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NAC1 Regulates Somatic Cell Reprogramming by Controlling *Zeb1* and *E-cadherin* Expression

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SUMMARY

Reprogramming somatic cells to induced pluripotent stem cells (iPSCs) is a long and inefficient process. A thorough understanding of the molecular mechanisms underlying reprogramming is paramount for efficient generation and safe application of iPSCs in medicine. While intensive efforts have been devoted to identifying reprogramming facilitators and barriers, a full repertoire of such factors, as well as their mechanistic actions, is poorly defined. Here, we report that NAC1, a pluripotency-associated factor and NANOG partner, is required for establishment of pluripotency during reprogramming. Mechanistically, NAC1 is essential for proper expression of *E-cadherin* by a dual regulatory mechanism: it facilitates NANOG binding to the *E-cadherin* promoter and fine-tunes its expression; most importantly, it downregulates the *E-cadherin* repressor ZEB1 directly via transcriptional repression and indirectly via post-transcriptional activation of the *miR-200* miRNAs. Our study thus uncovers a previously unappreciated role for the pluripotency regulator NAC1 in promoting efficient somatic cell reprogramming.

INTRODUCTION

The discovery of induced pluripotent stem cells (iPSCs) marked a milestone in the development of strategies in regenerative medicine (Takahashi and Yamanaka, 2006). However, the generation of iPSCs is a lengthy and inefficient procedure that requires many processes such as global remodeling of chromatin and resetting of the epigenome (Apostolou and Hochedlinger, 2013; Papp and Plath, 2013; Watanabe et al., 2013). In recent years, many efforts have been focused on the identification of important players that could either facilitate (Theunissen and Jaenisch, 2014) or hinder (Winzi et al., 2014) the reprogramming process, leading to the discovery of NANOG as one of the reprogramming facilitators (Silva et al., 2006, 2009). Although NANOG accelerates the induction of pluripotency, its mechanisms of action are only partially understood (reviewed in Saunders et al., 2013).

In our pursuit to identify pluripotency and reprogramming factors that may modulate NANOG functions in reprogramming, we examined additional components of the NANOG interactome (Costa et al., 2013; Wang et al., 2006). In particular we identified nucleus accumbens-associated protein 1 (NAC1), a stem cell-enriched factor that also interacts with OCT4 (Ding et al., 2012) and SOX2 (Ding et al., 2015). NAC1 belongs to the bric-a-brac tramtrac broad complex/pox virus and zinc-finger (BTB/POZ) family of transcription factors (Mackler et al., 2003), and it is a ubiquitously expressed protein originally identified in the nucleus accumbens of the rat brain as a cocaine-inducible gene (Cha et al., 1997). Subsequently, NAC1 has been shown to play a role in the behavioral responses to psychostimulants (Mackler et al., 2000). In ESCs, NAC1 is a common interacting partner (Wang et al., 2006) of, and upstream modulator (Kim et al., 2008) for, many pluripotency factors and epigenetic regulators. However, its mechanistic actions in pluripotency are not defined. Besides being upregulated in pluripotent cells, NAC1 overexpression is also a hallmark of several type of cancers, including ovarian, cervical, and uterine (Ishikawa et al., 2010; Shih et al., 2011; Yeasmin et al., 2012). At the molecular level, NAC1 possesses a POZ domain N-terminally, and a BEN domain at the C terminus. The NAC1 POZ domain interacts with many factors, but is unique in that it does not contain a zinc-finger DNA-binding domain such as other POZ transcription factors. Therefore, it is believed that the NAC1 C-terminal BEN domain can mediate its binding to chromatin similarly to other BEN-containing transcriptional repressors (Dai et al., 2013).

We have begun to investigate the role of NAC1 in the maintenance and establishment of pluripotency and demonstrated that *Nac1* was surprisingly dispensable for early embryo development (Yap et al., 2013). Not unexpectedly, thereafter we were able to derive *Nac1* knockout

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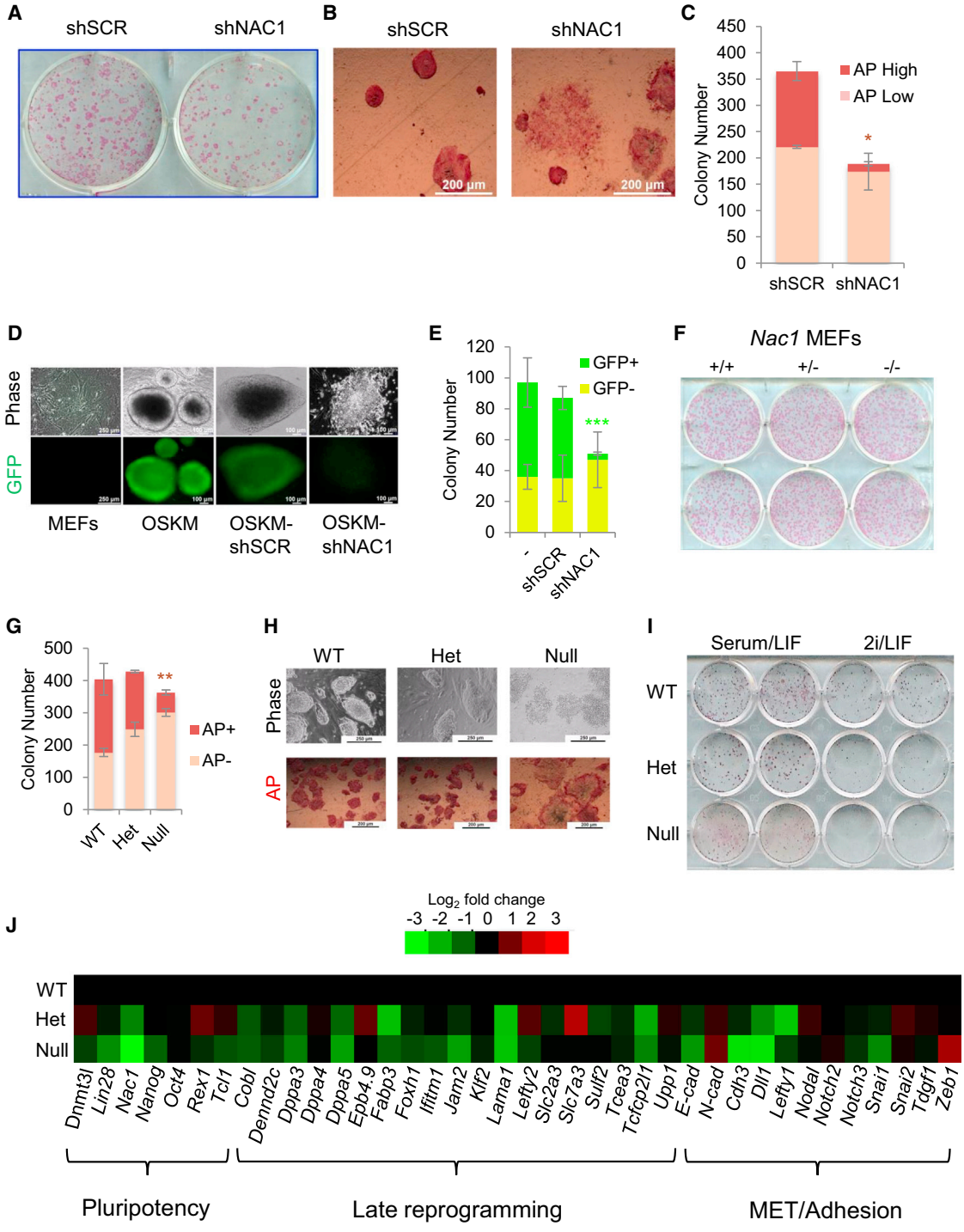

Figure 1. *Nac1* Is Required for Somatic Cell Reprogramming

(A) Images of AP-stained wells for MEF-derived iPSCs upon control and *Nac1* KD.

(B) Images of AP-stained iPS colonies upon control and *Nac1* KD.

(C) Quantification of control and *Nac1* KD iPS colonies scored based on intensity of AP staining.

(D) Images in bright field and GFP fluorescence for iPS colonies upon control and *Nac1* KD MEF reprogramming.

(E) Quantification of control and *Nac1* KD iPS colonies scored for GFP expression.

(F) Representative pictures of wells of AP-stained iPS derived from *Nac1* WT (+/+), het (+/−), and null (−/−) MEFs.

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(KO) mouse embryonic stem cells (mESCs), which undergo normal self-renewal and maintain pluripotency (our unpublished data). In this study, we dissected the functional contribution of NAC1 in establishing pluripotency during somatic cell reprogramming. We identified a critical role for NAC1 in transcriptionally and post-transcriptionally modulating *E-cadherin* and *Zeb1* expression during the generation of iPSCs. In the absence of NAC1 functions, reprogramming is diverted to an anomalous state that can be fully rescued with the re-expression of E-CADHERIN, but not NANOG or ESRRB. Our data thus uncover a previously unappreciated reprogramming factor that plays an indispensable role, beyond the mesenchymal-to-epithelial transition (MET), in controlling *E-cadherin* expression and establishing the *bona fide* pluripotency of iPSCs.

RESULTS

NAC1 Depletion Impairs Somatic Cell Reprogramming

Several pluripotency factors, including NANOG, TET1, and TET2, are essential for somatic cell reprogramming, while dispensable for stem cell maintenance once pluripotency is established (Golipour et al., 2012). Although NAC1 functions in the maintenance of pluripotency in ESCs were mostly superfluous (our unpublished data), we decided to explore whether NAC1 could play a role in the establishment of pluripotency during somatic cell reprogramming.

To test the effects of NAC1 on reprogramming, we knocked down its expression in mouse embryonic fibroblasts (MEFs) harboring an *Oct4* distal enhancer-driven GFP reporter that is only expressed in fully pluripotent iPSCs (Yeom et al., 1996). Subsequently, we transduced the four Yamanaka factors, as depicted in Figure S1A. *Nac1* knockdown (KD) was efficient (Figure S1D, top) and minimally altered MEF proliferation (Figure S1B). However, it drastically affected the total number and morphology of alkaline phosphatase (AP) positively stained iPS colonies, as well as the intensity of the staining (Figures 1A–1C). When scoring for GFP-positive colonies, we found that NAC1 downregulation not only diminished total GFP-positive populations (Figure S1C), but also compromised the morphology of iPS colonies, compared with scramble small hairpin RNA (shRNA) control (shSCR) (Figure 1D). Data from three independent reprogramming experiments revealed that the majority of the iPS colonies upon *Nac1* KD were GFP negative (Figure 1E).

Since the *Nac1* KO mouse was not embryonic lethal, we were able to derive *Nac1* wild-type (WT), heterozygous (het), and null MEFs (Figure S1D, bottom). We then employed these fibroblasts in our reprogramming assays. As shown in Figures 1F and 1G, there was minimal difference in total number of iPS colonies upon AP staining among WT, het, and null cells. However, *Nac1* null colonies stained less efficiently for AP, due to their pre-iPS-like morphology (Figures 1G and 1H) compared with WT and het cells. We also crossed our *Nac1*$^{+/-}$ mice with the *Oct4*-GFP reporter mouse and derived *Nac1* mutant MEFs harboring the GFP reporter (Figure S1E, top). Consistent with *Nac1* KD experiments, *Oct4*-GFP expression in reprogrammed colonies, which were less compact and with a disintegrated morphology, was lower in the absence of *Nac1* (Figure S1E, bottom).

To assess whether *Nac1*-depleted iPSCs were indeed not fully reprogrammed, we switched the medium from serum/leukemia inhibitory factor (LIF) to 2i/LIF and allowed the reprogrammed cells to grow further for 10 days, to select for fully reprogrammed iPS colonies and kill partially reprogrammed cells (Silva et al., 2008). As depicted in Figure 1I, about 50% of *Nac1* WT iPSCs survived in the 2i/LIF medium. In contrast, null cells showed significantly lower rates of survival, suggesting that the vast majority of *Nac*1 null colonies were not fully reprogrammed (Figure 1I). In addition, the typical pre-iPS morphology of *Nac1* null iPSCs was not due to a slower reprogramming process, because this morphology persisted for more than 15 passages in serum/LIF conditions (data not shown).

To analyze the effects of *Nac1* depletion at the gene expression level during reprogramming, we picked several morphologically good *Nac1* WT and het iPS colonies, and abnormal null iPS colonies under serum/LIF culture. We then investigated the expression of markers for pluripotency, early and late reprogramming, typical pre-iPS to iPS, and MET/cell adhesion, by qRT-PCR analyses. We found incomplete upregulation of a number of late-acting pluripotency genes including *Nanog, Lin28, Tcl1, Dnmt3l*, and *Rex1*, when *Nac1* was deleted (Figure 1J). When we examined cell adhesion and MET genes, we found that epithelial cell-adhesion markers such as *E-cadherin* and

(G) Quantification of *Nac1* WT, het, and null iPS colonies based on AP staining.
(H) Images of representative *Nac1* WT, het, and null iPS colonies in bright field (top panel) and after AP staining (bottom panel).
(I) Pictures of duplicated wells for *Nac1* WT, het, and null iPS colonies stained with AP upon incubation in serum/LIF or 2i/LIF medium.
(J) Average qPCR gene expression profiling for three *Nac1* WT, three het, and nine null clonal iPSC lines. Indicated are selected pluripotency markers, late reprogramming markers, and MET/cell-adhesion genes. *E-/N-cad* stands for *E-/N-cadherin*.
Results in (C), (E), and (G) are averages ±SD from three independent experiments; *p < 0.05, **p < 0.01, ***p < 0.001. Statistical significance is relative to shSCR AP High (C), shSCR GFP+ (E) and WT AP+ (G). See also Figure S1.

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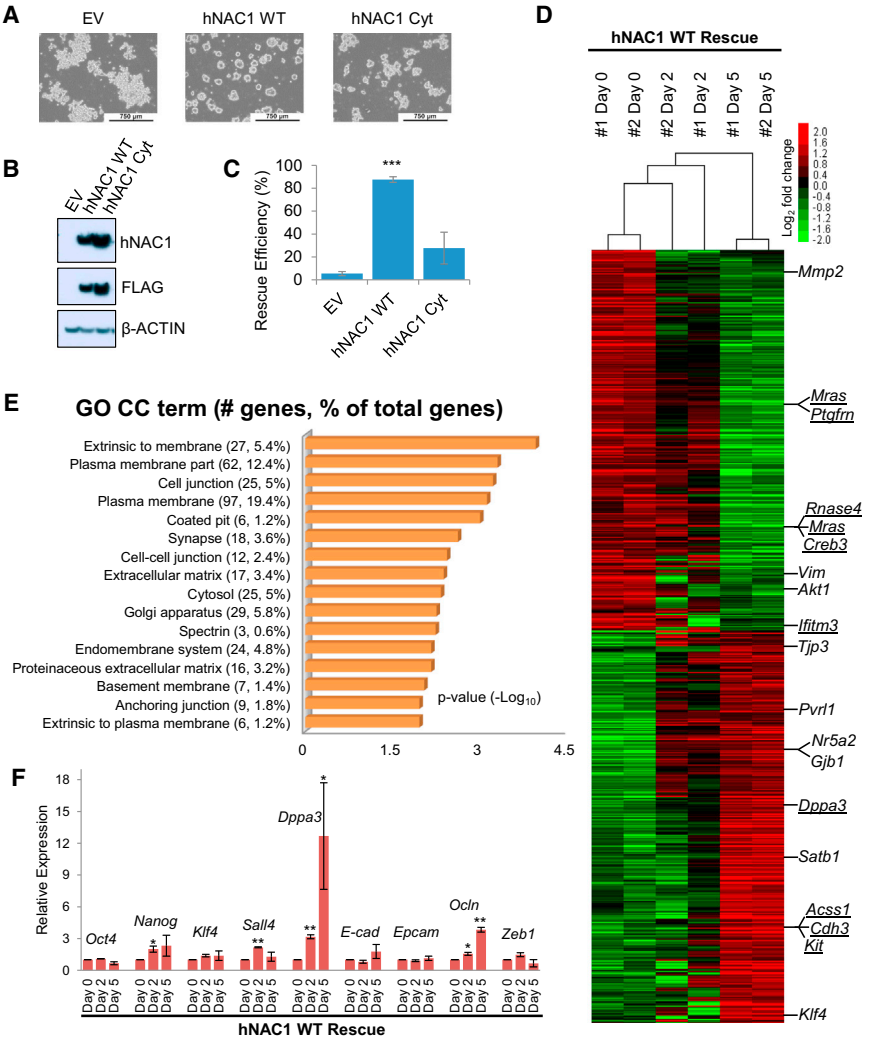

Figure 2. NAC1 Re-introduction Rescues the Null iPS Phenotype

(A) Bright-field images of *Nac1* null iPSCs transfected with empty vector (EV), hNAC1 WT, and hNAC1 Cyt (cytosolic only).

(B) Western blot (WB) analyses showing overexpression of NAC1 WT and mutant.

(C) Quantification of NAC1 WT and mutant rescue efficiencies based on iPS colony morphology. Data are average percentages ±SD of three independent experiments; ***p < 0.001. Statistical significance is relative to EV control.

(D) Heatmap of time course microarray analyses for two *Nac1* null atypical iPS clones during hNAC1 overexpression rescue. Indicated are genes known to have a role in reprogramming, and/or involved in MET/cell adhesion. Underlined are putative NAC1 target genes in mESCs.

(E) Gene ontology (GO) analyses of differentially regulated genes during the hNAC1 rescue. CC stands for cellular compartment.

(F) qRT-PCR analyses of selected pluripotency and MET markers during the rescue. Data for *Dppa3* are extracted from Figure S2D. Results are from three independent experiments with two different iPS lines and plotted as average ± SD; *p < 0.05, **p < 0.01. Statistical significance is relative to day 0. See also Figure S2.

Cdh3 were downregulated, whereas mesenchymal markers such as *N-cadherin* and *Zeb1* were upregulated in cells depleted of *Nac1*, relative to WT control (Figure 1J). Many other late reprogramming markers previously defined (Sridharan et al., 2009) were also not properly regulated in *Nac1* null iPSCs (Figure 1J). Taken together, these results suggest that reprogramming of *Nac1*-depleted cells is halted at an either intermediate or atypical reprogramming stage.

hNAC1 Overexpression Rescues the Null iPS Phenotype

To confirm that the *Nac1* null abnormal iPS phenotype we obtained was due to lack of *Nac1* functional contribution and not other unknown reasons, we overexpressed human NAC1 (hNAC1) in null atypical iPS clones that had been passaged extensively but yet retained their aberrant iPS morphology. As shown in Figure 2A, re-introduction of the hNAC1 protein quickly and completely rescued the iPS morphology, suggesting that incomplete or abnormal reprogramming was due to the absence of NAC1. Since NAC1 can also function as a cytosolic protein in cancer (Wu et al., 2011; Yap et al., 2012), we tested whether this could be also true in pluripotent cells. Nuclear/cytosolic fractionation of mESCs, followed by western blot analyses, indeed revealed that NAC1 was abundantly expressed in both nuclear and cytosolic extracts (Figure S2B). To examine whether cytoplasmic functions of NAC1 may have contributed to the observed rescue of *Nac1* null iPSC morphology, we overexpressed a form of hNAC1 mutated in its nuclear import sequence, which has been reported to be exclusively cytosolic (Okazaki et al., 2012). As depicted in Figures 2A and 2C, the cytosolic version of hNAC1 (hNAC1 Cyt), based on overall colony morphology, seemed to only slightly rescue the phenotype, despite its expression being higher than the WT

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counterpart (Figure 2B). However, a closer examination of the individual colonies revealed that hNAC1 Cyt-rescued colonies were more like the empty vector (EV) condition than the WT one (Figure S2A). These results indicate that NAC1-dependent nuclear activities are required and mostly responsible for NAC1 functions during somatic cell reprogramming. To identify downstream target genes that could mediate NAC1 nuclear functions, we performed a time course hNAC1 rescue of the null phenotype and analyzed the gene expression profiles from day 0 to day 5 in two different clonal lines. After 5 days, the rescue was evident morphologically (Figure S2C). To assess the validity of the approach, we also checked the expression of a known NAC1 target gene in ESCs (Kim et al., 2008), *Dppa3*, during the time course of rescue by qRT-PCR. As shown in Figure S2D, the *Dppa3* expression pattern was consistent with it being a NAC1 target and demonstrated that the hNAC1-dependent rescue became evident around day 2 and increased over time.

To examine gene expression at a global level during rescue, we selected day 0, 2, and 5 samples for microarray analyses in biological duplicates. As shown in Figure 2D, hundreds of genes were differentially regulated during the rescue (see Table S3 for a list of all the genes). We noticed that a number of genes differentially regulated in *Nac1* WT and null iPS clones analyzed by qRT-PCR (Figure 1J) were not present in the heatmap. This likely reflects the dynamic regulation of these genes during the reprogramming process. Alternatively, the 2-fold cutoff stringency of the heatmap and/or the increased sensitivity of qRT-PCR analyses may have caused this discrepancy. Nevertheless, it is important to point out that two mesenchymal genes (*Mmp2* and *Vim*), previously reported to be repressed during MET (reviewed in Esteban et al., 2012) and several pre-iPS genes (*Akt1, Mras, Ptgfrn, Rnase4, Creb3*, and *Ifitm3*), known to be downregulated during the pre-iPS to iPS transition (Sridharan et al., 2009), are all repressed upon the rescue of *Nac1* null atypical iPSCs with WT hNAC1. Conversely, many genes which were reported to be stimulated during the transition from pre-iPS to iPS (Golipour et al., 2012; Samavarchi-Tehrani et al., 2010; Sridharan et al., 2009) are also upregulated upon WT hNAC1 rescue. These include *Acss1* and *Kit*, the adhesion genes *Tjp3, Pvrl1, Gjb1*, and *Cdh3*, and the pluripotency genes *Nr5a2, Dppa3, Satb1*, and *Klf4*, many of which are putative NAC1 target genes in mESCs (Kim et al., 2008) (underlined in Figure 2D). Interestingly, gene ontology (GO) assessments revealed that the main categories were factors involved in membrane functions (Figure 2E).

To validate the microarray results, we also investigated the expression of several genes during the rescue by qRT-PCR. Besides genes from the heatmap of Figure 2D, we selected a few additional ones involved in MET/cell-adhesion and membrane functions, and several pluripotency and reprogramming factors known to be putative NAC1 targets or regulators of MET genes. Consistent with the microarray results and/or data in Figure 1J, we found upregulation of *Nanog, Klf4, Sall4, Dppa3, E-cadherin*, and *Occludin* (*Ocln*), and downregulation of *Zeb1* (Figure 2F). To complement the time course gene expression during the ectopic hNAC1 rescue, we also checked a number of pluripotency, late reprogramming, and cell-adhesion markers in four additional *Nac1* null iPS clones, stably transfected with EV or WT hNAC1. As presented in Figure S2E, among the pluripotency factors tested, endogenous *Oct4* expression was not significantly dependent on hNAC1 presence. In contrast, *Nanog* and *Rex1* were appreciably upregulated upon ectopic hNAC1 expression. More interestingly, late reprogramming markers such as *Dppa3* and *Lefty2*, and the two most abundantly expressed cadherins in ESCs, *E-cadherin* and *Cdh3*, were considerably activated upon hNAC1 rescue. These data further imply that hNAC1 might rescue the reprogramming defects by completing the requisite MET process (reviewed in Shu and Pei, 2014) started in the early stage of reprogramming and/or activating late-acting pluripotency gene expression during the final stages of reprogramming. To test the requirement of NAC1 function beyond the MET stage in reprogramming, we generated NAC1 null neural progenitor cells (NPCs) from ESCs for reprogramming assays (see Figures S2F and S2G for NPC characterization). We found that most of the *Nac1* null NPC-derived iPS colonies still retained the *Nac1* null atypical phenotype observed during MEF reprogramming (see Figure S2H for morphology, and Figure S2I for quantification).

Together, these results suggest that the *Nac1* null abnormal iPS morphology is not simply due to an incomplete MET process, but more likely to the partial/failed reactivation of both MET genes as well as pluripotency genes beyond the MET process.

Ectopic Expression of E-CADHERIN Is Sufficient to Rescue the *Nac1* Null iPS Phenotype

To further investigate the molecular mechanisms by which NAC1 regulates reprogramming, we tested whether known or potential NAC1-downstream target genes could recapitulate NAC1 functions. Among the pluripotency factors we chose *Nanog, Esrrb*, and *Klf4* for the following reasons. *Nanog* was previously demonstrated to be a NAC1-regulated gene in mESCs (Kim et al., 2008). Moreover, our gene expression analyses during reprogramming revealed that *Nanog* could not be fully activated in the absence of NAC1 (Figures 1J, 2F, and S2E). ESRRB has been shown to recapitulate NANOG functions during reprogramming (Festuccia et al., 2012). KLF4 is the major transcription factor regulating genes involved in cell-cell adhesion during

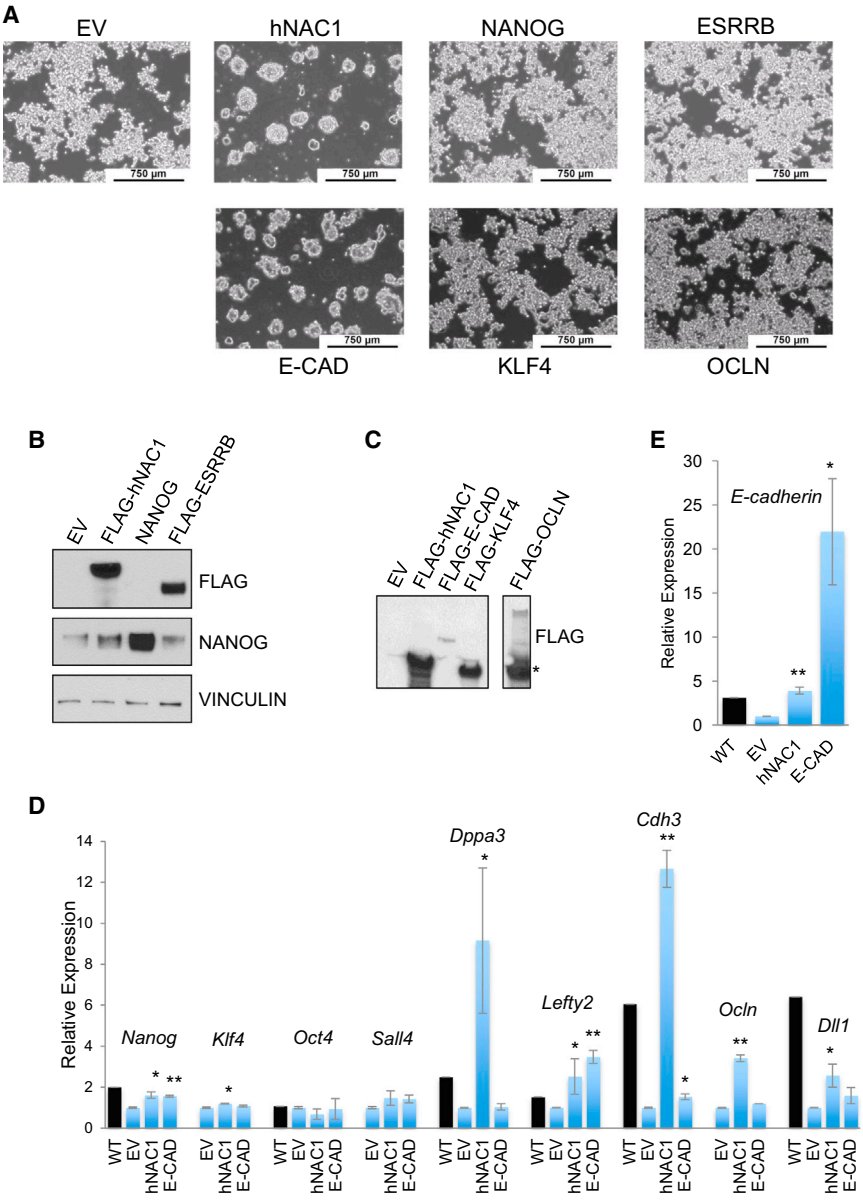

Figure 3. E-CADHERIN Overexpression Rescues the *Nac1* Null iPS Phenotype

(A) Images of *Nac1* null iPS morphology upon rescue with hNAC1, NANOG, ESRRB, E-CADHERIN (E-CAD), KLF4, and OCLN. Note that only hNAC1 and E-CAD rescued.

(B) WB analyses with indicated antibodies showing the overexpression of the factors used in the rescue experiments. VINCULIN serves as loading control. *Nanog* transgene is not FLAG-tagged. Equal amounts of whole-cell lysate were loaded in each lane.

(C) WB analyses with FLAG antibody showing the overexpression of the factors used in the rescue experiments. Equal amounts of whole-cell lysate were loaded in each lane. * Denotes OCLN-specific band.

(D) mRNA levels for selected genes by qRT-PCR, upon hNAC1 and E-CAD rescues, relative to control.

(E) qRT-PCR analyses of *E-cadherin* expression upon the succeeded rescues shown in (A).

Black bars in (D) and (E) represent WT controls. Data in (D) and (E) are from three independent experiments in triplicates, with two different iPS lines, plotted as averages ± SD; *p < 0.05, **p < 0.01. Statistical significance is relative to EV.

reprogramming (Li et al., 2010). Interestingly, ectopic expression of none of the transcription factors mentioned above was able to rescue the *Nac1* null abnormal iPS phenotype, compared with ectopic hNAC1-dependent rescue (Figure 3A). We confirmed that those ectopic factors were properly expressed (Figures 3B and 3C). These data suggest that during reprogramming *Nac1* lies downstream of *Nanog, Esrrb*, and *Klf4* action in promoting *bona fide* pluripotency. Alternatively, there may exist NAC1-specific targets that are not controlled by those pluripotency factors. In addition, our results indicate that *Nac1*$^{-/-}$ aberrant iPSCs are distinct from *Nanog*$^{-/-}$ pre-iPSCs (Festuccia et al., 2012; Silva et al., 2009), and that *Nac1* has regulatory functions other than, or downstream of, the activation of pluripotency genes during reprogramming.

To uncover such unique NAC1 functions in reprogramming, we decided to test the transgenic rescue of the *Nac1*$^{-/-}$ atypical iPSC phenotype by overexpressing E-CADHERIN and OCLN, two factors involved in cell-cell adhesion. This choice was based on our findings that membrane- and cell-adhesion-related terms were enriched in the GO analysis of the differentially expressed genes upon hNAC1 rescue (Figure 2E), and that *E-cadherin* and *Ocln* were fully upregulated under our reprogramming settings only in the presence of NAC1 (Figures 1J, 2F, and S2E). In addition, E-CADHERIN has been shown to be vital

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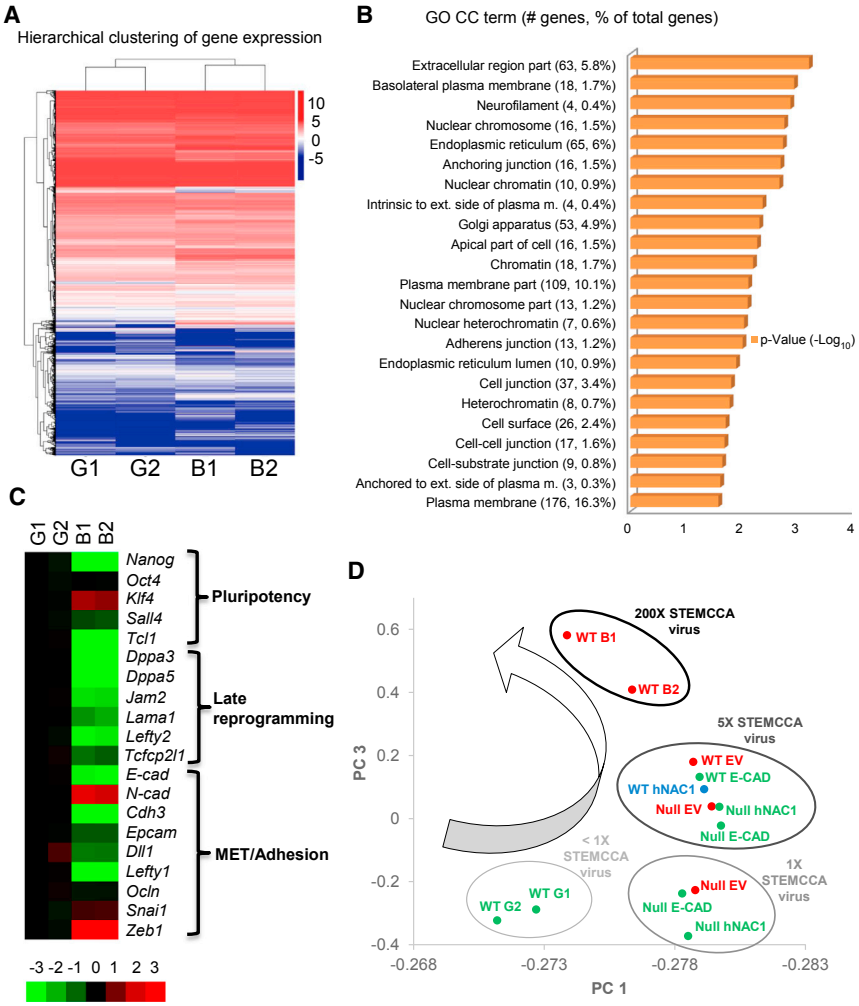

Figure 4. *Nac1* Protects Reprogrammed Cells from Acquiring Abnormal iPS States upon High Expression of the Reprogramming Factors

(A) Heatmap showing differential gene expression between two *Nac1* WT iPS colonies with normal morphology (G1 and G2) and two with aberrant morphology (B1 and B2).

(B) GO analyses of the differentially expressed genes in (A).

(C) qRT-PCR analyses of selected pluripotency, late reprogramming, and MET/adhesion markers in G1, G2, B1, and B2 WT iPS cells.

(D) Principal-component (PC) analyses of various *Nac1* WT and null iPS samples before and after indicated rescues. Note how the position in the 2D space is mostly determined by the amount of the reprogramming STEMCCA virus used. Color code: red, bad morphology; green, good morphology; blue, differentiated morphology. See also Figure S3 and Movie S1.

during somatic cell reprogramming (Chen et al., 2010; Redmer et al., 2011). Remarkably, E-CADHERIN overexpression alone phenocopied hNAC1 overexpression to generate typical dome-shaped compact iPS colonies (Figure 3A), despite its ectopic expression being much lower than hNAC1 overexpression (Figure 3C). In contrast, OCLN did not rescue the *Nac1* pre-iPS phenotype (Figure 3A). qRT-PCR analyses showed that *Nanog* (Figure 3D) together with *E-cadherin* (Figure 3E) were upregulated during hNAC1 and E-CADHERIN-mediated rescues, as expected. In contrast, expression of other pluripotency genes, such as endogenous *Oct4, Klf4,* and *Sall4*, was not affected (Figure 3D). Additional pluripotency and adhesion factors, i.e., *Dppa3, Cdh3*, and *Ocln*, were rescued only by hNAC1, whereas *Lefty2* was rescued by both hNAC1 and E-CADHERIN overexpressions (Figure 3D). These results establish *E-cadherin* as a critical target of NAC1 in reprogramming and further suggest that proper regulation of *E-cadherin* expression beyond the early MET stage can be a critical molecular event leading to efficient and complete somatic cell reprogramming.

NAC1 Protects Reprogramming Cells from Acquiring Aberrant Pluripotent States in Response to High Transgene Expression

During the reprogramming experiments described in Figures 1 and S1, we noticed the appearance of a few *Nac1* WT iPS colonies with abnormal morphology. To assess whether those rare WT colonies were pre-iPS or colonies with an atypical pluripotent state similar to the majority of *Nac1* null colonies, we harvested two of them (together with two iPS colonies with normal morphology) and performed global gene expression analyses by RNA sequencing (RNA-seq). As shown in Figure 4A, many genes were differentially regulated between morphologically good (G1 and G2) and bad (B1 and B2) WT colonies. When we scored these genes for GO, we found similar categories as those for *Nac1* null iPS colonies after and before

hNAC1-dependent rescue (Figure 4B versus Figure 2E). Moreover, we analyzed the expression of a few pluripotency, late reprogramming, and MET/adhesion markers, and found the trend of their expression between *Nac1* WT good and bad colonies was strikingly similar to the one between *Nac1* WT and null colonies (Figure 4C versus Figure 1J), and between *Nac1* null colonies after and before hNAC1-driven rescue (Figure 4C versus Figures 2F, S2E, 3D, and 3E). These results suggest that the *Nac1* WT and null aberrant iPS colonies underwent similar pathways toward alternative pluripotent states, reminiscent of both pre-iPS and F-class cells (Tonge et al., 2014). However, *Nac1* WT MEFs required higher expression of transgenes to be diverted toward those morphologically abnormal iPS colonies, compared with null cells (data not shown), indicating a protective role of NAC1 for proper iPSC formation. In addition, hNAC1 overexpression did not rescue the WT bad morphology phenotype (Figure S3A), but rather triggered differentiation, indicating that very high levels of NAC1, in conjunction with high expression of the Yamanaka factors, may be deleterious for the self-renewal abilities of iPSCs. Also, unsuccessful rescue by hNAC1 was not due to failed hNAC1 overexpression but likely to the inadequate increase of the levels of endogenous *E-cadherin* (Figures S3B and S3C). Conversely, ectopic E-CADHERIN rescue was successful, confirming a major role for E-CADHERIN in controlling iPSC morphology.

We next assessed whether the atypical morphology in *Nac1* WT and null iPSCs was associated with similar gene expression signatures. We performed RNA-seq analyses of *Nac1* WT and null iPS colonies with abnormal morphology, upon rescue with EV, hNAC1, or E-CADHERIN. We also included the four RNA-seq samples analyzed in Figure 4 (WT iPSCs with good morphology, G1 and G2, and bad morphology, B1 and B2). As depicted in Figures S3D and S3E, the heatmap of differentially regulated genes did not illustrate striking differences among the samples. To our surprise, samples appeared to cluster according to the amount of the STEMCCA reprogramming viruses employed during iPS generation, more than the overall iPS colony morphology, or the genetic background (WT versus null). Moreover, principal-component (PC) analyses clearly demonstrated a correlation between the position in the PC 2D space and the amount of the viruses over other parameters (Figure 4D). However, the presence of NAC1 significantly reduced the number of iPS colonies with atypical morphology for each amount of the virus (data not shown and Figures 1G and 1H), indicating a protecting role for NAC1 in preventing reprogramming toward abnormal iPS states.

NAC1 Collaborates with NANOG in Regulating *E-cadherin* Expression during Reprogramming

To dissect how NAC1 might transcriptionally control *E-cadherin* expression during reprogramming, we tested whether *E-cadherin* was a direct transcriptional target of NAC1. We transfected *Nac1* null aberrant iPSCs with hNAC1 and successfully confirmed its binding to the *E-cadherin* promoter (Figure 5A) by chromatin immunoprecipitation (ChIP)-qPCR assays. However, reporter assays in both *Nac1* null ESCs and heterologous HEK293T cells indicate a minimal or negative effect of ectopic hNAC1 expression on *E-cadherin* promoter activity (Figure 5B). These results suggest that the transcriptional action of NAC1 on target gene regulation during reprogramming may be different from the one in self-renewing ESCs and/or require additional factors that are not present in 293T cells.

Since *E-cadherin* upregulation is a hallmark of reprogramming, and it has been detected as a downstream target of other pluripotency regulators (see the ChEA website (http://amp.pharm.mssm.edu/lib/chea.jsp) and references therein), we thus hypothesized that NAC1 may control *E-cadherin* expression in cooperation with other stem cell factors. To identify such potential players, we turned our attention to *Nanog* due to: (1) its upregulation upon NAC1 rescue (Figures 2F and 3D); (2) the previous identification of *Nanog* as a downstream target of NAC1 in ESCs (Kim et al., 2008); and (3) the interaction between NANOG and NAC1 (Costa et al., 2013; Wang et al., 2006). Indeed, luciferase reporter assays in ESCs showed a NANOG-dependent activation of the *E-cadherin* promoter, which was counteracted by concomitant hNAC1 expression (Figure 5B, top). We also confirmed NANOG binding at the *E-cadherin* locus (Figure 5C blue bars). Importantly, we detected an enhancement in NANOG recruitment at the *E-cadherin* proximal promoter upon hNAC1 expression, compared with negative control (EV) and E-CADHERIN-mediated rescues (Figure 5C, green bars with pound signs versus blue and red bars). This was despite *Nanog* being similarly upregulated by E-CADHERIN and hNAC1 ectopic expression (Figure 3D). In addition, we detected NAC1 binding at the −4.7 kb enhancer region of the *Nanog* locus in rescued iPSCs (Figure 5D, green bars). In contrast, E-CADHERIN did not bind there (Figure 5D red bars). The specific requirement of NAC1 for enhanced NANOG binding to the *E-cadherin* promoter, together with the transcriptional activation of *Nanog* (Figures S2E and 3D) and direct NAC1 binding to its regulatory locus, may explain why E-CADHERIN or hNAC1 can, but NANOG alone cannot rescue the reprogramming defect (Figure 3A).

Downregulation of *Zeb1* Can Fully Reprogram Stalled *Nac1* Null Pre-iPSCs

Since we noticed that *Zeb1*, a major *E-cadherin* repressor (Eger et al., 2005; Shirakihara et al., 2007), was not completely downregulated in the absence of *Nac1* (compare Figure 1J with 2F for *Zeb1* expression) during

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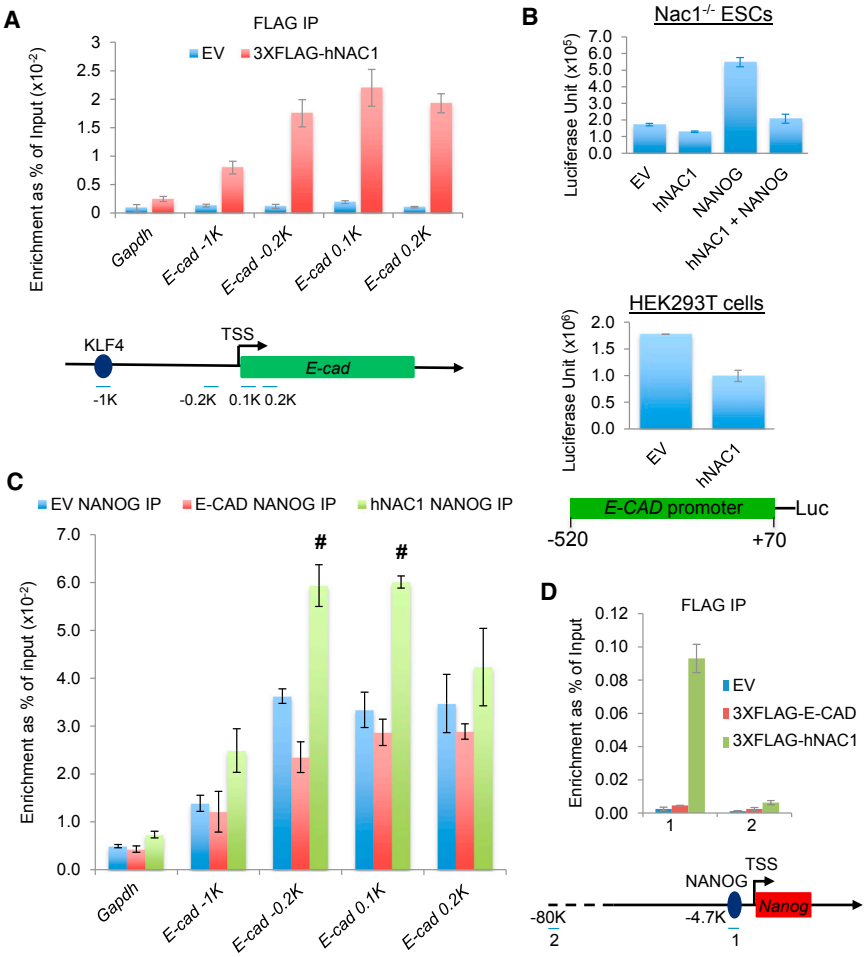

Figure 5. NAC1 Fine-Tunes the Expression of *E-cadherin* during Reprogramming

(A) ChIP assays showing hNAC1 binding at the *E-cadherin* proximal promoter in hNAC1-rescued null iPSCs. Top panel is the FLAG ChIP. Bottom panel depicts a schematic representation of the *E-cadherin* locus and the position of the qPCR primer sets used.

(B) Luciferase assays of hNAC1 and NANOG effects on the E-CADHERIN proximal promoter in Nac1$^{-/-}$ ESCs (top panel) and HEK293T cells (bottom panel).

(C) NANOG recruitment on the *E-cadherin* promoter upon empty, hNAC1, and E-CAD rescues of the null iPSC phenotype. The green bars with hash signs (#) denote increased NANOG binding upon hNAC1 overexpression, relative to controls.

(D) hNAC1 binding on the *Nanog* locus by ChIP. Bottom panel shows the location of the two qPCR primer sets employed.

Data in (A)–(D) are averages ± SD from representative experiments in triplicates.

reprogramming, we wondered whether NAC1 could also regulate *E-cadherin* indirectly via *Zeb1*. We first tested whether *Zeb1* depletion would mimic NAC1 and E-CADHERIN overexpression in rescuing the *Nac1* null pre-iPS phenotype. Indeed, as depicted in Figure 6A, two independent shRNAs against *Zeb1*, both of which significantly downregulated its expression (Figure 6B), rescued the *Nac1* null aberrant iPS morphology. Gene expression analyses upon shZEB1 rescues revealed that *E-cadherin* itself (Figure 6B), and *Nanog* (Figure S4A), were drastically upregulated compared with controls. Additional qRT-PCR experiments showed that the endogenous pluripotency genes, *Oct4* and *Klf4*, were not significantly upregulated (Figure S4A). These results confirm that OCT4 is not involved in any rescue and that KLF4 expression levels by themselves cannot completely explain the levels of *E-cadherin* expression before and after the rescues, despite the fact that KLF4 has been previously shown to be a major transcription factor regulating *E-cadherin* during reprogramming (Li et al., 2010) and in cancer cells (Koopmansch et al., 2013). In contrast, the pluripotency markers *Sall4*, *Dppa3*, and *Lefty2*, as well as the cell-adhesion molecule *Cdh3*, were significantly activated upon the rescues (Figures S4A), similarly to hNAC1- and E-CADHERIN-dependent rescues. This further highlights the critical functions of NAC1 in the transcriptional regulation of the MET-EMT-related genes for efficient reprogramming.

NAC1 Directly and Indirectly Represses *Zeb1* during Reprogramming

Our finding that knockdown of *Zeb1* was sufficient to recapitulate the NAC1 rescue of the null abnormal iPS phenotype (Figure 6A) promoted us to postulate a direct NAC1 role in repressing the *Zeb1* promoter. We first assessed the ability of NAC1 to bind to the *Zeb1* locus by ChIP assays. As shown in Figure 6C, hNAC1 was enriched at the *Zeb1* proximal promoter in *Nac1* null atypical iPSCs upon rescue. We then employed a 600-bp fragment of the human *ZEB1* promoter fused to the luciferase gene to determine whether NAC1 could repress *ZEB1* in HEK293T cells that are devoid of stem cell-specific factors. As shown in Figure 6D, hNAC1 repressed the *ZEB1* proximal promoter

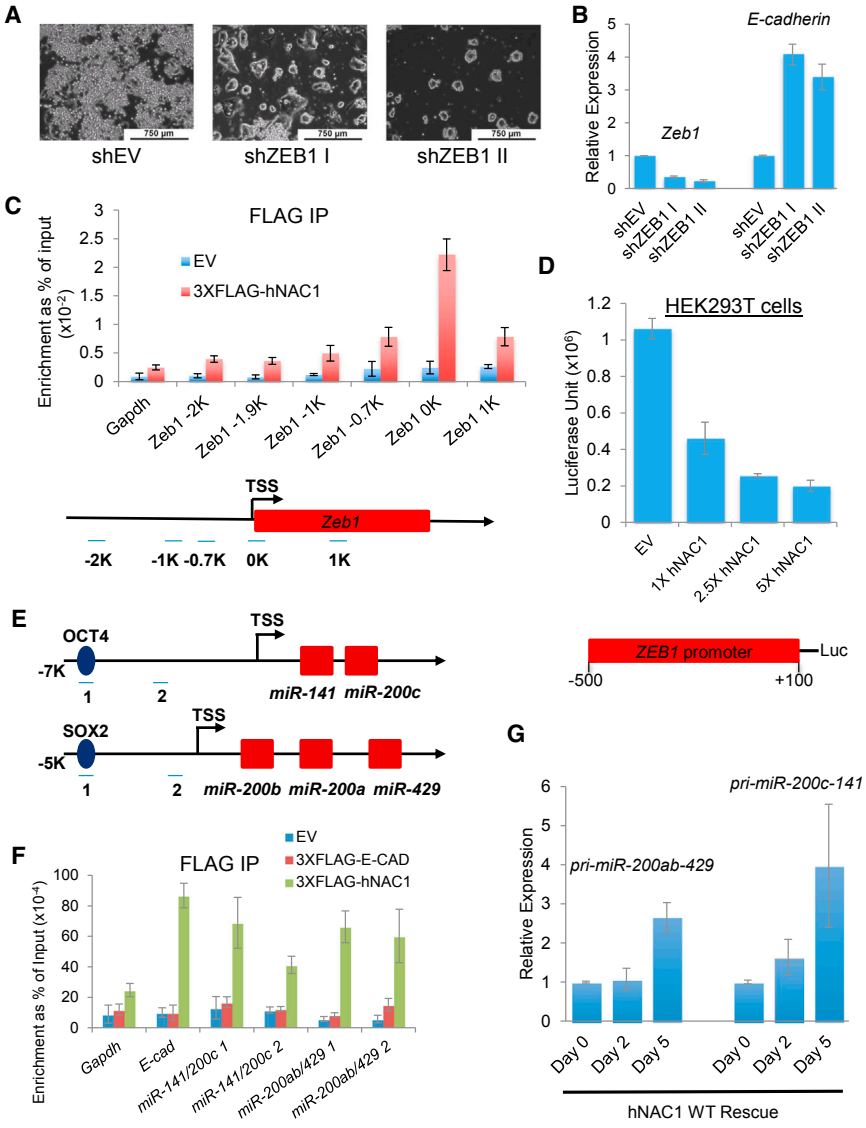

Figure 6. NAC1 Binds and Represses the *Zeb1* Promoter Directly, and Indirectly via *miR-200* during Reprogramming

(A) Images of *Nac1*$^{-/-}$ pre-iPS upon rescue with two shRNAs against *Zeb1*, and control sh-empty.

(B) qRT-PCR analyses for *Zeb1* and *E-cadherin* expression upon shZEB1 in *Nac1* null aberrant iPSCs.

(C) ChIP assays showing binding of hNAC1 on the *Zeb1* proximal promoter in rescued iPSCs (top panel). Bottom panel depicts the position on the *Zeb1* locus of the qPCR primer sets used.

(D) Luciferase assay showing hNAC1 amount-dependent repression of the *ZEB1* proximal promoter in HEK293T cells (top). Bottom panel shows the schematic of the luciferase reporter used.

(E) ChIP assays showing binding of hNAC1 on the *miR-200* loci in rescued iPSCs. E-CAD overexpression serves as a negative control, while the *E-cad* locus (−0.2 K primer set) serves as a positive control.

(F) Schematic depiction of the *miR-200* loci and location of the qPCR primer sets employed in ChIP.

(G) Expression of the *miR-200* pri-miRNAs during the hNAC1 WT rescue of the null iPS phenotype.

Data in (B)–(E) and (G) are averages ± SD of representative experiments in triplicates. See also Figure S4.

efficiently, demonstrating that NAC1 can directly repress *Zeb1* to favor *E-cadherin* expression.

The *miR-200* family of microRNAs (miRNAs) has been demonstrated to downregulate *Zeb1/2* during somatic cell reprogramming (reviewed in Leonardo et al., 2012). Therefore, we inspected whether NAC1 could also repress *Zeb1* indirectly by acting on those miRNAs. As shown by ChIP assays in Figures 6E and 6F, overexpressed hNAC1 bound to the *miR-200* family loci in rescued *Nac1* null pre-iPSCs, which correlated with higher expression of those pri-miRNAs during the reprogramming process (Figure 6G). These findings clearly indicate that both the transcriptional (direct) and post-transcriptional (indirect) regulations of *Zeb1* expression could contribute to NAC1 nuclear functions for efficient reprogramming. To assess the importance of the role of NAC1 in regulating the *miR-200* family during reprogramming, we attempted to rescue the abnormal *Nac1* null reprogrammed cells by overexpressing all the members of the *miR-200* family. As shown in Figure S4B, however, we were not able to rescue the null phenotype. These data suggest that the major role of NAC1 during reprogramming is to regulate *E-cadherin* expression via *Zeb1* repression.

DISCUSSION

Cell-cell adhesion, particularly the one mediated by E-CADHERIN, is fundamental for pluripotent stem cell biology because it regulates the degree of stemness,

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differentiation, and somatic cell reprogramming (reviewed in Pieters and van Roy, 2014). It has been also well established that a crucial step in iPS generation is the MET process (Esteban et al., 2012), exemplified by the upregulation of *E-cadherin*. In fact, if *E-cadherin* is not expressed, MET cannot complete and reprogramming is halted (Chen et al., 2010; Li et al., 2010; Redmer et al., 2011; Samavarchi-Tehrani et al., 2010). However, how *E-cadherin* is regulated during reprogramming is incompletely understood. Our study demonstrates a previously unappreciated role of the BTB-POZ transcriptional regulator NAC1 in direct transcriptional and indirect, via *miR-200* and ZEB1, post-transcriptional control of *E-cadherin* expression during the reprogramming process.

We have described previously how NAC1 participates in the intricate protein interaction and transcription regulatory networks that regulate stem cell maintenance and pluripotency, suggesting a potentially significant role for NAC1 in self-renewal and/or pluripotency (Costa et al., 2013; Ding et al., 2012; Kim et al., 2008; Wang et al., 2006). However, its functions and molecular mechanisms involved in the establishment and maintenance of pluripotency were poorly defined. Remarkably, here we found that NAC1 could function as a reprogramming factor and was critical for ground state pluripotency in reprogramming during and beyond the early MET stage. This reinforces the notion that many regulators of the late-maturation phase during somatic cell reprogramming can be dispensable for early development or stem cell maintenance, as reported previously (Golipour et al., 2012), which is exemplified by *Nanog* (Chambers et al., 2007), *Esrrb* (Martello et al., 2012), and *Klf4* (Katz et al., 2002; Segre et al., 1999).

During reprogramming NAC1 could not be replaced by NANOG, ESRRB, and KLF4, although NAC1 was thought to regulate their expression in pluripotent cells (Kim et al., 2008). Rather, NAC1 was essential for *Zeb1* repression and proper expression of *E-cadherin* to reach full pluripotency manifested by typical compact dome-shaped iPS morphology and reactivation of the *Oct4* distal enhancer. Our data indicate that proper induction of *E-cadherin* cannot be reached in the absence of NAC1, even when KLF4 is overexpressed (Figure 3), despite the fact that KLF4 can promote epithelial gene expression, and is essential for the MET process (Li et al., 2010). Since it has been previously shown that high levels of ZEB1/2 in cancer cells can displace KLF4 from the *E-cadherin* promoter with concomitant repression of transcription (Koopmansch et al., 2013), the regulatory action of KLF4 on *E-cadherin* expression during reprogramming may require NAC1-dependent downregulation of *Zeb1*. Our findings also argue against *Nac1* null atypical iPSCs being similar to the pre-iPSCs generated by the Silva group (Silva et al., 2009). Unlike Silva pre-iPSCs, our *Nac1* null abnormal iPSCs already expressed NANOG (Figure 3B, the EV lane), and their reprogramming to full pluripotency was not dependent on NANOG overexpression or the 2i/LIF medium. Another interesting aspect of NAC1 function during reprogramming is its protecting role in preventing reprogramming cells to be diverted toward altered pluripotent states, reminiscent of pre-iPS and F-like states, particularly when reprogramming transgene expression is high (Figure 4D).

In conclusion, our study identifies NAC1 as a reprogramming factor, critical for proper expression of *E-cadherin* during iPSC generation with a multifaceted regulatory mechanism (Figure 7). First, NAC1 binds and transcriptionally represses *Zeb1*, one of the main repressors of *E-cadherin*. Second, it stimulates the expression of the *miR-200* family of miRNAs to downregulate *Zeb1* post-transcriptionally. Third, NAC1 directly binds to the *E-cadherin* promoter and regulates co-factor (e.g., NANOG) binding to fine-tune its expression. These results enlighten our knowledge of the molecular mechanisms of somatic cell reprogramming and bring us a step closer to more efficient generation of iPSCs. Finally, our study will benefit our understanding of the role of NAC1 in cancer progression and metastasis. In that respect, our findings suggest that, in cancers where NAC1 is overexpressed, its repressor functions may be the driving force in the downregulation of *E-cadherin*, leading to enhanced EMT, cancer cell migration, and metastasis, as described previously (Gao et al., 2014).

EXPERIMENTAL PROCEDURES

Cell Culture

iPSCs were grown in standard serum/LIF condition unless otherwise specified.

qRT-PCR Assays

RNAs were extracted with the QIAGEN RNeasy Plus Kit and converted to cDNA with the qScript cDNA SuperMix (Quanta BioSciences). qPCR was performed as described previously (Fidalgo et al., 2011). Oligo sequences are listed in Table S1.

Microarray Profiling, RNA-Seq, and GO Analyses

RNAs from day 0 (control non-transfected), 2, and 5 samples of the hNAC1 time course rescue experiments from two different null iPSC lines, were analyzed on an Illumina MouseWG-6 v.2.0 Expression BeadChip at the Genomics Core Facility, Icahn School of Medicine at Mount Sinai.

Cellular compartment functional annotation for genes differentially regulated in the *Nac1* iPSC microarrays was performed by using David bioinformatics tools (Huang et al., 2009).

For RNA-seq analyses, total RNAs were extracted as above. RNA-seq libraries were prepared at Beijing Genomics Institute, and their quality and yield analyzed by an Agilent 2100 Bioanalyzer and ABI

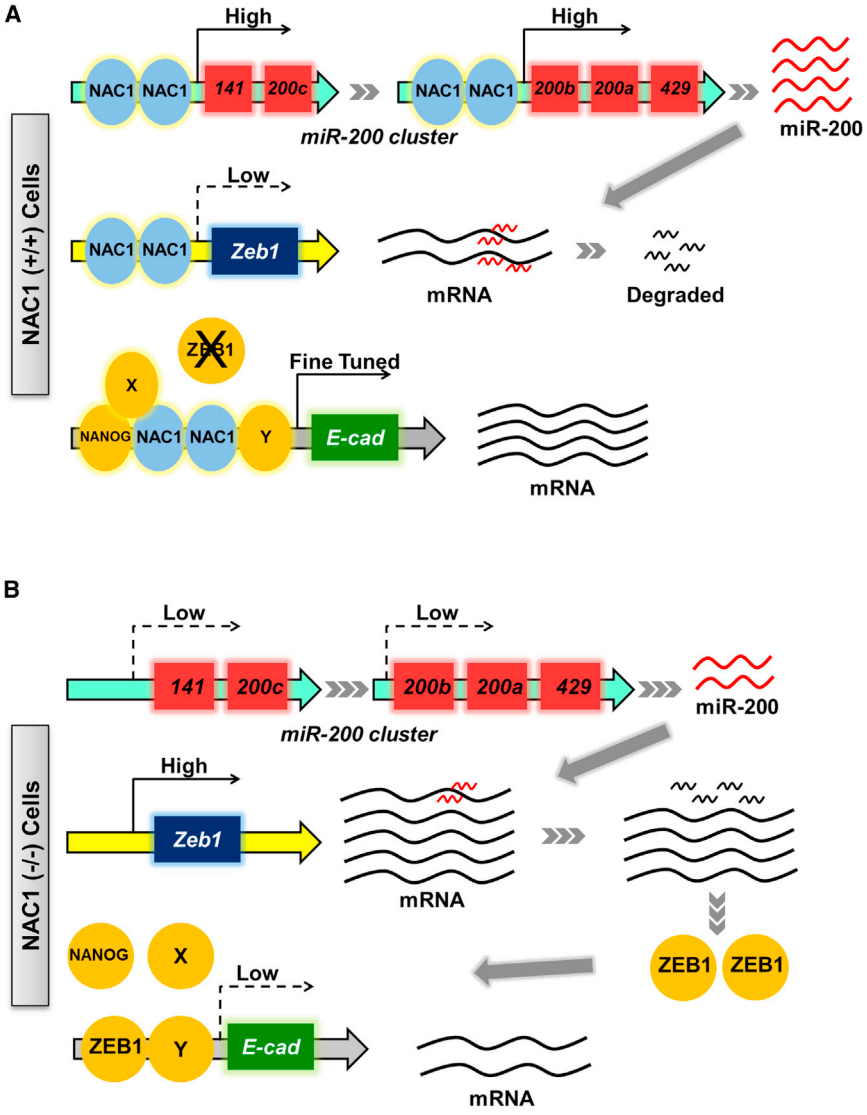

Figure 7. Working Model for NAC1 Roles during Somatic Cell Reprogramming

(A) When NAC1 is expressed, it can activate the expression of the *miR-200* cluster and also directly bind the *Zeb1* promoter. Both actions would lead to the downregulation of the ZEB1 protein, and consequently fine-tuning of *E-cadherin* expression by NAC1 itself, NANOG, and possibly other factors (X represents a putative activator, while Y a putative repressor).

(B) In the absence of NAC1, *Zeb1* expression is high, whereas the *miR-200* mRNAs are downregulated. As a consequence, there is very low post-transcriptional repression of *Zeb1*, and ZEB1 protein can displace transcription activators on the *E-cadherin* promoter and repress transcription.

StepOnePlusReal-Time PCR system, and sequenced on an Illumina HiSeq 2500/4000 instrument. Reads were filtered and then aligned to the reference genome with Bowtie2. Quantitative gene expression was determined by the RSEM software.

Reprogramming and iPSC Rescue Experiments

MEF reprogramming experiments were performed as published previously (Costa et al., 2013; Fidalgo et al., 2012) and described in Supplemental Experimental Procedures.

For rescue experiments (with clonal and/or bulk populations), iPSCs were transfected with piggybac-based expression vectors for indicated proteins and selected with 200 μg/mL hygromycin for a week. Colonies were then photographed and collected for gene expression analyses. For *Zeb1* KD rescue assays, cells where infected with pLKO-pim-based lentiviruses with two *Zeb1* shRNAs and one empty control, and selected with 1 μg/mL puromycin for a few days For miR-200 rescues, viruses were prepared and cells infected as described in the Supplemental Experimental Procedures.

ChIP-qPCR Assays

ChIP experiments were performed as in (Lee et al., 2006) with a few modifications described in Supplemental Experimental Procedures.

ACCESSION NUMBERS

The accession number for the microarrays is GEO: GSE100350. The accession numbers for RNA-seq are SRA: SRX2885263, SRX2896775, SRX2896971, SRX2897024, SRX2897194, SRX2899130, SRX2899145, SRX2900609-14.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, three tables, and one movie and can be

found with this article online at http://dx.doi.org/10.1016/j.stemcr.2017.07.002.

AUTHOR CONTRIBUTIONS

F.F., M.F., and J.W. designed the research, performed the experiments, and analyzed the data. N.Y. performed the experiments and analyzed the data. X.H. analyzed the microarray data. A.S., J.D., D.G., and B.D. provided technical assistance. F.F. wrote the manuscript draft. J.W. conceived the project and revised and approved the final manuscript.

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