaired two-tailed Student's t -test.

reprogramming factor combinations SKO/SKOM. The GFP-positive colonies were counted in SKO or SKOM reprogramming, respectively. The normalized relative reprogramming efficiency was calculated as reported [40] and shown in Fig 3A. We found that Gadd45a can improve the efficiency of reprogramming (Fig 3A). The resulting iPSC clones were characterized for the expression of several pluripotent markers (Appendix Fig S2A–E), and for their abilities to generate chimeric mice capable of germline transmission (Appendix Fig S2F).

The Gadd45 family comprises three members, Gadd45a, Gadd45b and Gadd45g, which have several conserved domains and similar functions [41,42]. Although the expression level of these three genes in reprogramming cells was similar to that in control MEFs (Appendix Fig S3A), all of them could greatly promote reprogramming (Fig 3A), but had no additive effects, indicating that they function in the same way (Fig 3B). We further showed that Gadd45a could not substitute Sox2, Klf4, or Oct4 among the reprogramming factors in generating iPSCs (Appendix Fig S3B).

To test whether endogenous Gadd45 proteins are required for reprogramming, we knocked down the three Gadd45 proteins using shRNAs (Fig EV3A). The reprogramming efficiencies, H1 dynamics, and pluripotency gene expression were not affected by neither individual nor combined knockdown them (Fig EV3B–D), suggesting paradoxically that either Gadd45 proteins are not the endogenous factors mediating heterochromatin relaxation or any residual Gadd45a might be sufficient during reprogramming.

We then investigated the time window of sensitivity to Gadd45a with a doxycycline (DOX)-inducible system and showed that Gadd45a is effective in the early and middle stages of reprogramming. Specifically, we showed that in SKO-mediated reprogramming, Gadd45a is effective between days 2 and 14, whereas in SKOM-mediated reprogramming, between days 2 and 8. When DOX was added at different time points, we found that Gadd45a does not have any effect on both SKO- or SKOM-mediated reprogramming after day 11 (Fig 3C). These results suggest that Gadd45a functions in a time-dependent manner to promote reprogramming at the early and middle stages when heterochromatin remodeling occurs.

Gadd45a G39 residue is crucial for both reprogramming and heterochromatin relaxation

The Gadd45 proteins are stress inducible and involved in multiple biological processes, such as cell cycle, senescence, tumor progression, DNA repair and active DNA demethylation [43–45]. To better understand the roles of Gadd45a in heterochromatin relaxation, we constructed and tested a series of point mutants of Gadd45a in somatic cell reprogramming. According to previous reports, residues G39 and K45 are conserved among three Gadd45 proteins and are critical for Gadd45 proteins to bind RNAs [46]. We designed substitutions for G39 with alanine, K45 with glutamate, and also a non-conservative residue R34 with glycine (Fig EV4A), which has no effects on structure [47]. We then showed that the R34G and K45E mutants had no effect on the functions of Gadd45a in both heterochromatin and euchromatin dynamics, as well as reprogramming (Fig 4A, B and E, and Appendix Fig S4A). However, the G39A mutant lost the ability to relax heterochromatin and euchromatin according to FRAP (Fig 4A and B, and Appendix Fig S4A). We further showed that G39A Gadd45a is no longer able to reduce the

HP1a foci as effectively as wild-type Gadd45a (Fig 4C), consistent with the FRAP results. To further confirm the FRAP result, we performed immuno-FISH to map the endogenous *Oct4* loci and HP1a foci in MEFs infected with wild-type or G39A Gadd45a. We observed that there was no association between the *Oct4* loci and HP1a foci in MEFs infected with wild-type Gadd45a. However, the *Oct4* loci remained co-localized with HP1a in MEFs infected with G39A Gadd45a, as in controls (Figs 4D and EV4B). Together, we conclude that G39 is required for Gadd45a to relax chromatin.

Next, we showed that G39A Gadd45a is no longer able to further loosen heterochromatin during reprogramming (Appendix Fig S4B). The increase of H3K9Ac and H3K27Ac and the reduction of H3K9Me2/3 and H3K27Me2/3 by wild-type Gadd45a were also impaired by G39A mutation (Fig EV4C). Consequently, G39A Gadd45a could not enhance reprogramming by SKO or SKOM (Fig 4E). These results indicate that G39 is critical for Gadd45a to relax heterochromatin and enhance reprogramming.

Gadd45a destabilizes histone–DNA interactions and facilitates binding of Yamanaka factors to their targets via G39

To explore the role of Gadd45a and its G39 residue in histone–DNA interaction, we purified recombinant wild-type Gadd45a and G39A Gadd45a proteins (Fig EV5A and B) and performed electrophoretic mobility shift assay (EMSA) with several probes amplified from the *Oct4* promoter (Appendix Fig S5A). As expected, the addition of core histones alone retarded the oligomer mobility (Fig 5A). Gadd45a did not change the mobility of the probes in the absence of core histones, indicating that Gadd45a does not interact directly with DNA (Fig 5A). In the presence of core histones, Gadd45a increased the mobility of DNA and counteracted the effect of histones (Fig 5A and Appendix Fig S5B–D), consistent with the observed interruption of the core histones and double-stranded DNA by Gadd45 [48]. On the other hand, G39A Gadd45a was inactive in the same assay system (Fig 5A and Appendix Fig S5B–D). We also tested a half-length probe compared with the previous probes and obtained similar results (Appendix Fig S5E). As both the *CMV* promoter and the *EGFP* gene showed the same results, Gadd45a appears to regulate the interactions between DNA and histones in a DNA sequence-independent manner (Appendix Fig S5F and G). Since core histones prepared by high-salt extraction may contain unknown nuclear components, we decided to repeat the same experiments with purified recombinant histones. To determine which protein Gadd45a acts on, we simply used the H2A/H2B heterodimer and the H3/H4 tetramer to perform EMSA (Fig 5B). We showed that the wild-type Gadd45a, but not the G39A mutant, blocked the mobility shift of the DNA in the presence of either the H2A/H2B heterodimer or the H3/H4 tetramer (Fig 5B).

We then performed ChIP-PCR and co-immunoprecipitation assays to characterize the interaction between chromatin and Gadd45a in living cells. As previously reported [48], we showed by co-immunoprecipitation that wild-type Gadd45a, not the G39A mutant, interacts with core histone H3 (Fig 5C). We then performed ChIP with two-step cross-linking, which use disuccinimidyl glutarate (DSG) to cross-link Gadd45a with core histones before ChIP-PCR [49]. We further showed that wild-type Gadd45a, but not G39A mutant, binds to the promoter regions of *Oct4* and *Nanog* (Figs 5D and EV5C and D).

Figure 3. Gadd45 proteins enhance reprogramming.

A Gadd45 proteins significantly improve reprogramming efficiency. The reprogramming efficiencies were compared with SKO or SKOM, respectively. We normalized the numbers of SKO+Factor to SKO+Flag (1 as control); also we normalized the numbers of SKOM+Factor to SKOM+Flag (1 as control). $^{**}P \leq 0.01$; $^{***}P \leq 0.001$; $n = 3$.

B The efficiencies of SKO- and SKOM-induced reprogramming were tested in overexpression of two or three Gadd45 proteins. The reprogramming efficiencies were compared with SKO or SKOM, respectively. $^{**}P \leq 0.01$, $^{***}P \leq 0.001$; $n = 3$.

C SKO-MEFs or SKOM-MEFs infected with DOX-inducible Gadd45a were either treated with DOX immediately after infection, and DOX was removed at different time points (left panel) or treated with DOX from different time points until the end of the experiment (right panel). The reprogramming efficiencies were compared with DOX-free treatment ($n = 3$).

Data information: In (A–C), data are presented as mean $\pm$ SD. P -values were calculated using an unpaired two-tailed Student's t -test.

Source data are available online for this figure.

Figure 4.

Figure 4. Mutational analysis of Gadd45a on heterochromatin dynamics and reprogramming.

- A Several Gadd45a mutations were tested revealing that only the G39A mutation lost the ability to increase the H1 dynamics of heterochromatin. More than 18 cells were analyzed for each group. *** $P \leq 0.001$.
- B The ratio of MF increased with wild-type Gadd45a or K45E and R34G Gadd45a, but not G39A Gadd45a at day 3. More than 18 cells were analyzed for each group. *** $P \leq 0.001$.
- C Immunofluorescence detection of HP1a foci in MEFs transfected with wild-type Gadd45a or G39A Gadd45a (upper panel). Quantitative analysis of the ratio of the HP1a foci area to the total nuclear area revealed by DAPI staining (dashed lines) shows that wild-type Gadd45a reduces the relative area of HP1a foci, whereas the G39A Gadd45a does not (lower panel). More than 20 cells were analyzed for each group. Scale bar: 5 μm . ** $P \leq 0.01$.
- D Immuno-FISH images showing the localizations of endogenous *Oct4* loci and HP1a foci in MEFs infected with wild-type or G39A Gadd45a. More than 72 cells were analyzed for each group. Scale bar: 5 μm .
- E Comparison of the reprogramming efficiencies of SKO-MEFs and SKOM-MEFs overexpressing wild-type or mutant Gadd45a proteins. The reprogramming efficiencies were compared with SKO or SKOM, respectively. ** $P \leq 0.01$, *** $P \leq 0.001$; $n = 3$.

Data information: In (A), data are presented as mean (SEM is not shown); in (B and C), data are presented as mean $\pm$ SEM; in (E), data are presented as mean $\pm$ SD. P -values were calculated using an unpaired two-tailed Student's t -test.

Source data are available online for this figure.

By disrupting the histone–DNA interactions, Gadd45a could potentially change the chromatin status and modify DNA accessibility. To test this idea, we analyzed the nuclease accessibility to endogenous *Oct4* and *Nanog* promoter regions and several binding sites of exogenous reprogramming factors during reprogramming. We showed that MEFs infected with SKO factors display an open structure in all the studied regions compared with MEFs infected with the Flag control (Fig 5E and Appendix Fig S6). We then showed that Gadd45a, but not its inactive G39A mutant, further increases the nuclease access (Fig 5E and Appendix Fig S6).

We then wished to test whether Gadd45a facilitates the binding of the Yamanaka factors *Oct4*, *Sox2*, and *Klf4* to their targets during reprogramming. By performing ChIP-PCR to assess the factor binding properties, we showed that on day 8 all three factors bound to their targets more readily when co-expressed with wild-type Gadd45a, but not G39A Gadd45a (Fig 5F). Consistently, a recent study has shown that chromatin opening by chromatin relaxers such as CAF-1 could promote binding of reprogramming factors to their targets [25]. Together, our data demonstrate that Gadd45a, via G39, disrupts histone–DNA interactions, opens heterochromatin, and enhances the binding of reprogramming factors to their targets.

Gadd45a activates pluripotency genes

To investigate the genes regulated by Gadd45a through chromatin relaxation during reprogramming, we analyzed the gene expressions in MEF cells undergoing reprogramming transfected with wild-type Gadd45a or G39A. Microarray assays were performed twice and the common data showed that Gadd45a promotes the expression of 335 genes involved in embryo development (such as *NF2* and *Spr1a*), germ cell development (such as *Rnf17* and *Ddx25*), amino acid modification (such as *Metap2* and *Map6d1*), among many (Fig 6A and Appendix Fig S7A and B). On the other hand, Gadd45a also appears to inhibit the expression of 96 genes involved in neurotransmitter transport (such as *Gad2* and *Trim9*) and several metabolic processes (such as *Stat5b* and *Cyt11*) (Appendix Fig S7A and B, and S8A). We also showed that pluripotency maintenance genes are upregulated by Gadd45a during reprogramming (Fig 6B). We then confirmed the expression of these pluripotency genes by qPCR and showed that whereas wild-type Gadd45a enhances the expression of pluripotency genes such as endogenous *Oct4*, *Nanog*, and *Sox2*

during reprogramming, G39A Gadd45a does not (Fig 6C and Appendix Fig S8B). The higher pluripotency gene expression could be the cause or the consequence of improved reprogramming. Of note, *Jhdm1b*, a factor that could greatly enhance reprogramming efficiency [9], does not promote the expression of pluripotency genes under similar settings (Appendix Fig S8C).

Discussion

In this report, we present a live cell imaging method, FRAP, to measure the dynamics of heterochromatin/euchromatin in cells undergoing reprogramming and demonstrated significant heterochromatin relaxation at the initial stage of reprogramming. Furthermore, by using the adapted FRAP as a screening tool, we identified Gadd45a as an unexpected heterochromatin relaxer as well as reprogramming enhancer. Similarly, it has been reported that the histone chaperone CAF-1 promotes reprogramming as well as increases chromatin dynamics [25]. The chromatin remodelers, such as *Brg1*/*BAF155* [12], *INO80* [50], have been also reported to be able to promote reprogramming. Thus, heterochromatin dynamics may serve as a critical process in regulating the initiating phase of somatic cell reprogramming.

Chromatin structure and dynamics can be analyzed by a number of methods, including electron microscopy [51] and ChIP-seq [52,53]. However, neither can be performed on live cells. In this study, we developed the live cell imaging method, FRAP, for concisely measuring the dynamics of heterochromatin/euchromatin, which can also be adapted as a screening tool for heterochromatin relaxer. By this method, we tested several factors including p53 and p21 whose overexpression leads to reduction in chromatin dynamics (Fig EV2A and B). Indeed, p53 activation and p21 activation have been reported to modulate cellular senescence, one important function of which is densely stained regions of chromatin [30–32]. FRAP is therefore able to identify not only enhancers but also barriers of reprogramming.

Our finding that Gadd45a is a heterochromatin relaxer capable of enhancing reprogramming may generate further interests mechanistically. As Gadd45a has been reported previously to play important roles in regulating cell cycle, senescence, apoptosis, DNA damage repair, DNA demethylation, and tumorigenesis [43,54,55], one may be intrigued if these reported functions are responsible for the observed impact of Gadd45a on reprogramming. However, we actually showed that Gadd45a inhibits MEF proliferation during

Figure 5. Gadd45a destabilizes histone–DNA interactions and facilitates binding of Yamanaka factors to their targets.

- A Recombinant Gadd45a protein and its G39A mutant were added to the histones–oligo–DNA mixture. The mixtures were separated by 4% non-denaturing PAGE and stained with ethidium bromide (EB). The oligo–DNA probes were amplified from the *Oct4* promoter. “Histone+” means in the presence of core histones.
- B Recombinant wild-type Gadd45a and G39A Gadd45a were added to the mixtures of oligo–DNA and the recombinant H2A/H2B heterodimer or H3/H4 tetramer. The mixtures were separated by 4% non-denaturing PAGE and stained with EB.
- C Co-immunoprecipitation of wild-type Gadd45a, G39A Gadd45a, and H3 shows that the interaction between Gadd45a and H3 is dependent on G39 residue.
- D Two-step cross-linking method identified Gadd45a could interact with chromatin in the promoter regions of *Oct4* and *Nanog*. Cells were cross-linked with DSG before ChIP–PCR analysis. ** $P \leq 0.01$; *** $P \leq 0.001$; $n = 3$.
- E The chromatin compaction of the indicated regions was detected by nuclease accessibility assay. Genomic DNA was purified from MEFs infected with Flag alone and SKO plus Flag, wild-type Gadd45a, or G39A Gadd45a on day 8. * $P \leq 0.05$; ** $P \leq 0.01$; $n = 3$.
- F ChIP–PCR analysis of the binding of Oct4, Sox2, and Klf4 to their targets individually in MEFs infected with SKO plus Flag, wild-type Gadd45a, or G39A Gadd45a on day 8. NC, negative control; *** $P \leq 0.001$; $n = 3$.

Data information: In (D–F), data are presented as mean $\pm$ SD. P -values were calculated using an unpaired two-tailed Student’s t -test. Source data are available online for this figure.

reprogramming (Appendix Fig S9), suggesting that it enhances reprogramming not through accelerating cell proliferation. On the other hand, the relationship between Gadd45 proteins and p53 is inconsistent as cells activate Gadd45a through p53 under ionic irradiation [43,48], yet p53 is a potent inhibitor of reprogramming [35–37] and all Gadd45 proteins have the same effects on reprogramming (Fig 3A). Therefore, it appears that Gadd45a promotes and p53 inhibits reprogramming through different mechanisms.

We did not demonstrate that endogenous Gadd45 proteins are required for reprogramming. We used shRNAs to knockdown Gadd45 proteins and showed that the knockdown has no effect on reprogramming. This could be due to the residual Gadd45 proteins after shRNA-mediated knockdown being sufficient for

reprogramming. As such, only genetic ablation of all three Gadd45 proteins may allow us to address this question in the near future. Nonetheless, our work shows that Gadd45, when overexpressed as an exogenous factor, disrupts histone–DNA interactions, opens up chromatin, and facilitates the binding of the Yamanaka factors to their targets. Gadd45a could be opening chromatin directly by disrupting histone–DNA interactions. There could also be alternative mechanism such as DNA methylation. Indeed, we have analyzed the total DNA methylation and hydroxymethylation levels at the early stage of reprogramming in the presence and absence of Gadd45a. Neither DNA methylation nor hydroxymethylation was affected by Gadd45a (Appendix Fig S10A and B). We also tested for the DNA methylation of the *Oct4* and *Nanog* promoter regions and

Figure 6. Gadd45a facilitates the activation of pluripotency genes.

A Summary of enriched Gene Ontology terms more potently upregulated by Gadd45a. The *P*-values represent the modified Fisher exact corrected EASE score.

B Heatmaps depicting the relative fold change of gene expression at 8 dpi by DNA microarray. Red and green colors indicate increased and decreased expression, respectively.

C qPCR analysis of endogenous *Oct4*, *Nanog*, or endogenous *Sox2* expression level during reprogramming with SKO plus Flag, wild-type Gadd45a, or G39A Gadd45a. ***P* ≤ 0.01, ****P* ≤ 0.001; *n* = 3.

Data information: In (C), data are presented as mean ± SD. *P*-values were calculated using an unpaired two-tailed Student's *t*-test.

found no obvious differences among GFP, SKO, and SKO plus Gadd45a at day 8 (Appendix Fig S10D). Besides, we tried to overexpress XPB and XPG, which were reported to be the DNA repair endonucleases required for Gadd45a to demethylate DNA [55,56], as well as TAF12, that could recruit Gadd45a and the nucleotide excision repair proteins (such as XPG) to demethylate the promoter of rRNA genes [45], and showed that these proteins are unable to enhance reprogramming (Appendix Fig S10C). Further studies would be needed to clarify the mechanism.

Interestingly, G39 has been reported to be a critical amino acid for the function and localization of Gadd45a [46]. Exchange of glycine residue at this position for alanine would lead to considerable steric clashes such as RNA binding defect. However, K45E Gadd45a still enhances reprogramming despite its inability to bind RNAs, indicating that the two properties of Gadd45a can be uncoupled. By analyzing the crystal structure of Gadd45a *in silico*, dimerization of Gadd45a does not seem to be affected by G39A [47]. This raises the possibility that G39A interferes with some other important property of Gadd45a, for example, interaction with core histones to loosen heterochromatin in our experiments.

Finally, heterochromatin dynamics could be a universal process in cell fate transitions. Factors identified by FRAP screening might affect not only somatic cell reprogramming, but also other cell fate transitions. To this end, we have shown that Gadd45a could relax heterochromatin in human fibroblasts (Appendix Fig S11). We believe that further exploration of factors that can impact heterochromatin dynamics with FRAP as shown here can lead to better practice and understanding of cell fate reprogramming.

Materials and Methods

DNA constructs, cell lines, and cell culture

All expression vectors were based on the retroviral pMX backbone as described [9]. The inducible Gadd45a was cloned into the pRLenti plasmid [40]. The shRNAs: shGadd45a (5'-ATGGCATCCGAATGGAAATAA-3'); shGadd45b (5'-TGAAGAGAGCAGAGGCAATAA-3'); shGadd45g (5'-GATCGACTTGGTGACACTCTA-3'), were cloned into pSuper plasmids [9]. Point mutation constructs were generated with pMXs-Gadd45a as the template by using synthetic oligonucleotides: For R34G, primers were forward (5'-CAAGGCTCGGAGTCAGGGCACCATTACGGTCGGCGTGT-3') and reverse (5'-ACACGCCGACCGTAATGGTGCCTGACTCCGAGCCTTG-3'). Primers for G39A were as follows: forward (5'-ACCATTACGGTCGCCGTGTACGAGGCTGCCAA-3') and reverse (5'-TTGGCAGCCTCGTACACGGCGACCGTAATGGT-3'); primers for K45E were as follows: forward (5'-GGTCGGCGTGTACGAGGCTGCCGAGCTGCTCAACGTAGACCCGATAACGTGGTA-3') and reverse (5'-TACCACGTTATCGGGGTCTACGTTGAGCAGCTCGGCAGCCTCGTACACGCCGACC-3').

OG2 MEFs were derived from E13.5 embryos that carry the Rosa26-lacZ allele and a transgenic *Oct4* promoter driving GFP expression and used for reprogramming within two passages as described [8]. MEFs and plat-E cells were maintained in DMEM/high glucose supplemented with 10% fetal bovine serum (FBS) (Hyclone). Mouse ESCs and iPSCs were maintained in a media containing DMEM/knockout (Gibco), 15% KSR (Gibco), NEAA (Gibco), GlutaMax (Gibco), and LIF with feeder cells as described

[8]. Human skin fibroblasts were cultured in DMEM (Hyclone) with 10% FBS (Hyclone) + non-essential amino acids (Gibco) + L-glutamine (Gibco) + penicillin/streptomycin (Hyclone). The cells were obtained with approval from the ethics committee of the Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences.

Virus infection

Retroviral vectors (pMXs or pSuper) carrying the factors or shRNAs were transfected into plat-E cells using PEI (PolyScience) transfection. Lentiviruses with inducible Gadd45a were prepared by transfecting lentivirus vector (pRLenti), psPAX, and pMD2G into 293T cells. The viral supernatants were collected and filtered prior to infecting MEFs with polybrene (Sigma) as described [8].

Immunofluorescence

MEFs were infected with viral vectors carrying the genes of interest and then stained with antibody directed against HP1a (CST, #2616), Nanog (R&D, AF2729), Rex1 (Santa Cruz, sc-50668), or Ssea-1 (R&D, MAB2155). A Zeiss LSM 710 confocal microscope was used for detection. The area of HP1a-positive foci or DAPI foci was measured in ImageJ using the Particle Analysis plug-in.

FRAP

MEFs infected with GFP-Histone1.4 and mCherry-HP1a were cultured in 35-mm dishes with glass bottom (WPI) and then infected with viral vectors carrying the genes under investigation. FRAP was performed at day 3 post-infection. Bleaching was accomplished with 100% power of 488 nm laser, and images were taken at 1 fps with a Zeiss LSM 710 confocal microscope at 512 × 512 resolution. The bleach was confined to oval areas of 25 pixels in diameter using 100× oil objectives. The FRAP curve was measured by ImageJ after stack-registered with StackReg plug-in and analyzed by GraphPad [57].

iPSC generation and characterization

Oct4, Sox2, Klf4, c-Myc, and other plasmids were transfected into plat-E cells using PEI (PolyScience) to generate viral stocks that infect OG2-MEFs cultured in medium containing 15% FBS (Gibco) + NEAA (Gibco) + GlutaMax (Gibco) + sodium pyruvate (Gibco) + β-mercaptoethanol (Invitrogen) + LIF as described [8]. iPSC colonies (GFP-positive colonies) were picked up and characterized as described [8,58]. Chimeras were generated by injecting iPSCs into blastocysts derived from ICR mice, followed by implantation into pseudopregnant ICR mice. F2 mice were then bred from chimeric mice and ICR mice for germline transmission as described [58]. DOX (Sigma) was added at indicated time frame for the inducible experiments. GFP⁺ iPSC colonies were scored at the indicated days for SKO or SKOM and normalized relative reprogramming efficiency as described [40]. VPA was purchased from Sigma and used at 1 mM.

Protein purification

The murine Gadd45a cDNA and its mutants were inserted into pGEX-4T2 plasmid (GE Healthcare) fusion with a GST tag and

expressed in *E. coli* strain BL21 (DE3). The fusion proteins were purified following the instruction of GST fusion protein purification. The GST tag is removed upon purification.

SDS-PAGE and native PAGE

SDS-PAGE was performed with 12% acrylamide/Bis gel in Tris-glycine buffer containing 0.1% SDS, and native PAGE with 10% acrylamide/Bis gel in Tris-glycine buffer (pH 8.3) minus the SDS, and stained with Coomassie Blue as described [59].

Western blot

The purified wild-type and G39A Gadd45a proteins were analyzed with SDS-PAGE and then transferred to PVDF membrane (Millipore). After incubated with antibodies, the membrane was exposed to X film. The antibody against Gadd45a was purchased from Santa Cruz (sc-797x).

EMSA

The full-length Gadd45a and the G39A mutant proteins were produced and purified as described above. The core histones were extracted from mouse embryonic stem cells and purified with salt elution at 2.5 M NaCl and diluted in the assay buffer. The recombinant H2A/H2B heterodimer and the H3/H4 tetramer were purchased from NEB. The EMSA was carried out with the Chemiluminescent EMSA Kit (Beyotime, Jiangsu, PR China). Gadd45a or G39A proteins (100 µg) were incubated with or without core histones (same amount as other two proteins). Biotin-labeled DNA oligomers (cloned from *Oct4* promoter, *CMV* promoter, or *EGFP* gene) were added to the reaction mixture, and the products were loaded onto a non-denaturing gel. Then, it was either stained with ethidium bromide (EB) or transferred to NC membranes followed by incubating with streptavidin-HRP and detected with X-ray film. The probes used are as followed: primers for *Oct4* probe, forward (5'-AAGTTGTCCCCAGGGGAGCCATC-3') and reverse (5'-TCTTGTTGTCCAGTTGGTAG-3'); probe A: forward (5'-GGTGGTTAGTGCTAATCTACCAAC-3') and reverse (5'-ACCACAAAGCCTGTTGGCACTGC-3'); probe B: forward (5'-GGACTGGAGGTGCAATGGCTGT-3') and reverse (5'-CCCAGGAGGCTTCATTTCAAC-3'); probe C: forward (5'-GGGCATCCGAGCAACTGGTTTGT-3') and reverse (5'-TTTACCTCTCCCTCCCAATCCCA-3'), probe D: forward (5'-AGTTTCTCCACCCCCACAGCTCT-3') and reverse (5'-CTTAGCCAGGTCGAGGATCCACC-3'); *CMV* promoter probe: forward (5'-TCAATGGGTGGACTATTTACGGT-3') and reverse (5'-TTGGAATCCCCGTGAGTCAAAC-3'); *EGFP* gene probe: forward (5'-GTTACCGGGTGGTGCCATC-3') and reverse (5'-AGAAGATGGTGCGCTCCTGAC-3').

Nuclease accessibility assay

Nuclease accessibility assay was performed with EpiQ Chromatin Analysis Kit (Bio-Rad). MEFs were infected with Flag, SKO, or SKO plus wild-type or G39A Gadd45a, then divided into two groups with one of which was digested with the EpiQ nuclease. The genomic DNA was purified and subjected to qPCR. The primers were designed from the *Oct4* promoter, *Nanog* promoter, and binding

sites of reprogramming factors. The nuclease accessibility index was calculated after normalization to an internal control. Primers for O1 were as follows: forward (5'-CTCTCGTCTAGCCCTTCCT-3') and reverse (5'-CCTCCACTCTGTATGCTCA-3'). Primers for O2 were as follows: forward (5'-CTGACCCTAGCCAACAGCTC-3') and reverse (5'-TGCTCCTACACCATGCTCTG-3'). Primers for O3 were as follows: forward (5'-CTTAGTGTCTTTCCGCCAGC-3') and reverse (5'-TCCCCTCACACAAGACTTCC-3'). Primers for O4 were as follows: forward (5'-GCACTTCTCTGGGTCTCTG-3') and reverse (5'-TGAACCCAGTATTTAGCCC-3'). Primers for O5 were as follows: forward (5'-CTGTAAGGACAGGCCGAGAG-3') and reverse (5'-CAGGAGGCCTTCATTTTCAA-3'). Primers for O6 were as follows: forward (5'-CACGAGTGGAAAGCAACTCA-3') and reverse (5'-TTGGTTCCACCTTCTCCAAC-3'). Primers for N1 were as follows: forward (5'-ATCGCCTTGAGCCGTTGG-3') and reverse (5'-CAGGGAAGGGATTCTG-3'). Primers for N2 were as follows: forward (5'-ATGGTGGCTGTGGTGGC-3') and reverse (5'-GGTTGTGGTGTGTTTGA-3'). Primers for N3 were as follows: forward (5'-GGCAGTGGAAGAAGGGAA-3') and reverse (5'-AGCCACCATACTACTGTCTC-3'). Primers for *Fbxo15* were as follows: forward (5'-GCCCTTAGTCCCAGATG-3') and reverse (5'-CTCACCTTCAAGTCCTCAA-3'). Primers for *Dppa5* were as follows: forward (5'-GCGATAGCCCAAGAAGT-3') and reverse (5'-ACAGAGATTGAAGCAGACAT-3'). Primers for *Lefty* were as follows: forward (5'-GTCCAGACAGGCTTTTGTGT-3') and reverse (5'-AGTCTGCGGAGGAATGGTA-3'). Primers for *Chd1* were as follows: forward (5'-CCATGTTAAATGTCATTTA-3') and reverse (5'-TGGAGTTACAAAGGACTTTA-3'). Primers for *Tert* were as follows: forward (5'-ACTTTGGTTGCCCAATGC-3') and reverse (5'-AAGGAAAGGTGCGCAGGT-3'). Primers for *Mixl* were as follows: forward (5'-GAATAATCGCTTCCGCTGAC-3') and reverse (5'-AGAGGGGGTCTGTCAAGT-3'). Primers for *GAPDH* were as follows: forward (5'-TGC GACTTCAACAGCAACTC-3') and reverse (5'-CTTGCTCAGTGTCTTCTGTG-3'). Primers for *HBB* were as follows: forward (5'-GAGTGGCACAGCATCCAGGGAGAAA-3') and reverse (5'-CCACAGGCCAGACAGCAGCCTTC-3').

ChIP

Cells were cross-linked with 1% formaldehyde for 15 min at room temperature and then washed three times with PBS and then harvested by scraping with a spatula. Cells were lysed in SDS buffer (1% SDS, 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, and protease inhibitor cocktail) for 10 min at 4°C and sheared by sonication. Sheared chromatin was diluted with ChIP IP buffer (0.01% SDS, 1% Triton X-100, 2 mM EDTA, 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and protease inhibitor cocktail) by 10 times. Antibodies were coupled to Dynabeads with protein A or G (Invitrogen) for more than 3 h at 4°C in PBS supplemented with 0.01% Tween-20, and beads were washed with PBS supplemented with 0.01% Tween-20. Diluted chromatin was incubated with antibodies overnight at 4°C. After immunoprecipitation, beads were washed with low-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.0), and 150 mM NaCl), high-salt wash buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.0), and 500 mM NaCl], LiCl wash buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, and 10 mM Tris-HCl (pH 8.1)], and TE buffer [10 mM Tris-HCl and 1 mM EDTA (pH 8.0)]. DNA was extracted with

Chelex 100 and used for analysis [60]. ChIP assays using anti-Oct4 (Santa Cruz, sc-8628), anti-Sox2 (Millipore, 17-10256), and anti-Klf4 (R&D, AF3158) antibodies were performed on day 8 during SKO-mediated reprogramming. ChIP assays using anti-H3K9Me2/3 (Abcam, ab1220 and ab8898), anti-H3K9Ac (Abcam, ab10812), anti-H3K27Me2/3 (Abcam, ab24684 and ab6002), and anti-H3K27Ac (Abcam, ab4729) antibodies were performed on day 3 of SKO-mediated reprogramming. Primers for *GAPDH* were as follows: forward (5'-CCTTCATTGACCTCAACTACA-3') and reverse (5'-TAGACTCCACGACATACTCA-3') [19]. Primers for *Oct4* were as follows: forward (5'-ATACTTGAAGTGTGGTGGAG-3') and reverse (5'-GCTATCATGCACCTTTGTTAT-3'). Primers for *Nanog* were as follows: forward (5'-CAGGTGGGAAGTATCTATGG-3') and reverse (5'-ACGGCTATTCTATTCAGTGG-3'). Primers for *Sox2* were as follows: forward (5'-TTTATTCAGTTCCTCAGTCCAA-3') and reverse (5'-TTATTCCTATGTGTGAGCAAGA-3'). Primers for negative control (NC) were as follows: forward (5'-AGCATGTGTTCTTCTTACCA-3') and reverse (5'-GTTAGTTCATATTATTGTTCCACCTATA-3'). The negative control corresponds to NC_000074.5/*Mus musculus* strain C57BL/6J chromosome 8, MGSCv37 C57BL/6J; the locus is from 44,386,388 to 44,386,527, which does not belong to any promoter nor gene body. Other primers were the same in the nuclease accessibility assay.

To perform ChIP with two-step cross-linking, cells were cross-linked with 2 mM DSG (Thermo) in PBS supplemented with 1 mM MgCl₂ for 45 min at room temperature. After washing three times with PBS, cells were cross-linked with 1% formaldehyde and continued for ChIP assay as described above. ChIP assays using anti-Gadd45a antibodies (Santa Cruz, sc-797x) were performed on day 8 during SKO plus WT or G39A Gadd45a-mediated reprogramming. Primers used were the same in the nuclease accessibility assay.

Immuno-FISH

The probes for *Oct4* were generated from the BAC as RP24-248K18 (Children's Hospital Oakland, USA) containing the *Oct4* genome sequence and the probes from empty vector were used as controls. They were labeled with Atto550-dUTP by using a nick translation mix (ATTO-TEC GmbH, Siegen, Germany).

MEF cells were cultured on 12-well cell plates and infected with retrovirus encoding *Oct4* or other genes described in the text. The cells were transferred onto coverslips on day 3 and next day fixed with 2% formaldehyde and permeabilized with 0.5% Triton X-100 for 10 min each. Samples were denatured with 70% formamide/2× SSC for 15 min at 76°C and then hybridized with 10 μl of hybridization mix containing 10 ng probes with 65% formamide/2× SSC at 37°C for 24 h. After three washes with 50% formamide/2× SSC, the immunostaining of HP1a was performed as described above.

Real-time quantitative PCR (qPCR)

Total RNA was extracted with TRIzol (Invitrogen) and 3 μg RNA was used to generate complementary DNA. The expression levels of genes were determined using Premix Ex Taq (Takara) and analyzed with CFX96 Real-Time System (Bio-Rad). Primers for *Gadd45a* were as follows: forward (5'-TGAGCTGCTGCTACTGGAGA-3') and reverse (5'-TCCCGGCAAAAACAAATAAG-3'). Primers for *Gadd45b*

were as follows: forward (5'-CACCTGATCCAGTCGTTCT-3') and reverse (5'-TGACAGTTCGTGACCAGGAG-3'). Primers for *Gadd45g* were as follows: forward (5'-AGTCTGAATGTGGACCCTG-3') and reverse (5'-TCAACGTGAAATGGATCTGC-3'). Primers for endogenous *Oct4* were as follows: forward (5'-TAGGTGAGCCGTCTTCCAC-3') and reverse (5'-GCTTAGCCAGGTTCCGAGGAT-3'). Primers for endogenous *Sox2* were as follows: forward (5'-AGGGCTGGGAGAAAGAAGAG-3') and reverse (5'-CCGCCATTGTGTGATTAGT-3'). Primers for *Nanog* were as follows: forward (5'-CTCAAGTCCTGAGGCTGACA-3') and reverse (5'-TGAAACCTGTCCTTGAGTGC-3'). Primers for *Rex1* were as follows: forward (5'-CCCTCGACAGACTGACCCTAA-3') and reverse (5'-TCGGGGCTAATCTCACTTTCAT-3'). Primers for *Dppa3* were as follows: forward (5'-TGTGGAGAACAAGAGTGA-3') and reverse (5'-CTCAATCCGAA-CAAGTCTT-3'). Primers for *Dnmt3 l* were as follows: forward (5'-CGGAGCATTGAAGACATC-3') and reverse (5'-CATCATCATACAGGAAGAGG-3'). Primers for *Esrrb* were as follows: forward (5'-GCACCTGGGCTCTAGTTGC-3') and reverse (5'-TACAGTCTCGTAGCTCTTGC-3'). Primers for *Lin28* were as follows: forward (5'-CCAAGATTACTGAACCTACC-3') and reverse (5'-CGTTGCTAGAGACCATTC-3'). Primers for *Sall4* were as follows: forward (5'-CCCTGGGAAGTGCATGAAG-3') and reverse (5'-TCAGAGAGACTAAAGAACTCGGC-3'). Primers for *Trim6* were as follows: forward (5'-ATGACTTCAACAGTCTTGGTGG-3') and reverse (5'-TTCCCAGGCTGATAGGAGGTC-3'). Primers for exogenous *Gadd45a* were as follows: forward (5'-GGGTGGACCATCCTCTAGAC-3') and reverse (5'-CTTGGCAGCCTCGTACACGC-3'). Primers for exogenous *Sox2* were as follows: forward (5'-GGGTGGACCATCCTCTAGAC-3') and reverse (5'-GGGCTGTTCTTCTGGTTG-3'). Primers for exogenous *Klf4* were as follows: forward (5'-GGGTGGACCATCCTCTAGAC-3') and reverse (5'-GCTGGACGCAGTGTCTTCTC-3'). Primers for exogenous *Oct4* were as follows: forward (5'-GGGTGGACCATCCTCTAGAC-3') and reverse (5'-CCAGTTTCGAGAATCCAC-3'). Primers for *Actin* were as follows: forward (5'-TGCTAGAGCCAGAGCAGTA-3') and reverse (5'-AGTGTGACGTTGACATCCGT-3').

Co-immunoprecipitation

EGFP, wild-type *Gadd45a*, and G39A *Gadd45a* were constructed with Flag tag and transduced into MEFs. MEFs were lysed with buffer (50 mM pH 7.4 Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, protease inhibitors) for 30 min. Lysates were incubated with the anti-Flag resin (Sigma) for 4 h. After immunoprecipitation, resin was washed with lysis buffer six times and was boiled for 10 min to elute Flag fusion proteins. The eluent was analyzed by Western blot with antibodies against Flag (Sigma, F1804) and H3 (Abcam, ab1791).

DNA microarrays

DNA microarrays were performed using Agilent Whole Mouse Genomic Oligo Microarray chip (Shanghai Biotechnology). Microarray data were extracted with Feature Extraction software 10.7 (Agilent technologies). All the raw data were normalized by the Quantile algorithm, Gene Spring Software 11.0 (Agilent technologies). The experiment and data analysis were performed by Shanghai Biotechnology. Genes showing significant expression changes (FC > 2) upon

overexpression of wild-type Gadd45a compared to Flag or G39A Gadd45a in SKO-induced reprogramming were selected and further analyzed. The Gene Ontology analysis was performed using DAVID database (<https://david.ncifcrf.gov>). The *P*-values represent the modified Fisher exact corrected EASE score.

Bisulfate genomic sequencing

Genomic DNA (700 ng) was isolated and bisulfate converted using 50.6% sodium bisulfate (Sigma) and 10 mM hydroquinone (Sigma) overnight at 56°C. The promoter regions of *Oct4* and *Nanog* were amplified by PCR. The PCR products were cloned into the pMD18T vector and sequenced.

Global DNA methylation and hydroxymethylation status detection

Genomic DNA was isolated and analyzed with MethylFlash™ Methylated DNA Quantification Kit (Colorimetric) or MethylFlash™ Hydroxymethylated DNA Quantification Kit (Colorimetric) according to the manufacturer's instructions (Epigentek Group Inc.).

Statistics

DNA microarrays were performed twice and all the other experiments were performed more than three times. FRAP data analysis used two-tailed Student's *t*-test and expressed as mean ± SEM, which refers to previous reports [20,27,61]. Other data used Student's *t*-test and expressed as mean ± SD. *P* ≤ 0.05 was considered statistically significant.

Accession numbers

The accession number for the DNA microarrays gathered in this study is GSE56944.

Expanded View for this article is available online.

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Author contributions

DP supervised the project. DP, KC, and TW initiated wild-type and mutant Gadd45a effects on reprogramming efficiency and pluripotent gene expression, XinL and QL initiated setting up FRAP method to reveal heterochromatin dynamics in reprogramming and identify Gadd45a as a heterochromatin relaxer. DP, XinL, KC, and QL designed and performed FRAP, FISH, EMSA, ChIP, co-IP, nuclease accessibility assay, and microarray assays to show Gadd45 loosening chromatin by destabilizing histone-DNA interactions, facilitating binding of Yamanaka factors to their targets via G39, and regulating downstream multiple gene expression. DZ and YW participated in cell culture and ChIP experiments, YZ, JQ, SL, and YuL participated in immunofluorescence and FISH experiments, XiYL participated in EMSA and ChIP experiments, CC, XZ, JY, ZZ, and WQ participated in plasmid construction and iPSC identification, YinL and XH provided recombinant proteins, DQ, JC, GP, HRS, and GX provided suggestions. DP, XinL, KC, and QL wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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