

# Role of the Murine Reprogramming Factors in the Induction of Pluripotency

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## SUMMARY

Induced pluripotent stem (iPS) cells can be obtained from fibroblasts upon expression of Oct4, Sox2, Klf4, and c-Myc. To understand how these factors induce pluripotency, we carried out genome-wide analyses of their promoter binding and expression in iPS and partially reprogrammed cells. We find that target genes of the four factors strongly overlap in iPS and embryonic stem (ES) cells. In partially reprogrammed cells, many genes co-occupied by c-Myc and any of the other three factors already show an ES cell-like binding and expression pattern. In contrast, genes that are specifically co-bound by Oct4, Sox2, and Klf4 in ES cells and encode pluripotency regulators severely lack binding and transcriptional activation. Among the four factors, c-Myc promotes the most ES cell-like transcription pattern when expressed individually in fibroblasts. These data uncover temporal and separable contributions of the four factors during the reprogramming process and indicate that ectopic c-Myc predominantly acts before pluripotency regulators are activated.

## INTRODUCTION

Reprogramming of human and mouse fibroblasts to induced pluripotent stem (iPS) cells has been achieved by the expression of only four transcription factors, Oct4, Sox2, Klf4, and c-Myc, subsequently referred to as the “four factors” (Maherali et al., 2007; Okita et al., 2007; Takahashi et al., 2007; Takahashi and Yamanaka, 2006; Wernig et al., 2007; Yu et al., 2007). iPS cells hold great promise for the study and therapy of human diseases (Dimos et al., 2008; Park et al., 2008) because they are highly

similar to embryonic stem (ES) cells in their ability to self-renew and give rise to all three germ layers. Molecularly, reprogramming results in the remodeling of the somatic cell transcription and chromatin programs to the ES-like state, including the reactivation of the somatically silenced X chromosome, demethylation of the *Oct4* and *Nanog* promoter regions, and genome-wide resetting of histone H3 lysine 4 and 27 trimethylation (Maherali et al., 2007; Takahashi et al., 2007; Wernig et al., 2007). A key question raised by transcription factor-induced reprogramming is how the four factors act to bring about this change.

Two studies demonstrated that reprogramming of murine fibroblasts is a gradual process that follows a defined series of molecular events (Brambrink et al., 2008; Stadtfeld et al., 2008). Upon retroviral transduction of the factors fibroblast-specific genes become repressed, exemplified by the downregulation of the somatic marker *Thy1*, followed first by the activation of embryonic markers such as alkaline phosphatase (AP) and SSEA1 and later by the induction of pluripotency genes such as *Nanog* and *Oct4*. While *Thy1* downregulation occurs in the majority of cells, gain of SSEA1 and activation of pluripotency regulators happens with very low efficiency, suggesting that as yet unknown epigenetic barriers need to be overcome to induce pluripotency (Huangfu et al., 2008; Meissner et al., 2007; Mikkelsen et al., 2008).

In ES cells, chromatin immunoprecipitation (ChIP) experiments have demonstrated that Klf4, Sox2, and Oct4 together with other ES-specific transcription factors such as *Nanog* often co-occupy target genes, including their own promoters, which suggests that they cooperate in regulatory feedback loops to maintain self-renewal and pluripotency (Boyer et al., 2005; Chen et al., 2008; Jiang et al., 2008; Kim et al., 2008; Loh et al., 2006). These transcription factors can function both as activators of self-renewal and pluripotency genes and as repressors of lineage commitment genes, suggesting that they could activate the ES-specific transcription program and repress

fibroblast expression during reprogramming. Based on limited target overlap in ES cells, it was proposed that the function of c-Myc differs from that of Oct4, Sox2, and Klf4 (Chen et al., 2008; Kim et al., 2008). However, to date no studies have been performed to analyze how the four factors bind and function in iPS cells. It is conceivable that they have a different binding pattern in these cells, given their diverse origin. It is also possible that they fulfill transient roles during reprogramming that cannot be inferred from the analysis of the pluripotent end state and necessitate the study of intermediate stages of the process. Understanding the contribution of each factor to the different steps of reprogramming and an in-depth characterization of the iPS state should shed light on the molecular nature of reprogramming.

Here, we studied the function of the four factors at three stages of the reprogramming process: the initial phase, an intermediate step represented by partially reprogrammed cell lines, and the final iPS cell stage. Partially reprogrammed cells express markers of the intermediate reprogramming stage and have failed to transcriptionally activate pluripotency regulators but can be converted to a pluripotent state with small molecules that affect chromatin modifications or modulate signal transduction (Silva et al., 2008; Mikkelsen et al., 2008). By comparing binding of the factors and expression changes at the three stages, our analysis provides insight into the specific contribution of each factor to the reprogramming process.

## RESULTS

### The Four Factors Bind Similar Sets of Target Genes in iPS and ES Cells

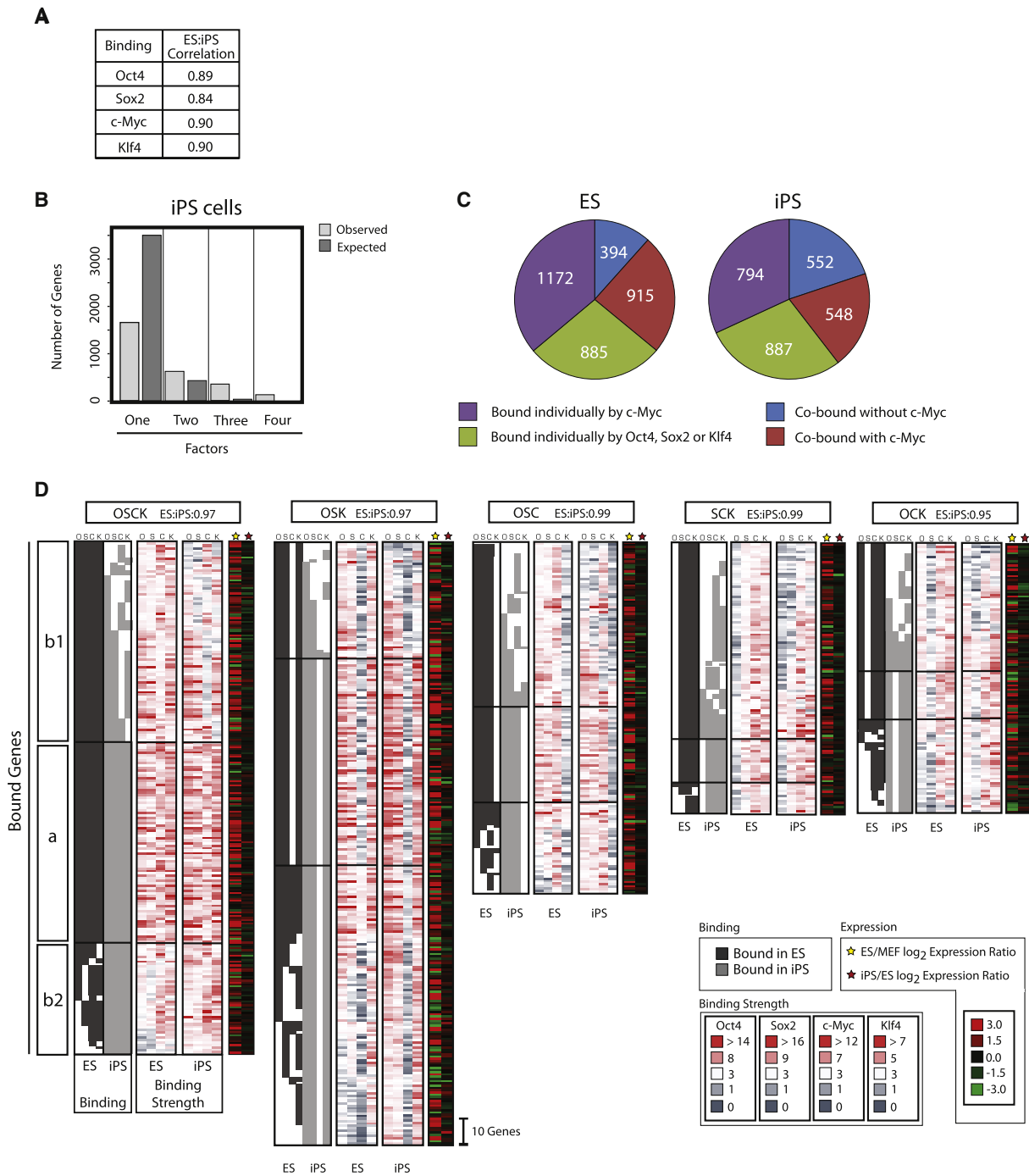
To characterize the completely reprogrammed state, we used ChIP and microarrays to determine the promoter regions that are bound by the four factors in two ES and two iPS cell lines and compared binding patterns between these cell types. The iPS cell lines analyzed here (clones 1D4 and 2D4) have completely suppressed retroviral transgene expression and entered a self-sustaining pluripotent state that can give rise to adult chimeras with germline contribution (Maherali et al., 2007). For each factor, binding between the two iPS cell lines is as highly correlated as between the two ES cell lines (Table S1). Hence, the replicate data sets were averaged before determining bound genes for each factor and cell type (all averaged binding data are provided in Table S2 and raw data files are summarized in Table S3, available online). A gene was called bound using an algorithm that considers information of neighboring probes (Experimental Procedures; Boyer et al., 2005). It should be noted that genes that fall below the threshold for being called bound might still be targeted by the various factors, perhaps transiently, reflecting weaker binding events. However, we use the phrase “bound” gene to refer to only those genes that passed our binding criteria. The reliability of our binding calls was validated by a comparison of our ES cell targets to previously published observations (Boyer et al., 2005; Chen et al., 2008; Jiang et al., 2008; Kim et al., 2008; Loh et al., 2006). For example, a gene ontology (GO) analysis indicated that c-Myc targets in ES cells include many metabolic regulators while Oct4, Sox2, and Klf4 targets are skewed toward transcriptional regulators of

development and differentiation; and the known DNA-binding motifs for Oct4, Sox2, Klf4, and c-Myc are highly enriched in the promoter regions that are targeted by these factors (data not shown). Furthermore, real-time PCR analysis confirmed strong enrichments at loci that were called bound (Figure S1), and bound targets identified in merged data sets were reliably found in the individual replicates indicating high reproducibility of the data (Figure S2A).

The four factors exhibit overall similarity in their binding between ES and iPS cells at the genome-wide level (Figure 1A). The number of target genes co-occupied by three or all four factors in iPS cells is much higher than expected by random chance (476 genes), and the number of targets bound by just one factor is much lower (1681 genes,  $\chi^2$  test  $p = 0.0057$ , Figure 1B), like in ES cells (Chen et al., 2008; Kim et al., 2008). Notably, while we found 794 genes bound by c-Myc alone, approximately half of the genes that are occupied by multiple transcription factors (548 genes) are also targets of this factor (Figure 1C), suggesting that, surprisingly, c-Myc coregulates many genes together with the other three factors in iPS cells ( $\chi^2$  test  $p = 1.9 \times 10^{-291}$ ).

To analyze co-occupancy by the four factors in more detail, we classified target genes according to the combination of factors bound. This categorization yielded a total of 15 groups, with one group containing genes bound by all four factors (single letter code OSCK for Oct4, Sox2, c-Myc, and Klf4), four groups with genes bound by different combinations of three factors (OSC, OSK, OCK, and SCK), six groups of genes that are bound by two factors (OS, OC, OK, SC, SK, and CK), and four gene groups that are bound by only one (Figure S3). Next, we asked whether genes that are bound by a particular combination of factors in iPS cells are associated with the same set of factors in ES cells and vice versa (Figures 1D and S4). Across all 15 groups, the binding pattern is similar between the two cell types. Focusing on the groups of genes co-bound by three or four factors (Figure 1D), up to 64% of ES cell targets are bound by the same sets of factors in iPS cells (Figure 1D, section a). However, the binding pattern is not identical because there are genes that are bound by fewer factors in iPS cells than in ES cells (Figure 1D, section b1), as there are genes bound by fewer factors in ES cells than in iPS cells (Figure 1D, section b2). Nevertheless, only very few genes that are associated with three or four factors in ES cells are not bound by any factor in iPS cells and vice versa. Indeed, when allowing differential binding by one factor between ES and iPS cells, up to 87% of the genes are bound by comparable sets of factors. These findings also apply to genes bound by two factors (Figure S4A). Interestingly, genes that are bound by a specific factor in one cell type but not in the other have a lower binding strength than those genes that are bound in both cell types (Figures 1D, S2, and S4A), supporting the notion that binding patterns in iPS and ES cells are highly conserved. Furthermore, target genes are similarly expressed in both cell types, suggesting that differences in factor binding are too small to dramatically affect transcription (Figures 1D and S4A).

The similarity of binding events between both cell types is further reflected by analogous functional classifications for genes in the 15 binding groups in iPS and ES cells (Figure S3).



**Figure 1. Target Overlap between ES and iPS Cells**

(A) Pearson correlation of binding strength at the probe level for each factor between iPS and ES cells across all genes.  
 (B) Bar chart indicates the number of genes observed to be bound by only one, any combination of two or three, and all four factors in iPS cells (light gray) and the expected number of target genes calculated assuming that each factor binds independently of the others (dark gray).  
 (C) Pie charts indicate the number and proportion of target genes bound by single or multiple factors in iPS or ES cells, divided according to the presence of c-Myc.  
 (D) Comparison of binding patterns in ES and iPS cells for genes bound by the indicated combination of three or four factors (denoted by single letter nomenclature with O = Oct4, S = Sox2, C = c-Myc, and K = Klf4). The three segments of each cluster are based on the overlap of binding events between ES and iPS cells: section (a) depicts genes bound by the same set of factors in ES and iPS cells, (b1) and (b2) contain genes that have binding in additional and/or fewer factors in either cell type. Binding of a gene is marked by a dark (ES) or light gray block (iPS). Heat maps plot the corresponding binding strength. For each factor, the lowest maximum

For both cell types, GO analysis demonstrates that genes associated with c-Myc, by itself or in combination with the other factors, are significantly enriched for regulators of metabolic processes including the control of translation, RNA splicing, cell cycle, and energy production, while genes bound by combinations of Oct4, Sox2, and Klf4, in the absence of c-Myc, are mainly implicated in transcriptional control of development. Together, these data indicate that Oct4, Sox2, Klf4, and c-Myc binding patterns in iPS cells resemble those of ES cells, suggesting that pluripotency relies on binding of the four factors to a conserved set of target genes. The extensive co-binding among all four factors indicates that c-Myc participates in the transcriptional network formed by Oct4, Sox2, and Klf4 much more than expected.

### Extensive Failure to Activate ES-like Expression in Partially Reprogrammed Cells

To begin to understand events that occur during the reprogramming process, we decided to analyze partially reprogrammed cell lines. These cells emerge at intermediate stages of reprogramming, when reprogrammed cells are selected based on morphology or reporter expression, and have been informative for understanding the barriers of the reprogramming process (Meissner et al., 2007; Mikkelsen et al., 2008; Takahashi and Yamanaka, 2006). We selected two stable, clonal, partially reprogrammed cell lines for our analysis (1A2 and 1B3) that were previously derived from female fibroblasts carrying a GFP/puromycin resistance reporter cassette under the control of the endogenous *Nanog* promoter (Maherali et al., 2007; Figure S5). These partially reprogrammed cells have an ES-like morphology surrounded by a few round cells, are positive for SSEA1, and, similar to ES cells, have a doubling time of 13 hr (Figure S5). The four factors are expressed from the exogenous retroviral constructs, and endogenous pluripotency regulators such as *Nanog* and *Oct4* and the inactive X chromosome are not reactivated (Figure S5). These cell lines can be maintained in culture without a change in morphology, and the expression profile of a pool of 10 colonies is highly similar to that of the population demonstrating homogeneity (Figure S6). A small fraction of GFP-positive, i.e., highly *Nanog*-expressing, cells (~1%) that support chimerism stochastically arises in these cell lines (Figure S6; Maherali et al., 2007), and upon addition of a specific MEK inhibitor used in recent reprogramming experiments (Silva et al., 2008) an efficient transition to GFP-positive colonies is observed (Figure S6). These data demonstrate that our partially reprogrammed cells reflect an intermediate stage of the reprogramming process that can transition to the completely reprogrammed iPS state.

We first characterized the gene expression pattern of the two partially reprogrammed cell lines. They are highly similar in expression to those analyzed recently by Mikkelsen et al. (Mikkelsen et al., 2008), even when derived from somatic cells other than fibroblasts, supporting the idea that partially reprogrammed

cells are trapped at a common intermediate state (Figure S6). Fibroblast-specific genes are more efficiently downregulated in partially reprogrammed cells (correlation  $_{ES/MEF: partial/MEF} = 0.628$ ) than ES-specific genes are activated (correlation  $_{ES/MEF: partial/MEF} = 0.324$ ; Figure S7, Table S4). A GO analysis revealed that ES-specific metabolic regulators are more completely activated in partial reprogrammed cells than transcriptional regulators (Figure S7), in agreement with the observation that key transcriptional regulators of pluripotency are turned on only during the final steps of the reprogramming process (Brambrink et al., 2008; Stadtfeld et al., 2008).

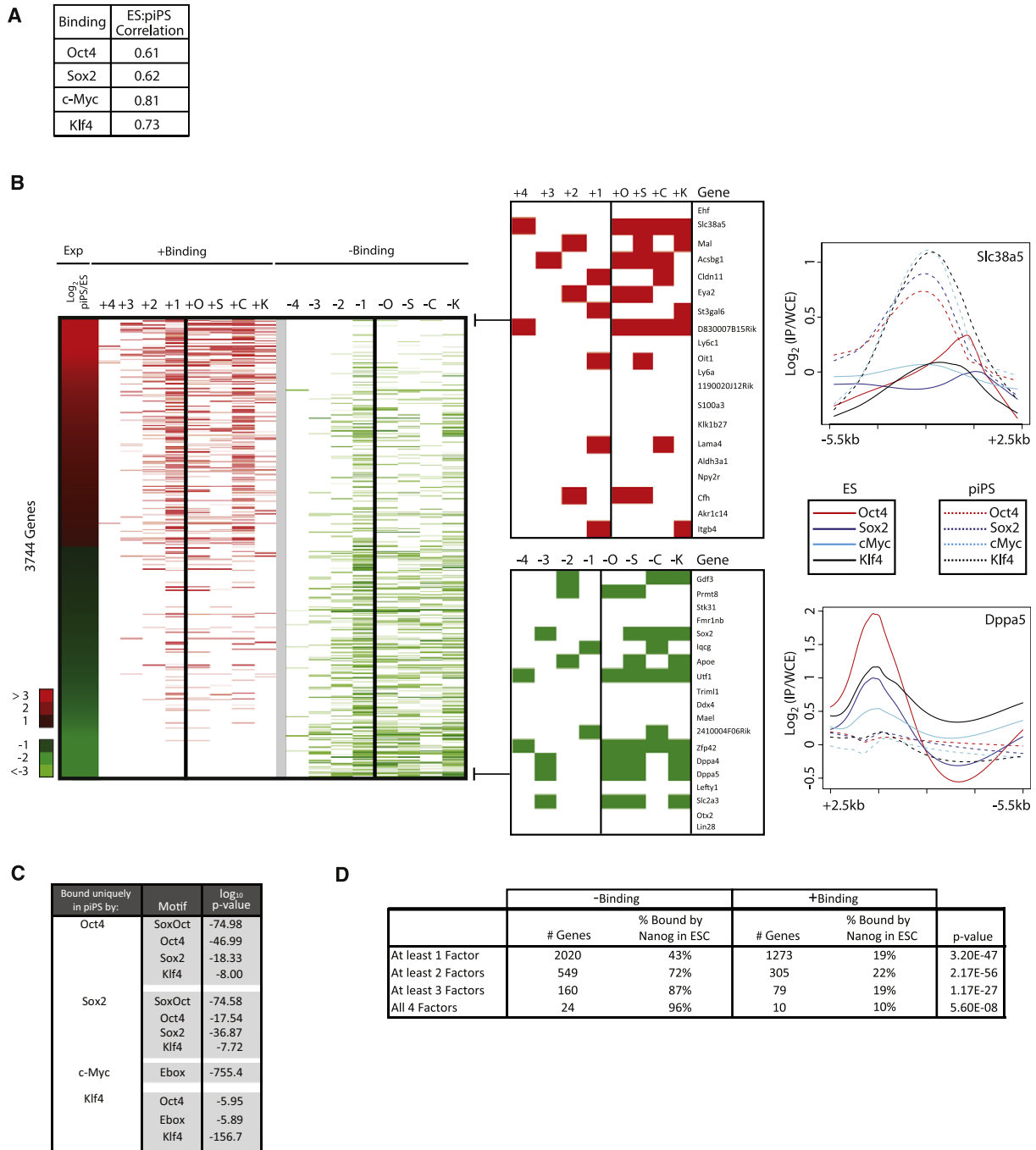
### Four Factor Binding in Partially Reprogrammed Cells

To address whether differential gene expression between partially reprogrammed cells and ES cells is due to binding of the four factors to different sets of genes, we determined their target genes in both partially reprogrammed cell lines. Analogous to expression, binding is highly correlated between these cell lines supporting the notion that these cells represent a stable and homogenous intermediate reprogramming state (Table S1). We therefore merged binding data from both cell lines to generate the set of bound genes and found that the genome-wide binding of each factor is much less conserved between ES cells and partially reprogrammed cells than between iPS and ES cells (Figure 2A, compare with 1A).

To study the relationship of factor binding and expression in detail, we focused on the 3744 genes that are more than 2-fold differentially expressed between partially reprogrammed cells and ES cells (Figure 2B, Table S1). Approximately one-third of these genes (1139 genes) are direct binding targets of the factors. Genes that are more highly expressed in partially reprogrammed cells than in ES cells are often bound by more factors in the intermediate state than in ES cells. In contrast, genes that are more highly expressed in ES cells often are bound by fewer factors in partially reprogrammed cells than in ES cells (Figure 2B). The higher the amplitude of differential expression the more factors tend to be differentially bound between ES and partially reprogrammed cells (Figure 2B), suggesting that binding by multiple factors leads to stronger transcriptional activation. These data imply that genes are more strongly expressed in partially reprogrammed cells compared to ES cells due to targeting of the four factors to promoter regions that they do not normally bind in ES cells, and conversely that the failure to activate ES cell-specific genes results from the lack of binding of the factors in partially reprogrammed cells. Differential binding of the factors in partially reprogrammed cells is also reflected by a different spectrum of functional classifications for target genes in partially reprogrammed cells (Figure S3).

It is critical to understand why the four factors target different genes in partially reprogrammed cells than in ES cells. We found that promoter regions bound uniquely in partially reprogrammed cells contain the known DNA-binding motif for the occupying factor, suggesting that targeting to these genes in partially

binding strength among all genes and cell types analyzed in this study was set to the darkest red color. For each binding cluster, expression ratios of ES cells/fibroblasts (MEFs) (yellow star) and iPS/ES cells (red star) and the Pearson correlation coefficient comparing expression of the genes within a cluster between ES and iPS cells are given (top).



**Figure 2. Differential Binding between Partially Reprogrammed Cells and ES Cells Correlates with Expression Differences**

(A) Pearson correlation of binding strength at the probe level for each factor between ES cells and partially reprogrammed cells across all genes. (B) The heat map in the Exp column depicts the  $\log_2$  expression ratio between partially reprogrammed cells (piPS) and ES cells for genes more than 2-fold differentially expressed between these cell types. For these genes, +Binding columns illustrate binding events in partially reprogrammed cells that were not found in ES cells (in red), with +1 to +4 indicating the number of factors that were bound in partially reprogrammed cells but not in ES cells and the letter indicating the factor (Oct4 [+O], Klf4 [+K], Sox2 [+S], or c-Myc [+C]). Similarly, in -Binding columns, genes that were bound in ES cells but not in partially reprogrammed cells are marked in green, with -1 to -4 indicating the number of factors that were bound in ES cells and not in partially reprogrammed cells and the letter indicating the factor. Binding differences for the most differentially expressed genes between ES and partially reprogrammed cells are presented in a zoomed view to the right and graphs to the far right plot  $\log_2$ (IP/input) ratios of probes over the 8 kb promoter region for one gene from each subgroup. (C) For binding events found in partially reprogrammed cells but not ES cells, bound regions were scanned for the presence of the DNA-binding motifs of the four factors (with the Ebox motif being bound by c-Myc). Significantly enriched motifs are indicated.

reprogrammed cells is not simply due to nonspecific or indirect binding but instead may be guided by direct interactions with their respective DNA-binding motif (Figure 2C). Given that the expression of the four factors is 3- to 8-fold higher in partially reprogrammed cells than in ES or iPS cells, these sites are perhaps low-affinity binding sites. In contrast, promoter regions that are bound in ES cells but not in partially reprogrammed cells may need other proteins that are not expressed in partially reprogrammed cells to allow cooperative binding of the factors. One such candidate is the transcription factor Nanog, which colocalizes with Oct4 and Sox2 at a subset of promoter regions in ES cells (Boyer et al., 2005; Chen et al., 2008; Kim et al., 2008) and is not expressed in our partially reprogrammed cell lines (Figure S5). Indeed, we found that genes that lack ES-like binding by the factors in partially reprogrammed cells are more often targets of Nanog in ES cells than genes where binding uniquely occurs in partially reprogrammed cells (Figure 2D). In particular, genes that are bound by three or four factors in ES cells and completely lack binding in partially reprogrammed cells are often Nanog targets in ES cells (Figure 2D). Thus, the absence of Nanog, and likely that of other transcription factors, could contribute to the lack of ES-specific binding in partially reprogrammed cells.

#### Oct4, Sox2, and Klf4 Targeting Is Particularly Impaired in Partially Reprogrammed Cells

Partially reprogrammed cells have fewer bona fide ES cell targets than iPS cells for Oct4 (38% of its ES cell targets are bound in partially reprogrammed cells versus 79% in iPS cells), Sox2 (35% versus 54%), and Klf4 (38% versus 67%), while c-Myc binds 67% in partially reprogrammed cells and 59% of its ES targets in iPS cells. Thus there is a widespread difference in binding between ES cells and partially reprogrammed cells that impinges more on Oct4, Sox2, and Klf4 than on c-Myc. This observation led us to address whether c-Myc co-occupancy affects binding of the other three factors. We found that Oct4, Sox2, and Klf4 binding in partially reprogrammed cells is more similar to their binding in ES cells when c-Myc is co-bound at the promoter (Figure 3A). For example, 48% of Oct4 ES cell targets are bound in partially reprogrammed cells when the target is co-bound by c-Myc in ES cells, compared to only 32% when co-binding in ES cells occurs with Klf4 and/or Sox2. Thus, c-Myc association divides ES cell target genes of the four factors into two groups and appears to be a measure of their targeting efficiency in partially reprogrammed cells. The difference in Oct4, Sox2, and Klf4 binding based on c-Myc co-occupancy is not observed when ES and iPS cell binding is compared (Figure 3A).

To further analyze these binding differences, we measured the average number of factors differentially bound between iPS cells and partially reprogrammed cells for genes in each ES cell binding group (Figure 3B). The most significant differences in binding between iPS and partially reprogrammed cells exist in

those genes that are co-occupied by Oct4, Sox2, and Klf4 (OSK), followed by genes associated with both Oct4 and Klf4 (OK). Genes bound by all four factors (OSCK) or Oct4 and Sox2 (OS) just pass the significance threshold. In general, genes in these groups also have the lowest correlation in expression between pluripotent and partially reprogrammed cells (Figure 3B). Genes bound by OSK are characterized by high binding strength in ES and iPS cells and largely unbound in partially reprogrammed cells (Figure 3C). Indeed, with only 24 OSK target genes in partially reprogrammed cells compared to 193 genes in iPS cells and 129 in ES cells, co-occupancy by OSK hardly exists in partially reprogrammed cells. These data show that there is widespread differential binding between iPS/ES cells and partially reprogrammed cells that most dramatically occurs in genes occupied by OSK. This finding was confirmed when differential binding between ES/iPS and partially reprogrammed cells was similarly analyzed for genes in each iPS-specific binding group (data not shown).

#### Oct4, Sox2, and Klf4 Co-occupancy Is Associated with Strong Transcriptional Activation

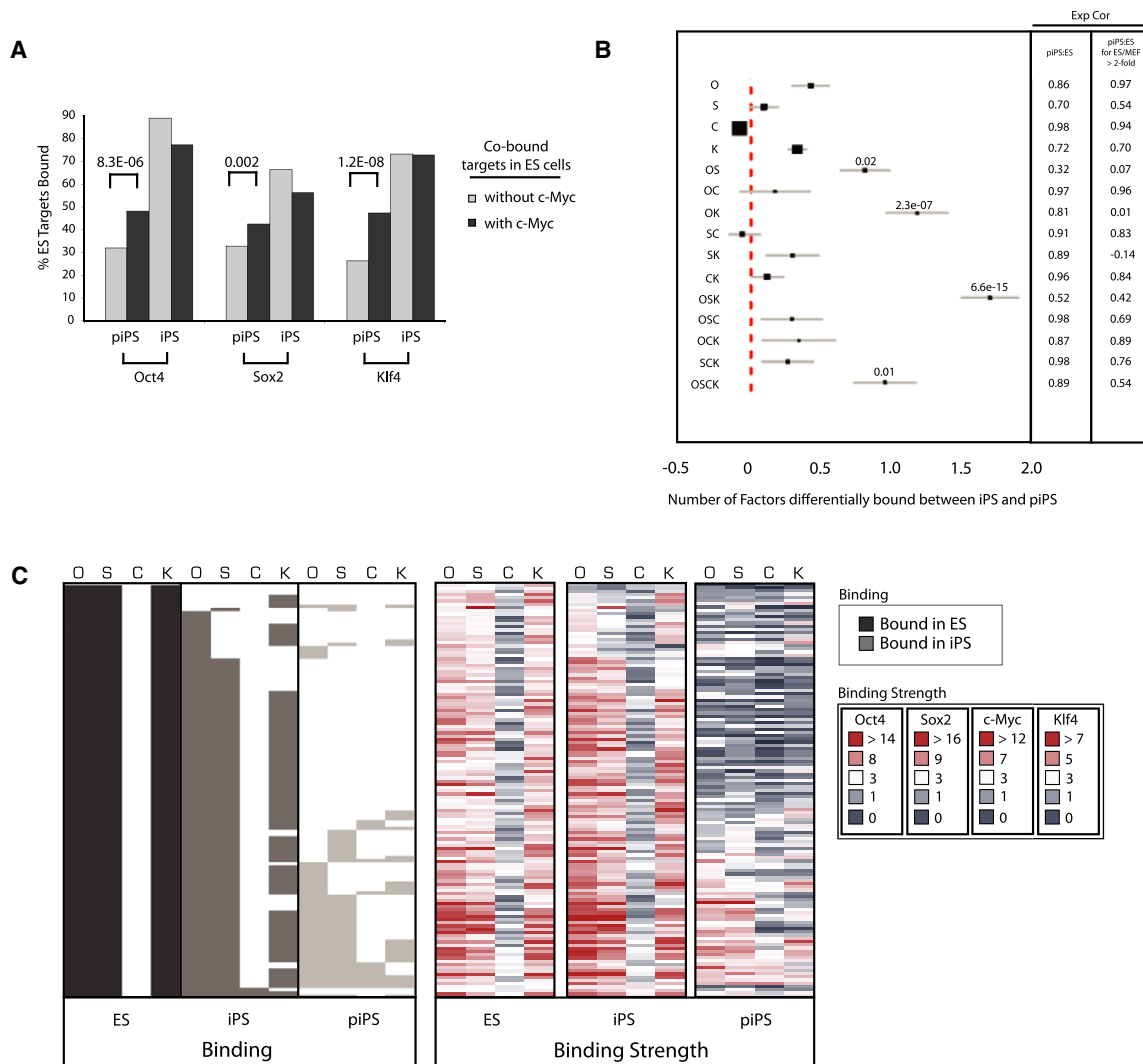
Genes co-occupied by OSK in ES cells, on average, have the highest increase in expression between iPS cells and fibroblasts, i.e., upon reprogramming (Figure 4A). As expected, these genes are not upregulated to the ES/iPS cell level in partially reprogrammed cells. In particular, OSK targets that are most activated in the transition of fibroblasts to iPS cells are the ones that fail to be induced (Figure 4B). These genes are not bound in partially reprogrammed cells and include highly expressed regulators of the pluripotent state (for example, *Tcl1*, *Dppa3*, *Dppa4*, *Dppa5*) and embryonic development (for example, *Foxh1*, *Lefty2*) (Figure 4C).

The expression analysis also revealed that the four factors appear to have a larger impact on the activation of gene expression because co-bound genes are usually more highly expressed in iPS and ES cells than in fibroblasts (Figure 4A). These findings also extend to the partially reprogrammed state (data not shown) indicating that an intrinsic property of reprogramming factor co-binding is to activate genes, irrespective of the cell type. Consistent with published observations (Chen et al., 2008; Kim et al., 2008), genes occupied by only c-Myc are activated more strongly than those bound singly by Oct4, Sox2, or Klf4.

#### Binding Is Incompatible with Low Histone H3K4 Trimethylation

We reasoned that the chromatin state of promoter regions could influence the binding properties of the factors during reprogramming. In general, transcribed genes are marked by the “activating” histone H3 lysine 4 trimethylation (H3K4me3), while repressed genes, especially those encoding developmental regulators, are associated with histone H3 lysine 27 trimethylation (H3K27me3). In repressed genes, H3K27me3 is often found in combination with H3K4me3 forming bivalent chromatin

(D) Genes bound by fewer factors in partially reprogrammed cells than in ES cells (–Binding) or by more factors (+Binding) were divided according to the number of factors differentially bound between these cell types. The number of genes per group and the fraction of these genes that is bound by Nanog in ES cells are presented. Significance was computed using the difference of proportions test.



**Figure 3. OSK Targets Are Most Differentially Bound between iPS Cells and Partially Reprogrammed Cells**

(A) Genes bound by two, three, or four factors in ES cells were divided into those co-occupied by c-Myc and those lacking c-Myc. For both groups of genes, the bar plot presents the fraction of Oct4, Sox2, or Klf4 ES targets that are bound by the respective factor in partially reprogrammed cells (piPS) or iPS cells. Significant differences for binding in piPS, computed using the difference of proportions test, are indicated.

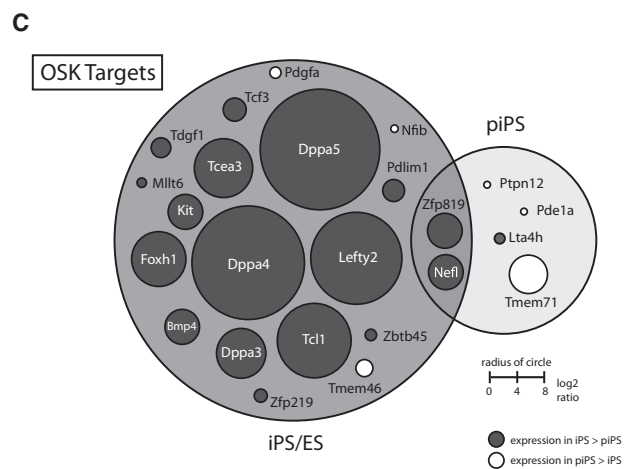
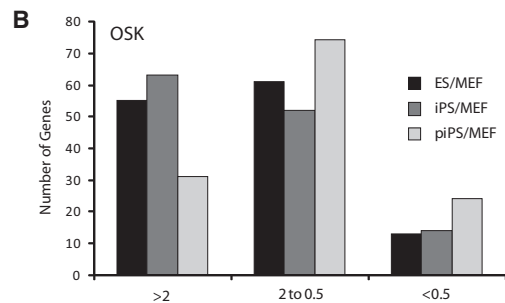
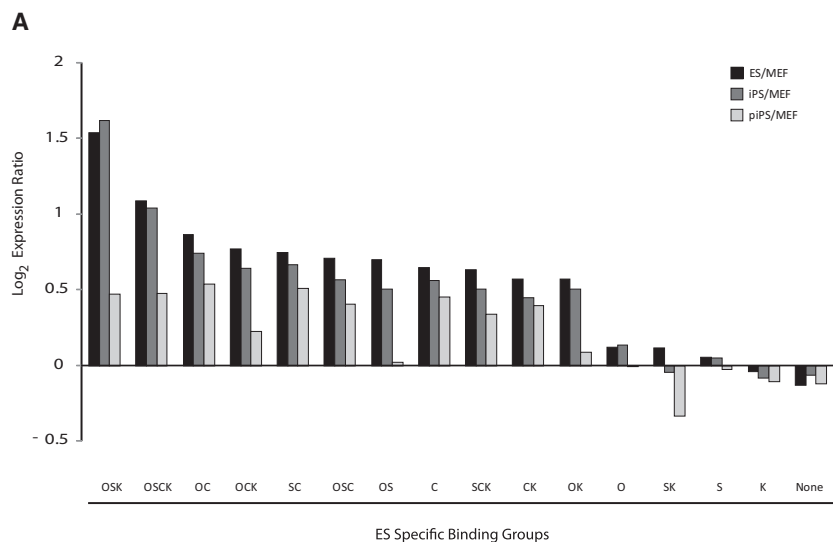
(B) The graph displays the average number of factors differentially bound between iPS and partially reprogrammed cells for target genes grouped according to their factor association in ES cells (given to the left), within a 95% confidence interval. Filled squares reflect the relative numbers of genes in each target group. Significance was determined using the binomial distribution. Pearson correlation coefficients (Exp Cor) for expression between ES and partially reprogrammed cells (piPS) were computed for genes in each group and for those genes in a given group that are expressed at least 2-fold higher in ES cells compared to MEFs.

(C) Binding calls and binding strength heatmaps of Oct4, Sox2, c-Myc, and Klf4 in ES cells, iPS cells, and partially reprogrammed cells (piPS, lightest gray) for OSK ES targets as described in Figure 1D.

domains (Bernstein et al., 2006). Our previous analysis demonstrated that these histone methylation marks are reset to the ES cell pattern in iPS cells (Maherali et al., 2007). To test the relationship between promoter histone methylation and binding of the four factors, we analyzed H3K4me3 and H3K27me3 patterns in fibroblasts, partially reprogrammed cells, and iPS and ES cells for all promoter regions bound by the four factors.

We found that the differential binding of the four factors between partially reprogrammed cells and ES/iPS cells cannot generally be explained by differences in K4 and K27 methylation

patterns between the pluripotent cells, partially reprogrammed cells, or fibroblasts. Specifically, the proportion of genes enriched for K27 methylation in ES cells and partially reprogrammed cells is similar for ES cells targets (Figure 5A) indicating that the presence of H3K27me3 per se does not interfere with binding of the factors. Similarly, the majority of ES cell targets of the four factors are methylated at K4 in ES cells, a pattern that is also observed in fibroblasts (Figure S8) and partially reprogrammed cells (data not shown). We noted that c-Myc, alone or with other factors, mainly associates with genes that have less



**Figure 4. OSK Targets Are Most Differentially Expressed between ES/iPS Cells and Partially Reprogrammed Cells**

(A) Bar chart depicting the mean of the log<sub>2</sub> expression ratio between the ES (black), iPS (dark gray), or partially reprogrammed cells (piPS, light gray) and fibroblasts (MEF) for genes bound by the indicated combination of factors in ES cells or for all unbound genes (None).

(B) The graph divides OSK targets of ES cells according to their expression ratio between indicated cell types.

(C) Venn diagram depicting genes bound by OSK in iPS and ES cells but not in partially reprogrammed cells (piPS) (left), genes bound in partially reprogrammed cells but not in ES and iPS cells (right), and genes bound by OSK in all three cell types (overlapping area). Circles are scaled and colored according to the expression difference between iPS cells and piPS.

lost H3K27me<sub>3</sub> in ES/iPS cells in accordance with their high transcriptional activation (Figure 5B, highlighted genes). In partially reprogrammed cells, this set of genes exhibits an intermediate pattern of histone methylation and has a severe lack of binding of the three factors (Figure 5B). A similar pattern of histone methylation change is also evident in a subset of OK, OS, and OSCK bound target genes that exhibit a strong induction of expression during reprogramming (data not shown and Figure S8). Together, these data indicate that highly expressed targets of the factors, which encode many important regulators of pluripotency and embryonic development (Figure 5B), are characterized by a specific histone H3K4/K27 methylation pattern that may be related to the lack of binding by the factors in partially reprogrammed cells.

**c-Myc Is a Major Contributor to Initial Reprogramming Events**

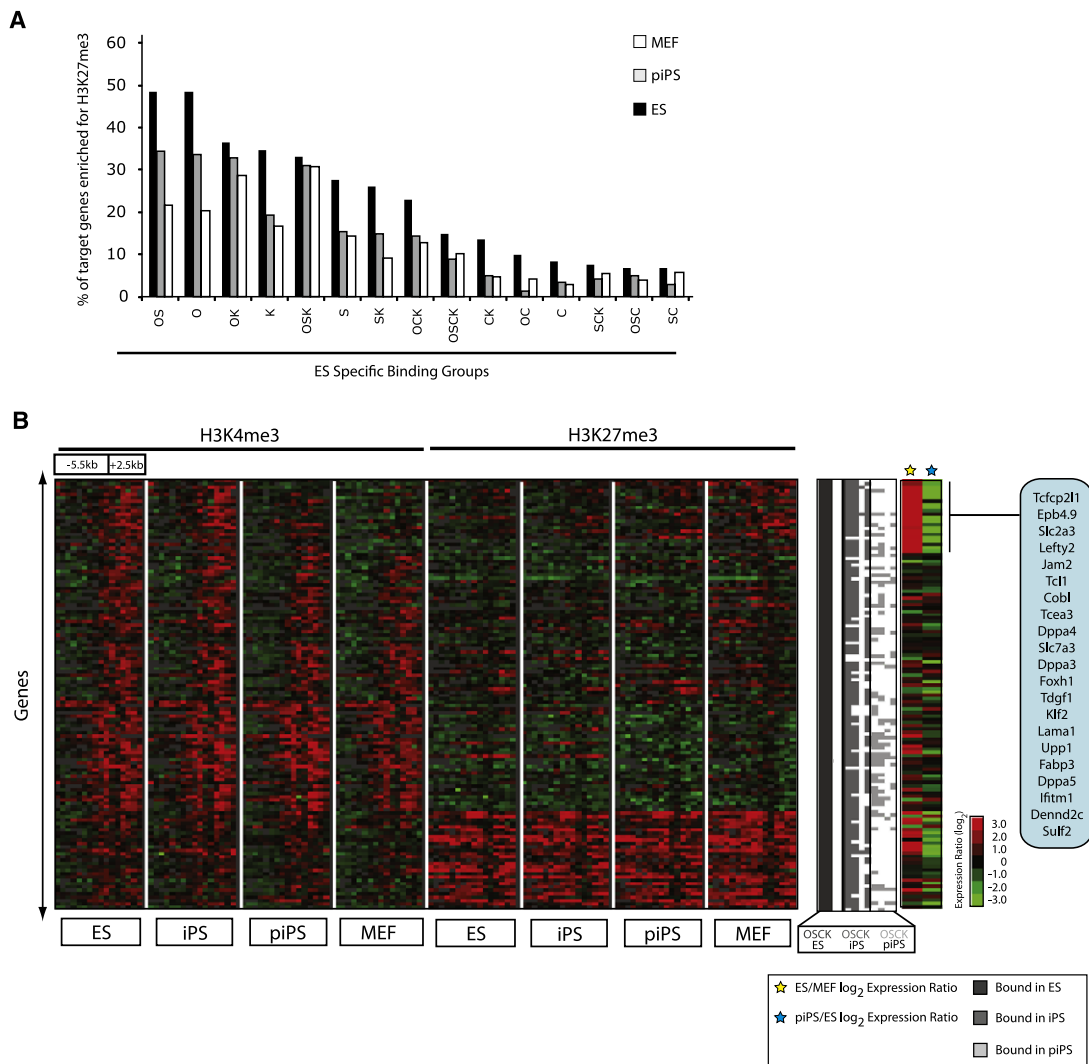
Our analysis of binding and expression in partially reprogrammed cells suggested that c-Myc does not greatly contribute to

repressive chromatin given that they are methylated at K4 and lack K27 methylation in all cell types including fibroblasts (Figures S8 and 5A). This result potentially explains why c-Myc binds in a more ES-like pattern in partially reprogrammed cells.

When specifically analyzing the methylation pattern in OSK cell ES targets (Figure 5B), we found a distinct histone methylation signature for genes that are most dramatically upregulated between ES/iPS cells and fibroblasts. Characterized by high H3K27me<sub>3</sub> and low H3K4me<sub>3</sub> enrichment in fibroblasts, these genes are strongly positive for H3K4me<sub>3</sub> and have

the activation of pluripotency regulators. Next, we wanted to directly test the contribution of each factor to the early phase of the reprogramming process and followed the levels of the somatic surface marker Thy1 upon retroviral expression of a single factor in fibroblasts. Within 2 days of infection, the population of Thy1-positive fibroblasts decreased most dramatically in cells expressing only c-Myc, and by day 4, more than half of those cells had lost the Thy1 marker (Figure 6A). This strong effect was not seen in fibroblasts infected with Klf4, Oct4, or Sox2 (Figure 6A) although the infection efficiency of each virus





**Figure 5. A Distinctive Histone Methylation Pattern for OSK Target Genes that Are Most Dramatically Upregulated in ES/iPS Cells Compared to Fibroblasts**

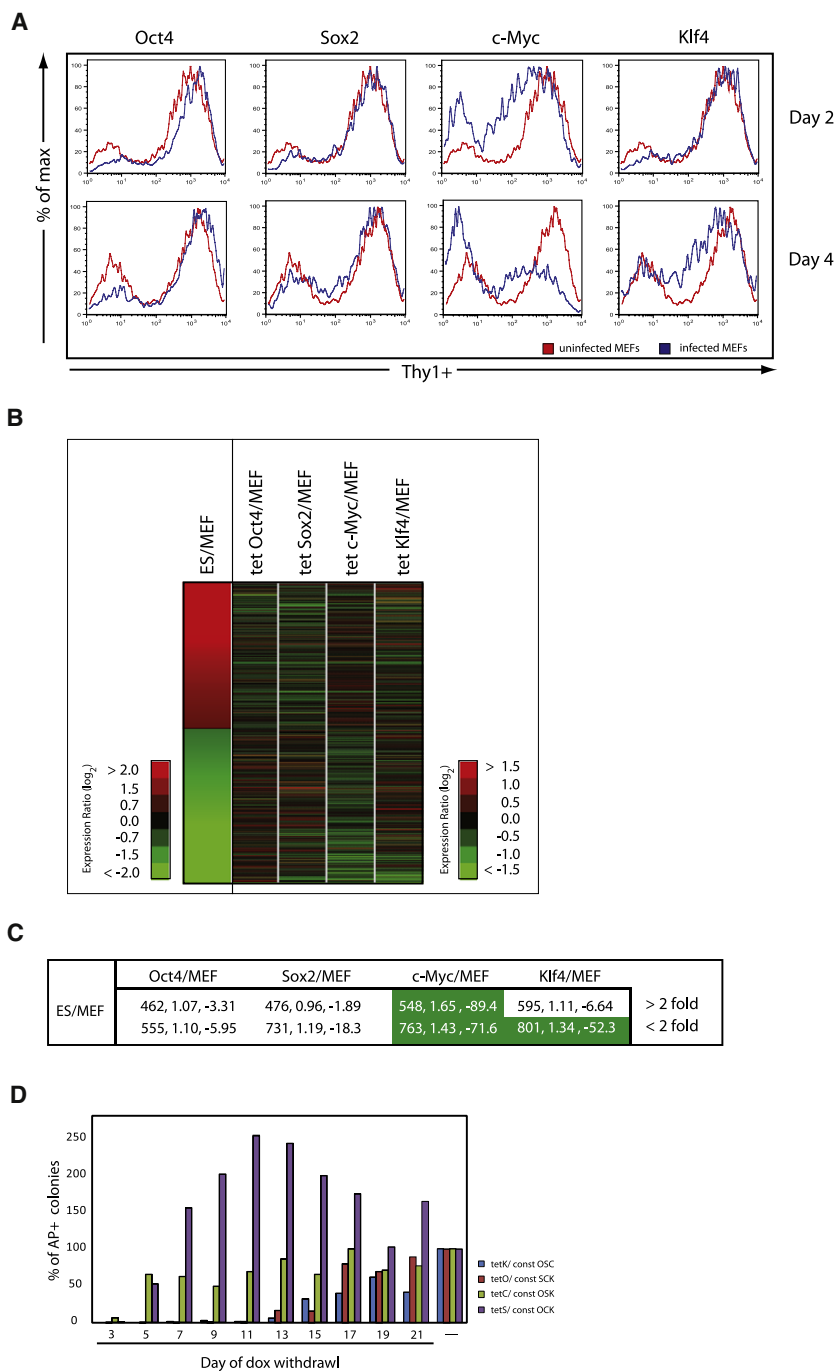
(A) Bar chart depicts the fraction of genes with enrichment of histone H3 K27 trimethylation in their promoter regions in different cell types, for genes that are bound by the indicated combination of factors in ES cells.

(B) Hierarchical clustering of histone H3 K4 and K27 trimethylation patterns in ES cells, iPS cells, partially reprogrammed cells (piPS), and fibroblasts for genes bound by OSK in ES cells. Each row represents the methylation pattern along the  $-5.5$  kb to  $+2.5$  kb promoter region relative to the transcription start site (TSS), reiterated eight times to present the data for each cell type and methylation mark. Each 8 kb promoter region is divided into sixteen 500 bp fragments that display the average log ratio of probe signal intensity with green, red, and gray representing lower-than-average, higher-than-average, and missing values for enrichment due to lack of probes in those regions, respectively. Binding of the factors in ES (dark gray), iPS (light gray), and partially reprogrammed cells (lightest gray) is presented to the right of the histone methylation data. Two sets of log<sub>2</sub> expression ratios are appended (ES/MEF and piPS/ES) and genes most highly expressed in ES cells relative to MEF are marked and listed.

was approximately the same (data not shown). When the four factors were added to fibroblasts in combinations, Thy1 downregulation was most affected when c-Myc and Klf4 were included in the mixture (Figure S9A). Collectively these results demonstrate that c-Myc mediates downregulation of the fibroblast marker most efficiently among the four factors.

To determine if this observation applies genome-wide, we investigated global expression changes that occur in fibroblasts upon individual expression of c-Myc, Klf4, Sox2, or Oct4. We first established an inducible expression system that allowed us to

start with a homogenous population of fibroblasts expressing one factor. The factors, under the control of a doxycycline-inducible promoter, were cloned into a retroviral vector that included a hygromycin resistance gene. Fibroblasts heterozygous for the reverse tetracycline transactivator in the constitutively active *Rosa26* locus (*R26* rTA/WT) were infected with individual inducible viruses, selected for hygromycin resistance, and 3 days after addition of doxycycline profiled for expression, along with an uninfected control. Addition of doxycycline resulted in the expression of the factors in almost 100% of the



**Figure 6. The Role of c-Myc in Early Steps of the Reprogramming Process**

(A) FACS plots showing Thy1 surface levels of fibroblasts 2 or 4 days post-infection with a retrovirus carrying the indicated factor (blue) in comparison to uninfected fibroblasts (red).

(B) Heatmap of expression differences between ES cells and fibroblasts (MEFs) and between fibroblast lines induced for c-Myc, Klf4, Oct4, or Sox2 expression for 3 days with doxycycline relative to an uninduced fibroblast control, for all genes more than 2-fold differentially expressed between ES cells and MEFs, ordered by decreasing expression ratio.

(C) Overlap of genes 2-fold up or down between ES/MEF and 2-fold up or down between MEFs induced to express Oct4, Sox2, Klf4, or c-Myc and uninduced MEFs. The first number presents the genes in the overlap, the second number the enrichment (described in Experimental Procedures), and the third number  $\log_{10}$  of the p value. Significant enrichments are highlighted in green.

(D) R26 rTA/WT fibroblasts were infected with three constitutively expressed retroviruses (const) and a dox-inducible retrovirus for the fourth factor (tet). Doxycycline (dox) was added until the indicated day and AP-positive colonies scored on day 25.

collagens and are involved in signaling and organ development. Together with the analysis of factor binding and expression in the partially and completely reprogrammed states, these results indicate that c-Myc enhances early steps of reprogramming by repressing fibroblast-specific expression and upregulating the metabolic program of the embryonic state.

Previously it was shown that reprogramming only occurs when all four factors are expressed for at least 8–10 days (Brambrink et al., 2008; Stadtfeld et al., 2008), but the temporal requirement for each individual factor remained untested. Based on our data, we wanted to test whether ectopic c-Myc expression is required for a shorter time period during reprogramming than that of Oct4, Sox2, and Klf4. R26 rTA/WT fibroblasts were infected with combinations of retroviruses that allowed the doxycycline-inducible expression of only one factor and

constitutive expression of the other three. Doxycycline was added 24 hr post-infection to induce expression of the “fourth” reprogramming factor and subsequently withdrawn at different time points, leading to efficient downregulation of induced transcripts within 24 hr as determined by real-time PCR (data not shown). AP-positive colonies were scored 25 days post-infection as a measure of reprogramming (Figure 6D). Fibroblasts induced to express c-Myc for 5 days gave rise to AP-positive colonies that only modestly increased in numbers when c-Myc expression was maintained for longer periods. In contrast,

cells (data not shown). In agreement with the Thy1 result, c-Myc-induced expression changes resembled those found between ES cells and fibroblasts most closely when compared to the effects of the other three factors (Figure 6B), for genes that become both up- and downregulated between ES cells and fibroblasts (Figure 6C). Furthermore, the expression changes upon ectopic c-Myc expression are most similar to those occurring in fibroblasts overexpressing all four factors, particularly for those genes that become repressed (Figure S9B). The genes downregulated in fibroblasts upon c-Myc expression include

induction of c-Myc for only 3 days resulted in dramatically fewer colonies, similar to reprogramming experiments in which c-Myc is completely omitted (Nakagawa et al., 2008; Wernig et al., 2008; data not shown), suggesting that a 5 day pulse of ectopic c-Myc expression is sufficient to obtain c-Myc-dependent reprogramming. In contrast, exogenous expression of Oct4 and Klf4 was required for at least 12 days. Surprisingly, ectopic Sox2 expression for only 5 days was enough to give rise to AP-positive colonies, although unlike for c-Myc, these colonies were more heterogeneous in morphology and greatly increased in number with prolonged exogenous expression, a result that deserves future investigation. Taken together, these data indicate that the four factors differ in their contribution to the reprogramming process and suggest that ectopic c-Myc is only required initially to attain a high reprogramming efficiency.

## DISCUSSION

This study aimed to uncover the contribution of the reprogramming factors to the induction of pluripotency. During the reprogramming process, fibroblast markers are repressed and early embryonic markers like SSEA1 are activated before expression of pluripotency regulators and the self-sustaining pluripotent state are attained (Brambrink et al., 2008; Stadtfeld et al., 2008). In agreement with these observations, fibroblast-specific genes are efficiently silenced in partially reprogrammed cells, while the embryonic program is not fully induced. Clones obtained in fibroblast reprogramming experiments with ectopic expression of only Oct4, Klf4, and c-Myc are also characterized by repression of fibroblast-specific genes although not to the same extent as in our four factor-induced partially reprogrammed cell lines (Figure S10). Thus, silencing of the somatic cell expression program appears to be an important initial step required for the induction of the ES-like expression program. Our data indicate a major contribution of c-Myc to this first step. We found that, of the four factors, c-Myc promotes the most ES-like expression changes, including the repression of fibroblast-specific genes (Figure 7A, i). Mechanisms by which c-Myc induces transcriptional repression are much less understood than its function as a transcriptional activator (Wanzel et al., 2003). Global repressive effects of c-Myc could be mediated through its interaction with the transcription factor Miz (Wu et al., 2003) or direct binding and activation of a transcriptional repressor. Interestingly, when murine fibroblasts were treated for 7 days solely with valproic acid (VPA), a histone deacetylase inhibitor that can replace c-Myc function during reprogramming, their expression started to resemble an ES-like state, including the repression of highly transcribed fibroblast-specific genes (Huangfu et al., 2008). Thus, c-Myc expression or VPA treatment may lay the framework for the efficient repression of the somatic expression and induction of the ES cell expression program.

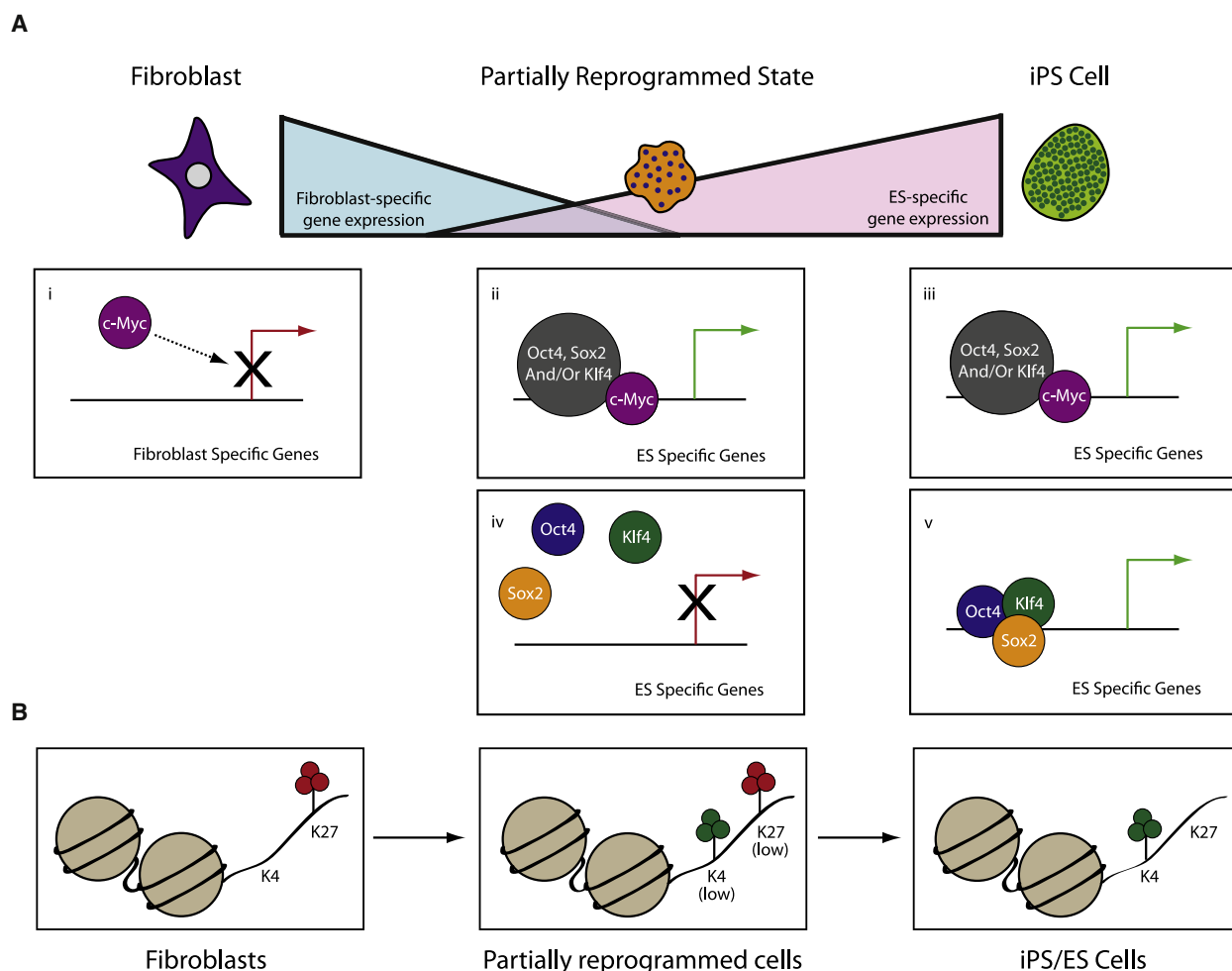
The comparison of binding patterns of the factors in iPS/ES cells and partially reprogrammed cell lines further strengthens the conclusion that the contribution of c-Myc to the reprogramming process is separable from that of the other three factors. The metabolism-related embryonic expression program is

induced by binding of combinations of all four factors (Figure 7A, ii and iii), while the activation of many regulators of the pluripotent state is dependent on co-binding of only Oct4, Klf4, and Sox2 (Figure 7A, v), which does not occur in partially reprogrammed cells (Figure 7A, iv). Indeed, ectopic expression of c-Myc is only required for the first few days of the reprogramming process. We propose that lack of Oct4, Sox2, and Klf4 co-binding in partially reprogrammed cells contributes to the failure to establish the pluripotent state. During reprogramming, Oct4, Sox2, and Klf4 can likely only stochastically overcome this binding block contributing to the low efficiency of this conversion.

An important question is why Oct4, Sox2, and Klf4 cannot associate with their ES cell target genes in partially reprogrammed cells. One explanation could be the absence of other factors, such as Nanog, that allow targeting of the reprogramming factors. These factors could form large complexes necessary for cooperative binding to target genes or could induce a conformational change of DNA allowing subsequent binding by Oct4, Sox2, and Klf4. Alternatively, the target promoters may have repressive chromatin structures that prevent appropriate binding of the factors. Indeed, our data demonstrate that a gain in histone H3K4 methylation is specifically associated with the activation of many pluripotency regulators (Figure 7B). In partially reprogrammed cells, the methylation status is not in an ES-like pattern and the reprogramming factors do not bind appropriately, suggesting that the establishment of the ES-like histone methylation pattern is either a requirement for or coincides with the recruitment of the factors. Elucidating how the methylation pattern transitions to an ES-like state and how Oct4, Sox2, and Klf4 are recruited to genes encoding pluripotency regulators should allow for the development of more efficient reprogramming strategies.

Interestingly, c-Myc is dispensable for fibroblast reprogramming as both human and murine iPS cells can be obtained in the absence of c-Myc, albeit with dramatically reduced efficiency and kinetics (Nakagawa et al., 2008; Wernig et al., 2008). The fact that activation of transcriptional regulators of pluripotency is largely independent of c-Myc binding provides an explanation for why c-Myc is not absolutely required for reprogramming. Our data indicate that reprogramming is delayed and inefficient in the absence of ectopic c-Myc expression because c-Myc greatly enhances the initial steps of reprogramming. The fact that Oct4, Sox2, and Klf4 are targeted in a more ES-like pattern in partially reprogrammed cells to genes that are co-bound by c-Myc also suggests that its presence facilitates the binding of the other factors. In addition, c-Myc normally fulfills many other functions, for example in regulating DNA replication (Dominguez-Sola et al., 2007) and global histone acetylation (Knoepfler et al., 2006), which may facilitate the reprogramming process more indirectly.

Once reprogramming is complete the binding pattern of the four factors is similar to that of ES cells, suggesting that a common binding signature has to be achieved to attain pluripotent gene expression. Differences in binding between these ES and iPS cells occur generally in genes that are bound more weakly. In the future, it will be important to determine whether any of these binding differences reflect an epigenetic memory of the starting cell population that may influence differentiation behavior.



**Figure 7. Role of the Four Factors during Reprogramming**

(A) c-Myc is a major contributor to the downregulation of fibroblast-specific genes at the beginning of the reprogramming process (i). In iPS cells, c-Myc binds and activates many ES cell-specific genes with roles in metabolic regulation, often with the other three factors (iii). For many of these target genes, binding and activation is also found in partially reprogrammed cells (ii). Target genes that are co-bound by Oct4, Sox2, and Klf4 in ES/iPS cells encode some of the most highly expressed regulators of pluripotency (v) and are not bound and activated at the partially reprogrammed state (iv). Our data suggest that the targeting of OSK to these genes represents a barrier to the reprogramming process and that c-Myc-bound genes largely become activated during earlier steps.

(B) State of histone H3K4 and K27 trimethylation within promoter regions of genes that become most highly activated during reprogramming and that are co-bound by Oct4, Sox2, and Klf4 in ES/iPS cells but not in partially reprogrammed cells.

## EXPERIMENTAL PROCEDURES

### Cell Culture and Reprogramming Experiments

ES (V6.5 and E14), iPS (1D4 and 2D4), partially reprogrammed cells (1A2 and 1B3), and three primary mouse embryonic fibroblast (MEF) lines derived from day (d) 14.5 embryos were cultured according to standard methods. Reprogrammed cell lines were grown in the presence of 1  $\mu$ g/ml puromycin. For new reprogramming experiments, the cDNAs of the four factors were cloned into the pMX (constitutive expression) and pRetroX-Tight-Hyg (Clontech, dox-inducible) retroviral vectors, which were individually transfected into PlatE packaging cells using Fugene (Roche). Viral supernatants harvested 48 hr post-infection were used to infect MEFs in the presence of 10  $\mu$ g/ml polybrene. Where indicated, MEFs heterozygous for R26 rtTA were used and cultures supplemented with 2  $\mu$ g/ml dox. Dox-containing medium was changed every 3 days until withdrawal. To obtain expression data upon induction of only one single factor, R26 rtTA MEFs were infected with the respective pRetro-virus and selected for 1 week in 100  $\mu$ g/ml hygromycin before dox induction.

### Flow Cytometry

Thy1-positive fibroblasts were sorted on a FACS ARIA (BD Biosciences) and subsequently infected. At the indicated time point, cells were harvested, passed through a 40  $\mu$ m cell strainer, incubated with PE-conjugated rat anti-Thy1 antibody (eBiosciences), and analyzed on an LSR cytometer (BD Biosciences) using the FloJo software (TreeStar).

### ChIP on Chip

Chromatin fragments associated with the respective transcription factor or histone mark were immunoprecipitated with specific antibodies (Oct4 [sc5279] and c-Myc [sc764] both SantaCruz, Sox2 [Chemicon 5603], Klf4 [R&D Biosystems AF3158], Nanog [Abcam 21603], H3K4 trimethylation [Abcam 8580], and K27 trimethylation [Upstate 07-449]) and hybridized on an Agilent promoter microarray (G4490) as described previously (Maherali et al., 2007). Probe signals were extracted and normalized using Agilent's Feature Extraction and ChIP Analytics software and binding calls made following a heuristic described previously (Boyer et al., 2005). Briefly, the algorithm takes the p values of two signal measures (normalized log ratio of the

signal intensity),  $X$  and  $\bar{X}$ , as input.  $X$  is the signal measure of an individual probe and  $\bar{X}$  the average signal measure of a probe and its closest upstream and downstream neighbors within 1 kb. Replicate samples were combined by taking the geometric mean of the  $X$  and  $\bar{X}$   $p$  values for each probe. A probe is called bound if the  $p$  value of  $\bar{X}$  is  $< .001$  and one of the following conditions is met: the  $p$  value of  $X < .001$  and the  $p$  value of either neighboring  $X$  is  $< 0.1$  OR the  $p$  value of at least two of three probes ( $X$  and its two neighbors) is  $< .005$ . A gene is called bound if any of its probes are called bound. The binding strength of a gene is defined as  $-\log_{10} p$  value( $\bar{X}$ ) of its most significantly bound probe. For histone methylation data, average probe signals were extracted in 500 bp windows as described (Maherali et al., 2007).

### Expression Data

RNA was extracted from duplicate samples of V6.5 and E14 ES cells, 1D4 and 2D4 iPS cells, 1A2 and a single sample of 1B3 partially reprogrammed cells, four samples of three different MEF lines, fibroblasts induced to express a single factor, and the uninduced control and analyzed on Affymetrix GeneChip Mouse Genome 430 2.0 arrays at the UCLA microarray core facilities. Quantile normalization was performed using the Affymetrix package (*affy*) (Bioconductor). To convert probe data into gene expression data, probes ending in “\_at” and “\_a\_at” were averaged for each gene. All gene expression data are provided in Table S4. Analysis combining averaged expression data with binding data was conducted on genes with expression  $>200$  in ES, iPS, pips, or MEF cells (provided in Table S2). Cluster 3.0 was used for hierarchical clustering and Java Treeview for visualization.

### Computational Methods

The expected number of binding events (Figure 1B) was computed assuming that binding by each factor is independent of that of the others. Individual binding probabilities were estimated from ChIP-chip data. Specific binding events are then the product of the probabilities (for example  $p(\text{OSK}) = p(\text{Oct4}) \times p(\text{Sox2}) \times p(\text{Klf4}) \times (1-p(\text{c-Myc}))$ ). The probability of binding by a specific number of factors is the sum of the probabilities satisfying that condition and the expected number for that condition is this probability multiplied by the number of genes on the array. For Figure 3B, the hamming distance,  $h$ , of a gene was defined as the number of mismatches in Oct4, Sox2, c-Myc, and Klf4 binding between ES and iPS cells or ES cells and partially reprogrammed cells, ranging from 0 for perfect match in binding to 4 for complete mismatch. The number of factors differentially bound between iPS and partially reprogrammed cells is the average of  $h(\text{iPS}, \text{ES}) - h(\text{piPS}, \text{ES})$ . Significance was determined using the binomial distribution with  $k$  equal to the number of times  $h(\text{piPS}, \text{ES})$  was greater than  $h(\text{iPS}, \text{ES})$  in a given ES binding cluster, (i.e.,  $p$  value =  $P(X > k)$ , where  $X \sim \text{Bin}(n, 0.5)$ ). Motif scanning was done using methods developed by Zhou et al. (2007; see Supplemental Data). Enrichment determined in Figure 6C is the conditional probability of a gene being 2-fold upregulated in the dox-induced MEF/uninduced MEF given that a gene is 2-fold up in ES/MEF divided by the marginal probability of being 2-fold up in dox-induced MEF/uninduced MEF. The hypergeometric distribution was used to determine significance.

### ACCESSION NUMBERS

Data are available at GEO under GSE14012.

### SUPPLEMENTAL DATA

Supplemental Data include a detailed description of the motif scanning method, 10 figures, and 7 tables and can be found with this article online at [http://www.cell.com/supplemental/S0092-8674\(09\)00007-5](http://www.cell.com/supplemental/S0092-8674(09)00007-5).

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