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The dosage of Patz1 modulates reprogramming process

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The acquisition of pluripotent cells can be achieved by combined overexpression of transcription factors Oct4, Klf4, Sox2 and c-Myc in somatic cells. This cellular reprogramming process overcomes various barriers to re-activate pluripotency genes and re-acquire the highly dynamic pluripotent chromatin status. Many genetic and epigenetic factors are essentially involved in the reprogramming process. We previously reported that Patz1 is required for maintenance of ES cell identity. Here we report that Patz1 plays an inhibitory role in OKSM-induced reprogramming process since more iPS colonies can be induced from Patz1^{+/-} MEFs than wild type MEFs; while the addition of Patz1 significantly repressed reprogramming efficiency. Patz1^{+/-} MEFs can surpass the senescence barrier of *Ink4a/Arf* locus, thus enhancing iPS colonies formation. Moreover, Patz1^{+/-} MEFs displayed higher levels of acetylated histone H3, H3K4me2, H3K4me3, H3K36me3 and lower levels of histone H3K9me3 and HP1 α , indicating that heterozygous knockout of *Patz1* results in a globally open chromatin which is more accessible for transcriptional activation. However, Patz1^{-/-} MEFs gave the lowest reprogramming efficiency which may result from cell senescence triggered by up-regulated *Ink4a/Arf* locus. Together, we have demonstrated that the dosage of Patz1 modulates reprogramming process via significantly influencing cell senescence, proliferation and chromatin structure.

Pluripotent cells possess the full potential to differentiate into all three germ layers of cell types. Recent studies have shown that the pluripotent state can be generated from mouse somatic cells by ectopic expression of transcription factors Oct4, Sox2, Klf4 and c-Myc (OKSM)¹. These induced pluripotent stem cells (iPSCs) resemble embryonic stem (ES) cells, possessing the abilities to self-sustain pluripotency and to differentiate into many cell types. This remarkable breakthrough endows stem cells with great application potentials in regenerative medicine. However, the acquisition of induced pluripotency remains a relatively slow and inefficient process. Furthermore, the comprehensive mechanism of reprogramming has not been fully elucidated.

There are cellular “barriers” for a somatic cell to overcome in order to be reprogrammed into a pluripotent stem cell. Understanding what regulate these reprogramming barriers can provide insight to better modulate reprogramming efficiency. Studies in past several years have revealed that the cell fate conversion from somatic cells to iPSCs is a dynamic process that involves a cascade of cellular events, such as silencing lineage-specific genes and reactivation of pluripotency genes, mesenchymal to epithelial transition (MET), overcoming cellular senescence and acquisition of cell immortality, reactivation of X-chromosome and resetting the chromatin signatures². Cellular senescence has been reported as a barrier which limits reprogramming efficiency at the initial stage³⁻⁵. The *Ink4a/Arf* tumor suppressor locus has a critical role in regulating cellular senescence in many types of cells⁶. For instance, silencing *Ink4a/Arf* locus, or ablation of its activator Jmjd3 had been shown to reduce cellular senescence and significantly improves reprogramming efficiency^{5,7}. Switch of epigenetic landscape is another layer of reprogramming barriers. Pluripotent cells possess a highly plastic chromatin structure which is globally open and decondensed with a higher ratio of active to repressive histone marks. The establishment of unique “bivalent” domains, which are marked by both active H3K4 trimethylation and repressive H3K27 trimethylation, is prerequisite for successful generation of iPSCs⁸. Hence the global repressive chromatin state in somatic cells is a major roadblock for reprogramming. It is noteworthy that recent studies highlighted the importance of the interplay between epigenetic factors and reprogramming transcription factors to facilitate the chromatin resetting



for pluripotency acquisition during reprogramming process. Inhibition of chromatin condensation, for example, ablation of NuRD/Mbd3 repressor complex, inhibition of HDAC activity or H3K4me3, strongly increases iPSC induction^{9–12}. Likewise, factors which have been proposed to promote active chromatin, such as H3K4me3 effector Wdr5 and H3K27me3 “eraser” Utx, facilitate the acquisition of pluripotency^{13,14}. Nevertheless, more studies are needed to fully elucidate the exact mechanism of how these epigenetic factors impact on the kinetics of OKSM-induced reprogramming.

Here, we report a regulator, Patz1 that modulates reprogramming efficiency dependent on cellular context. Patz1, also known as Zfp278 or MAZ-related factor (MAZR), is a POZ-, AT-hook, and Kruppel zinc finger protein. It belongs to POK (POZ and kruppel like zinc finger) family of transcription repressors. Interestingly, the biological functions of POK proteins are generally associated with other proteins interacting with the POZ domain¹⁵. Patz1 has been reported as a transcription regulator that can activate *Myc* through its interaction with Bach2, or act as a corepressor that attenuates RNF4-mediated androgen receptor-dependent transcription activation^{16,17}. Through binding with nuclear receptor corepressor (NCoR) complex, Patz1 negatively regulates CD8 expression¹⁸. Patz1 is also found to participate in BCL6-mediated transcription repression by direct interaction with BCL6¹⁹. Therefore Patz1 may function as an architectural transcription factor that can act either as activator or repressor depending on the protein it interacts with.

In addition, Patz1 is also involved in carcinogenesis, including colorectal, glioma, testicular and breast tumors^{20–23}. On the other instances, observations of rearrangement of *PATZ1* allele in small round cell sarcoma, tumor development in *Patz1*-knockout mice and its involvement in p53 pathway support a potential tumor suppressor role of Patz1^{19,24,25}. Besides, Patz1 has an emerging role of inhibiting cellular senescence in endothelial cells and MEFs^{25,26}. All these results indicate the function of Patz1 is solely context dependent.

Interestingly, Patz1 also has a critical role in embryo development. Majority of *Patz1*-knockout mice underwent prenatal death and the mice that survived showed a general growth retardation compared to WT mice^{19,27}. Given its predominant expression in ICM and ESCs, Patz1 has been identified as an important regulator of pluripotency that is required for maintaining ESC in undifferentiated state^{28,29}. Because of the versatility of Patz1 functions and its critical role in embryo development, we question its role in reprogramming process.

Here we show that overexpression of Patz1 inhibits OKSM-mediated reprogramming process, while partial depletion of *Patz1* enhances iPSC generation. Further examination revealed that the inhibitory role of Patz1 in the reprogramming process is possibly mediated by its potential role in c-Myc transcription regulation, cell senescence and chromatin regulation. Heterozygous knockout of *Patz1* in MEFs down-regulates repressive histone marks and up-regulates active histone marks, creating a more open chromatin accessible for transcriptional activation of pluripotency factors, thus facilitating the reprogramming. Moreover, *Patz1*^{+/-} MEFs appeared to surpass the senescence barrier of *Ink4a/Arf* locus, thus being easier to be reprogrammed. Complete loss of *Patz1*, however, resulted in cellular senescence that significantly blocks iPSC formation. Taken together, our data have revealed that Patz1, though a pluripotency factor in ES cells, exerts its function in reprogramming in a dosage-dependent way and possibly through its crosstalk with epigenetic factors. Our study thus shed light on how somatic Patz1 context affects reprogramming efficiency, opening a new route to reboot pluripotency via modulating the level of Patz1.

Results

Patz1 inhibits reprogramming process. To explore the role of Patz1 in the reprogramming process, we first added Patz1 together with the

canonical reprogramming factors OKSM to retrovirally infect MEFs. The MEFs harboring a *Pou5f1*-GFP transgene reporter, which will express GFP if *Pou5f1* promoter is reactivated, were used to identify putative iPSC colonies. The number of GFP⁺ colonies therefore served as an evaluation of reprogramming efficiency³⁰.

As shown in Figure 1a, the number of GFP⁺ colonies that were generated with OKSM infection is 70% less than OKSM control. Alkaline phosphatase (AP) staining also showed a significant reduction of iPSC colony formation with the addition of Patz1 (Fig. 1b). This indicates that Patz1 represses reprogramming process. To confirm the inhibitory role of Patz1 in reprogramming, *Patz1*-knockdown retrovirus was generated and infected MEFs together with OKSM. We observed that MEFs deficient in *Patz1* were reprogrammed with higher efficiency than in WT MEFs, as indicated by both the yield of GFP⁺ colonies and AP staining results (Fig. 1c,d).

iPSCs generated from OKSM and OKSMshP expressed pluripotency marker genes Oct4, Nanog and SSEA-1 (Supplementary Fig. 1a,c). The pluripotency of these iPSCs were next examined by embryoid body (EB) formation assays, showing that all these iPSCs were able to express all three germ layer markers upon differentiation (Supplementary Fig. 1b,d). These showed that OKSM and OKSMshP iPSC cells are pluripotent stem cells.

Addition of Patz1 blocks reactivation of *Pou5f1* promoter. To further investigate Patz1's inhibitory role during reprogramming process, we infected *Pou5f1*-GFP MEFs with Patz1 overexpression retrovirus 0 h, 24 h, 48 h and 72 h after OKSM infection. GFP⁺ colonies were counted daily until D12 when AP staining was performed. As shown in Figure 1e, compared to OKSM + P/0 h, there were more AP⁺ colonies formed if Patz1 was added at a later stage of reprogramming process. OKSM + P/72 h gave rise to the highest number of AP⁺ colonies, but it was still obviously less than OKSM control (Fig. 1f). This implies that Patz1 is a strong inhibitor for the initiation of reprogramming.

Similarly, number of GFP⁺ colonies was reduced with addition of Patz1 into OKSM and the efficiency tended to be further decreased when Patz1 was added 24 h, 48 h or 72 h later, suggesting that Patz1 can significantly repress *Pou5f1* promoter regardless of its addition point (Fig. 1g). Previous studies have revealed that AP marker can be gained during the initial stage while expression of endogenous-Oct4 is an indicator for a maturation phase of reprogramming³¹. Therefore, it could be inferred that Patz1 acts not only as an inhibitor for the initiation of reprogramming, but also as a potential roadblock for the progression from the early-intermediates to the matured iPSCs during reprogramming.

Heterozygous knockout of *Patz1* promotes reprogramming. Given that overexpression of Patz1 inhibits reprogramming while knockdown of *Patz1* enhances reprogramming, we surmised that lower Patz1 facilitates iPSC cell generation and deletion of *Patz1* may further significantly enhance reprogramming. To test our hypothesis, we next investigated the impact of heterozygous knockout and homozygous knockout of *Patz1* on cellular reprogramming.

Patz1^{+/-} MEFs and *Patz1*^{-/-} MEFs were generated as previously described²⁷. The expression levels of Patz1 were confirmed at both protein and mRNA levels (Fig. 2a,b). We infected these three types of MEFs with OKSM. As expected, *Patz1*^{+/-} MEFs produced the greatest number of Oct4⁺ colonies and AP⁺ colonies (Fig. 2c,d). Surprisingly, iPSC formation was significantly repressed in *Patz1*^{-/-} MEFs. Similar to previous observations, when Patz1 is overexpressed in WT MEFs, the generation of iPSC colonies was inhibited (Fig. 2e,f). This again reaffirmed the repressive impact of Patz1 in cellular reprogramming.

WT and *Patz1*^{-/-} MEFs derived iPSCs displayed characteristics of pluripotent cells. They expressed pluripotency markers and were able to differentiate to express all three lineage markers in EB formation

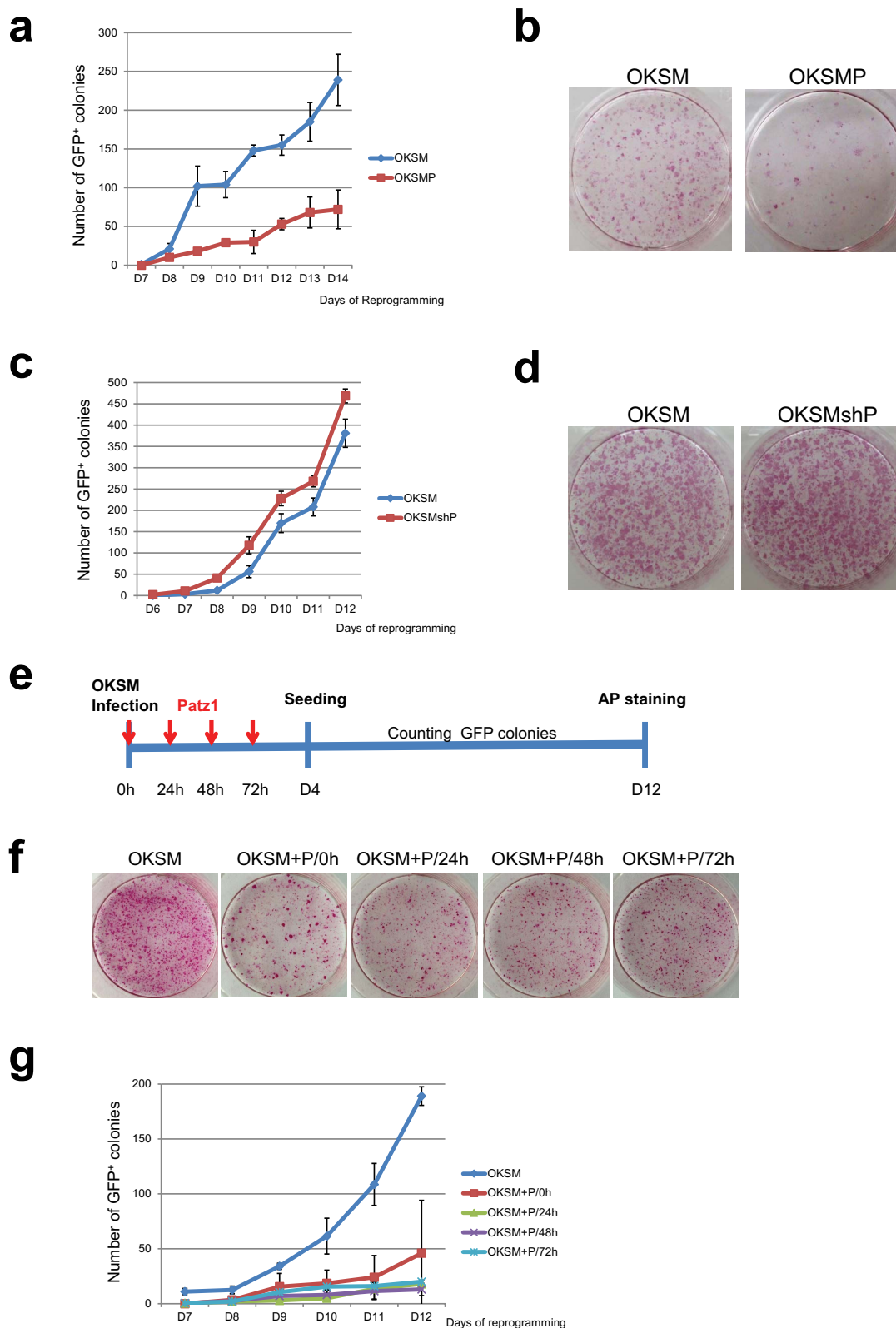


Figure 1 | Patz1 inhibits OKSM reprogramming process. (a) Addition of Patz1 significantly reduces GFP⁺ colonies induction in OKSM mediated reprogramming. The numbers of GFP⁺ colonies were counted daily from D6 till D12. Data represent means \pm SD of two independent experiments. (b) AP staining results indicate that there are less iPSC generated with OKSMP. (c) OKSMshP showed a higher reprogramming efficiency than OKSM control. (d) More AP colonies were obtained from OKSMshP than OKSM. (e) Schematic figure to show that *Pou5f1*-GFP MEFs were infected with Patz1 overexpressing retrovirus 0 h, 24 h, 48 h and 72 h after OKSM infection. The numbers of GFP⁺ colonies were counted everyday till D12, at which time point AP staining assays were performed. (f) Addition of Patz1 at different reprogramming points inhibits AP colony formation. (g) Overexpression of Patz1 represses activation of *Pou5f1* promoter in OKSM reprogramming, measured as GFP⁺ colonies number. Data represent means \pm SD of two independent experiments.

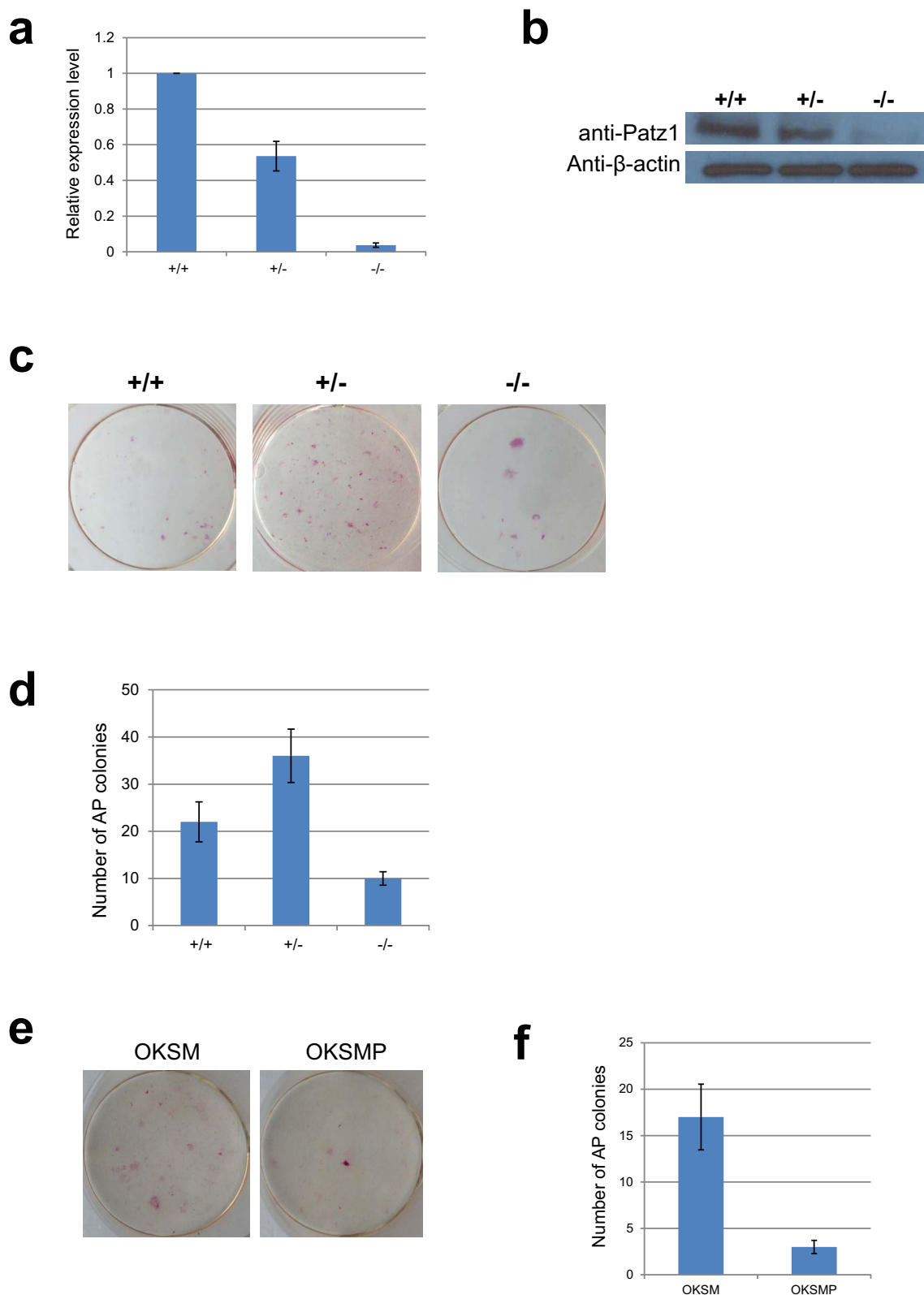


Figure 2 | Heterozygous knockout of *Patz1* promotes iPSC generation. (a) Relative mRNA level of *Patz1* in *Patz1*^{+/+}, *Patz1*^{+/-} and *Patz1*^{-/-} MEFs as demonstrated by real time PCR. The relative expression level was normalized against β -actin and *Patz1*^{+/+} MEFs were used as control. (b) *Patz1* protein expression in *Patz1*^{+/+}, *Patz1*^{+/-} and *Patz1*^{-/-} MEFs as assessed by western blot. β -actin protein levels served as a loading control. (c) AP staining results of iPSCs induced from *Patz1*^{+/+}, *Patz1*^{+/-} and *Patz1*^{-/-} MEFs. *Patz1*^{+/-} MEFs showed the highest reprogramming efficiency, while lowest number of iPSCs were generated from *Patz1*^{-/-} MEFs. (d) Quantification of AP colonies generated from *Patz1*^{+/+}, *Patz1*^{+/-} and *Patz1*^{-/-} MEFs. The numbers of AP colonies were counted under microscope after AP staining. Data represent means \pm SD of two independent experiments. (e) Representative results of AP staining of iPSC colonies generated by OKSM reprogramming in *Patz1*^{+/+} MEFs with or without overexpression of *Patz1* (f) Quantification of the experiment shown in (e). Data represent means \pm SD of two independent experiments.



assay (Supplementary Fig. 1e,f). However, the iPSCs derived from *Patz1*^{+/-} MEFs were difficult to passage and died gradually, indicating that a proper level of *Patz1* might be required for pluripotency induction in the later deterministic reprogramming stage. This is consistent with our previous report that depletion of *Patz1* impairs mESC pluripotency and self-renewal²⁹. Yet how *Patz1* dosage impacts on the derivation of healthy iPSCs is a critical issue that warrants further investigations.

***Patz1* acts through repression of *c-Myc* to inhibit iPSC induction.**

Previous studies have shown that *Patz1* acts either as an activator or a repressor for *c-Myc* transcription depending on the cellular context. Because *c-Myc*, the well-known reprogramming enhancer, regulates diverse cellular process during reprogramming^{31,32}, we therefore asked whether the inhibitory effect of *Patz1* is mediated through regulating *c-Myc*. We first infected *Pou5f1-GFP* MEFs with OKSP or OKSshP to examine the impact of *Patz1* on reprogramming in the absence of *c-Myc* retrovirus. As indicated in Figure 3, more iPSCs were generated from OKSshP and less iPSCs were derived from OKSP. In addition, the repressive effect of *Patz1* seems to be more striking in the absence of *c-Myc*. This suggests that *c-Myc* may counteract the inhibitory effect of *Patz1* overexpression during reprogramming.

Our previous ChIP-seq results in mESCs revealed that *c-Myc* is one of the binding targets of *Patz1*²⁹. ChIP using anti-*Patz1* antibody showed that indeed *Patz1* was bound to an intronic region of *c-Myc* in MEFs (Fig. 3e). We next examined the *c-Myc* level in *Patz1* OE, *Patz1*^{+/+}, *Patz1*^{+/-} and *Patz1*^{-/-} MEFs, showing that *c-Myc* was significantly repressed in *Patz1* OE MEFs, whereas it was obviously up-regulated in *Patz1*^{+/-} and *Patz1*^{-/-} MEFs (Fig. 3f). Interestingly, the level of p16, a major marker for cellular senescence, and p53 were up-regulated in *Patz1* OE MEFs (Fig. 3g). p53 and *c-Myc* are known to have opposite roles in diverse cellular processes³³⁻³⁶. Based on these results, it appears that the inhibitory effect of *Patz1* in the reprogramming process could be partially rescued by overexpression of *c-Myc*, implying that *Patz1* could act through repressing *c-Myc* to inhibit iPS cell induction.

Global gene expression analysis of *Patz1*^{+/-}, *Patz1*^{+/-} and *Patz1*^{-/-} MEFs. To better understand how *Patz1* is involved in the reprogramming process, we performed gene expression microarray analysis to examine the gene expression profiles in *Patz1*^{+/+} wild type (WT), *Patz1*^{+/-} and *Patz1*^{-/-} MEFs. As shown in Figure 4a, compared to WT MEFs, 1286 genes were down-regulated by more than 40% and 740 genes were up-regulated by more than 2 fold in *Patz1*^{+/-} MEFs. The same cutoff values gave 1353 down-regulated genes and 677 up-regulated genes in *Patz1*^{-/-} MEFs (Supplementary Fig. 2). These up-regulated or down-regulated genes were further grouped into 2 clusters according to their different levels in *Patz1*^{+/-} and *Patz1*^{-/-} MEFs respectively. Gene ontology (GO) analysis was conducted for each cluster of genes. Transcription regulation was one of the enriched terms in both up-regulated and down-regulated genes, which supports the role of *Patz1* as a transcription regulator. Many genes related to development, cell specification or differentiation were found to be altered, reaffirming the requirement of *Patz1* for a proper embryo development²⁷. Many neurophysiological terms are enriched, which is consistent with previous finding that *Patz1* is essentially involved in nervous system development and functions²⁷. Interestingly, a number of MET inducing genes were up-regulated and genes facilitating EMT were down-regulated in *Patz1*^{+/-} and *Patz1*^{-/-} MEFs, indicating that *Patz1* could be a potential regulator in MET induction during reprogramming process (Fig. 4b).

Moreover, some genes related to chromatin organization and epigenetic regulation of gene expressions were found within the up-regulated cluster. Interestingly, expressions of many histone deacetylases and acetylases were altered (Fig. 4c). Histone deacetylases,

such as *Hdac2*, *4*, *11*, were generally down-regulated upon *Patz1* loss, while histone acetylase *Hat1*, *Kat2a* were up-regulated. This implies that *Patz1* may crosstalk with many epigenetic factors and modulate chromatin modification.

It is noteworthy that there were a group of genes that were reversely changed between *Patz1*^{+/-} and *Patz1*^{-/-} MEFs, as indicated in cluster iii in Figure 4a. GO analysis indicated that these genes, which were down-regulated in *Patz1*^{+/-} MEFs but up-regulated in *Patz1*^{-/-} MEFs, were enriched in programmed cell death and apoptosis (Fig. 4d). Indeed previous studies have shown that *Patz1*^{-/-} MEFs usually underwent premature cellular senescence and grew significantly slower compared to their WT counterparts, while *Patz1*^{+/-} MEFs showed a much faster growth rate than WT MEFs (Supplementary Fig. 3). Therefore, we concluded that *Patz1*^{-/-} MEFs can be distinguished from *Patz1*^{+/-} MEFs by severe cell senescence.

***Patz1*^{+/-} MEFs surpass *Ink4a/Arf* locus barrier in reprogramming whereas knockout of *Patz1* induces cellular senescence.**

Among those reversely changed genes demonstrated in our microarray data, we found that *Cdkn2a* was significantly up-regulated in *Patz1*^{-/-} MEFs, but not in *Patz1*^{+/-} MEFs. *Cdkn2a* (also known as p16), encoded by *Ink4a*, is a key effector of cellular senescence. Recent studies have revealed that H3K27me3 level at *Ink4a/Arf* is decreased in response to expressions of reprogramming factors and silencing of this locus allows for an efficient reprogramming³⁻⁵. Therefore we hypothesized that *Patz1* may regulate this *Ink4a/Arf* locus to inhibit reprogramming process.

We examined the level of *Cdkn2a* in *Patz1*^{+/-} and *Patz1*^{-/-} MEFs by western blot. Indeed expression of *Cdkn2a* was reduced in *Patz1*^{+/-} MEFs, but was up-regulated in *Patz1*^{-/-} MEFs (Fig. 5a). Furthermore, our results of ChIP experiments showed that the active histone mark H3K4me3 was significantly decreased at *Ink4a* locus whereas the repressive mark H3K27me3 was much higher at *Ink4a* and *Arf* loci in *Patz1*^{+/-} MEFs (compared with wild type of *Patz1*^{+/+} MEFs) (Fig. 5b,c). We next harvested RNAs from *Patz1*^{+/+}, *Patz1*^{+/-} and *Patz1*^{-/-} MEFs 5 days after OKSM infection to examine the levels of *Ink4a* and *Arf* in response to reprogramming factors. We found that at day 5 of reprogramming *Ink4a* and *Arf* mRNA levels were significantly increased in *Patz1*^{-/-} MEFs compared to the WT counterparts, whereas their levels were clearly reduced in *Patz1*^{+/-} MEFs (Fig. 5d). This reinforced our hypothesis that the *Ink4a/Arf* locus is silenced in *Patz1*^{+/-} MEFs and the roadblock of *Ink4a/Arf* locus was surpassed in *Patz1*^{+/-} MEFs thus contributing to the faster cell division and higher reprogramming rate of these cells compared to wild-type controls. Therefore *Patz1*^{+/-} MEFs can then be easily reprogrammed.

***Patz1* negatively regulates reprogramming by modulating global histone modifications in MEFs.**

Patz1 belongs to POK family of transcription repressors and the POZ domain is known to interact with corepressor complexes to negatively regulate gene transcriptions. As a number of terms related to chromatin assembly, organization and epigenetic regulations of gene expressions were enriched among the up-regulated genes in *Patz1*^{+/-} and *Patz1*^{-/-} MEFs (Fig. 4a,c), we next explored the roles of *Patz1* in the epigenetic regulation.

Since *Patz1* has been shown to interact with *Ncor1* and *Sirt1*^{18,26}, we first examined the global histone acetylation levels. H3Ac level was higher in *Patz1*^{+/-} MEFs and was significantly repressed in *Patz1* OE MEFs (Fig. 6a,b). This suggested that the level of *Patz1* is negatively correlated with histone acetylation level. In addition, our microarray results revealed that a number of histone acetylases and deacetylases were altered in *Patz1*^{+/-} MEFs (Fig. 4c). Using ChIP analysis, we also found that *Patz1* could bind to many histone deacetylation related genes, indicating that *Patz1* may potentially regulate these genes to modulate histone acetylation (Supplementary Fig.

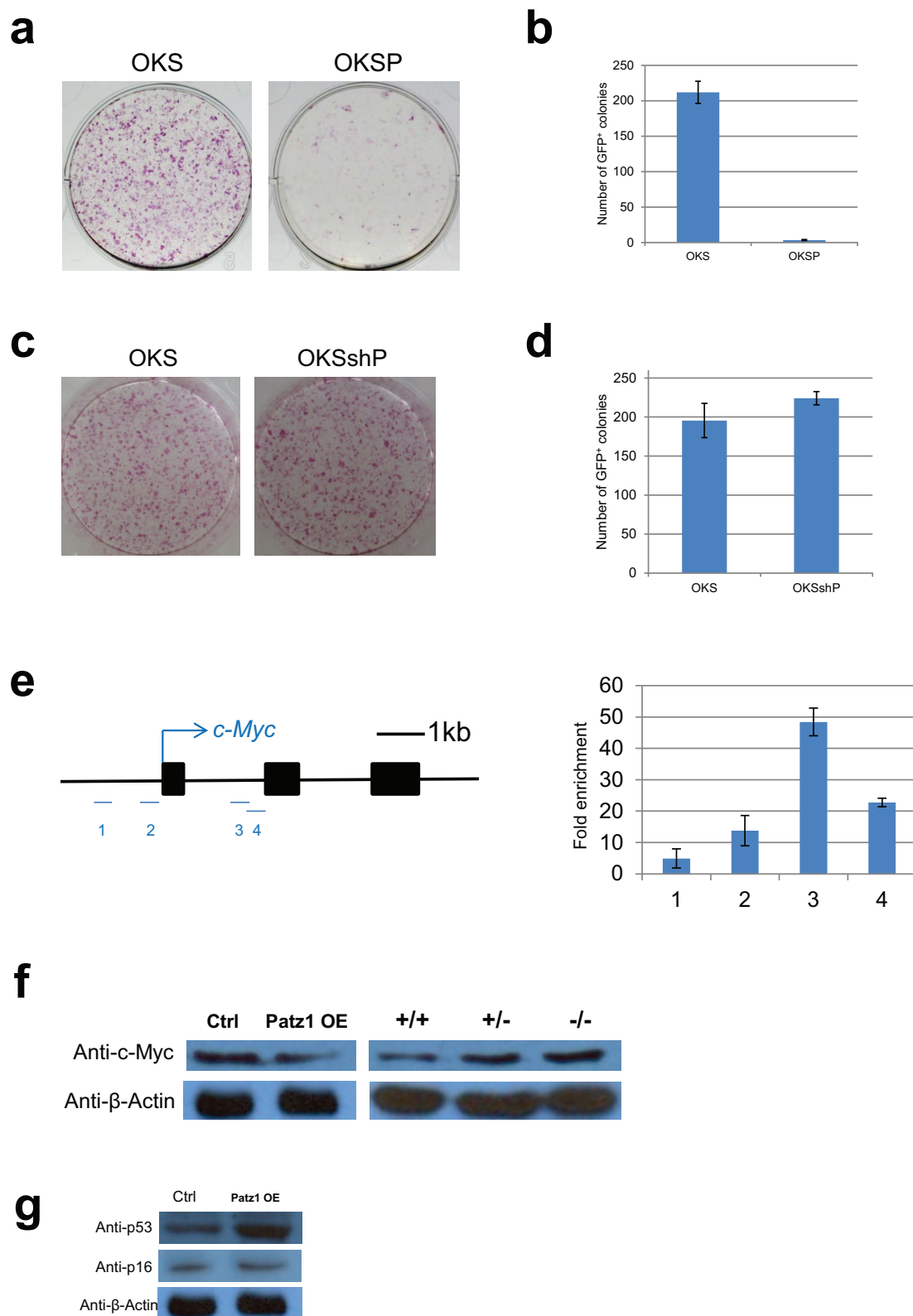
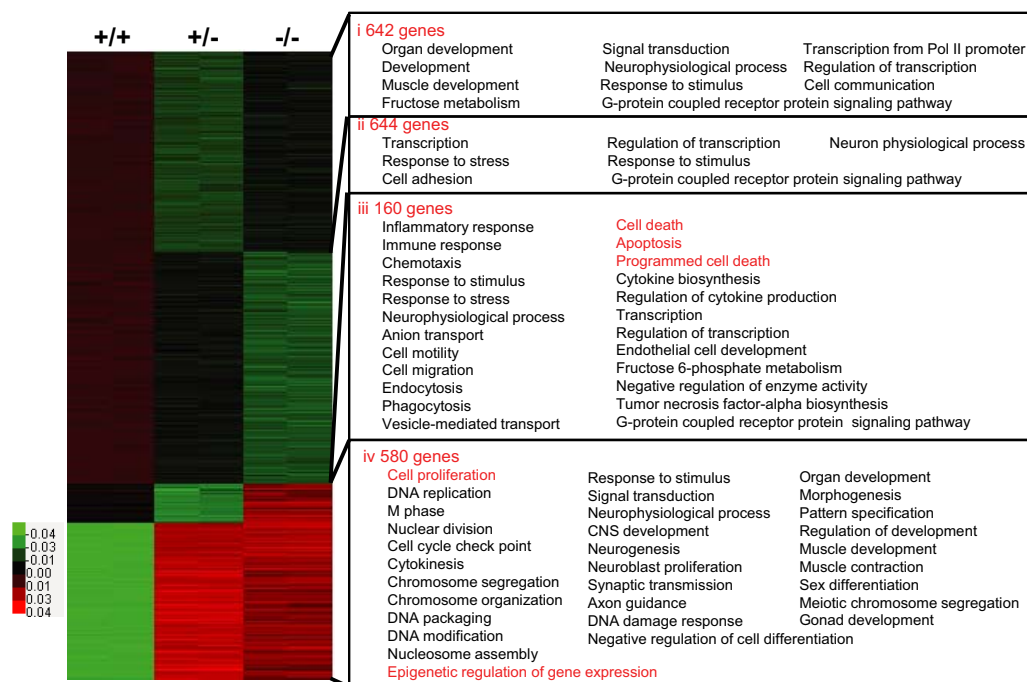


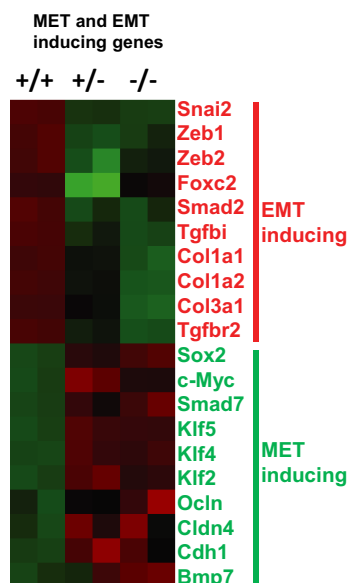
Figure 3 | **Patz1 inhibits reprogramming process possibly via regulation of *c-Myc*.** Representative results of AP staining after reprogramming of *Pou5f1-GFP* MEFs with OKS and OKSP. There were less iPSCs generated when Patz1 was added to the OKS cocktail. (b) *Pou5f1* promoter activity, as assessed by GFP⁺ colonies number, was highly decreased in OKSP versus OKS induced iPSCs. (c) *Patz1* knockdown increased AP colony formation in OKS reprogramming. (d) More GFP⁺ colonies were generated with OKSshP compare with OKS. (e) Patz1 binds to *c-Myc* in WT MEFs. (f) Cropped blots showing that overexpression of Patz1 repressed *c-Myc* protein level in MEFs; while *c-Myc* expression was up-regulated in Patz1^{+/-} or Patz1^{-/-} MEFs. (g) Cropped blots showing that overexpression of Patz1 induced up-regulation of p16 and p53. β-actin served as loading control.



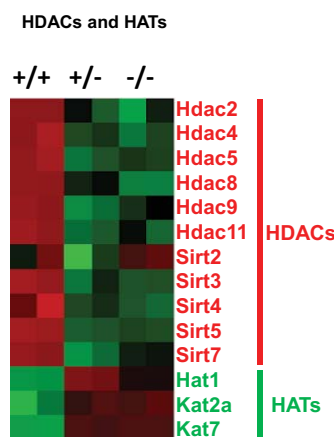
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b



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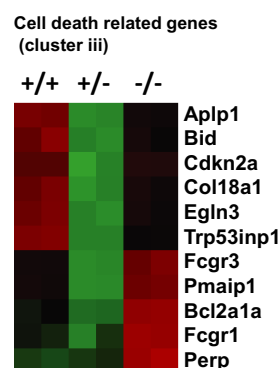


Figure 4 | Gene expression profiles in *Patz1*^{+/+}, *Patz1*^{+/-} and *Patz1*^{-/-} MEFs. (a) Heatmap of genes selected according to the fold change (>2.0 or <0.6) in *Patz1*^{+/-} MEFs compared to *Patz1*^{+/+} MEFs. Relative highly expressed genes are shown in red and low expressed genes in green. Compared to WT MEFs, 1286 genes (clusters i and ii) were down-regulated by more than 40% and 740 genes were up-regulated (clusters iii and iv) by more than 2 fold in *Patz1*^{+/-} MEFs. These up-regulated or down-regulated genes were further grouped into 2 clusters with Cluster 3.0 according to their different levels in *Patz1*^{+/-} and *Patz1*^{-/-} MEFs respectively. Heatmaps were visualized using Java Treeview. GO analysis (GATHER) was performed with each cluster of genes and enriched GO terms ($p < 0.05$) were selected and classified into groups accordingly. (b) List of EMT or MET inducing genes which expressions were changed in *Patz1*^{+/-} and *Patz1*^{-/-} MEFs compared to WT MEFs. The trend indicates that EMT inducing genes were generally repressed whereas MET inducing genes were up-regulated upon *Patz1* loss. (c) List of histone deacetylase and acetylase genes, whose expression is changed in *Patz1*^{+/-} and *Patz1*^{-/-} compared to WT MEFs. Histone deacetylases were generally down-regulated, while histone acetylases were up-regulated, in *Patz1*^{+/-} and *Patz1*^{-/-} MEFs compared to WT control. (d) List of cell death related genes differentially expressed in *Patz1*^{+/-} and *Patz1*^{-/-} compared to WT MEFs. All these genes belong to the cluster iii, i.e. up-regulated in *Patz1*^{-/-} MEFs whereas down-regulated in *Patz1*^{+/-} MEFs compared to WT MEFs. Each selected gene was taken as individual tiles from the thumbnail-dendrogram duplicates.

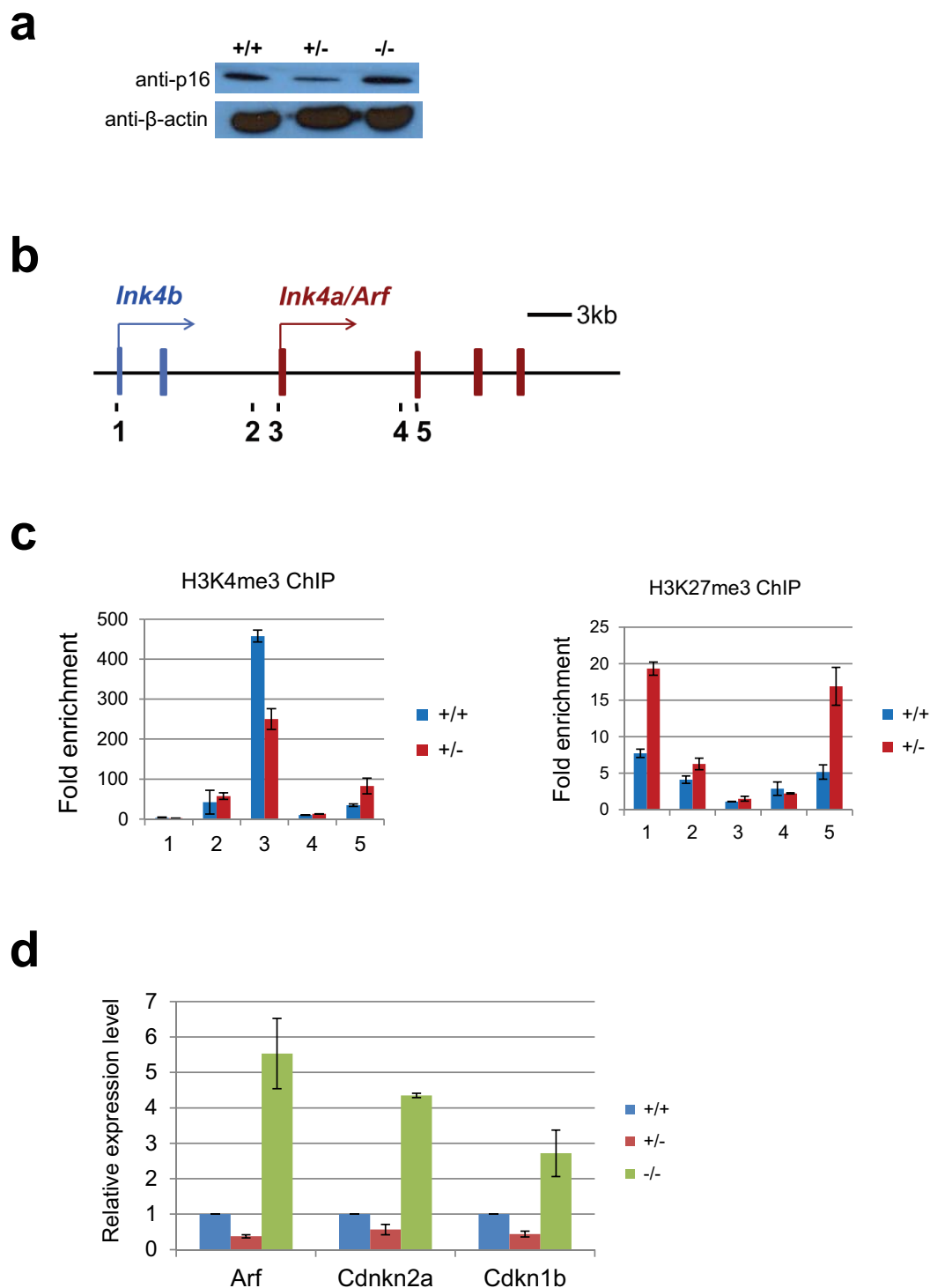


Figure 5 | *Ink4a/Arf* locus is activated in *Patz1*^{-/-} MEFs. (a) Expression levels of *Cdkn2a* in *Patz1*^{+/+}, *Patz1*^{+/-} and *Patz1*^{-/-} MEFs. WB results showed increased expression of *Cdkn2a* in *Patz1*^{+/-} MEFs and repressed expression in *Patz1*^{-/-} MEFs. Cropped blots were used here. The uncropped blot image was available in Supplementary Figure 7. (b) Schematic presentation of location of real time PCR primers at *Ink4a/Arf* locus. (c) ChIP results showed a reduced level of active histone mark H3K4me3 and elevated level of repressive histone mark H3K27me3 in *Patz1*^{+/-} MEFs. Realtime PCR were conducted to quantify the fold enrichment as relative to input, and the fold changes were normalized against an intragenic control region on chromosome 17. (d) Relative mRNA levels of *Ink4a*, *Ink4b* and *Arf* in *Patz1*^{+/+}, *Patz1*^{+/-} and *Patz1*^{-/-} MEFs 5 days after OKSM infection. Note that at day 5 of reprogramming *Ink4a*, *Ink4b* and *Arf* mRNA levels were significantly increased in *Patz1*^{-/-} MEFs compared to the WT counterparts, whereas their levels were clearly reduced in *Patz1*^{+/-} MEFs.

4). Indeed when *Patz1* was overexpressed, the level of *Hdac2*, *Hdac3* and *Sin3a* were significantly increased, suggesting that *Patz1* overexpression increases histone deacetylation via activation of these epigenetic factors (Fig. 6b).

We also examined the level of marks associated with euchromatin such as H3K36me3 and H3K4me3, and the repressive histone marks H3K9me3, H3K27me3 in *Patz1*^{+/+}, *Patz1*^{+/-} and *Patz1*^{-/-} MEFs. As shown in Figure 6c, the levels of active histone marks were generally

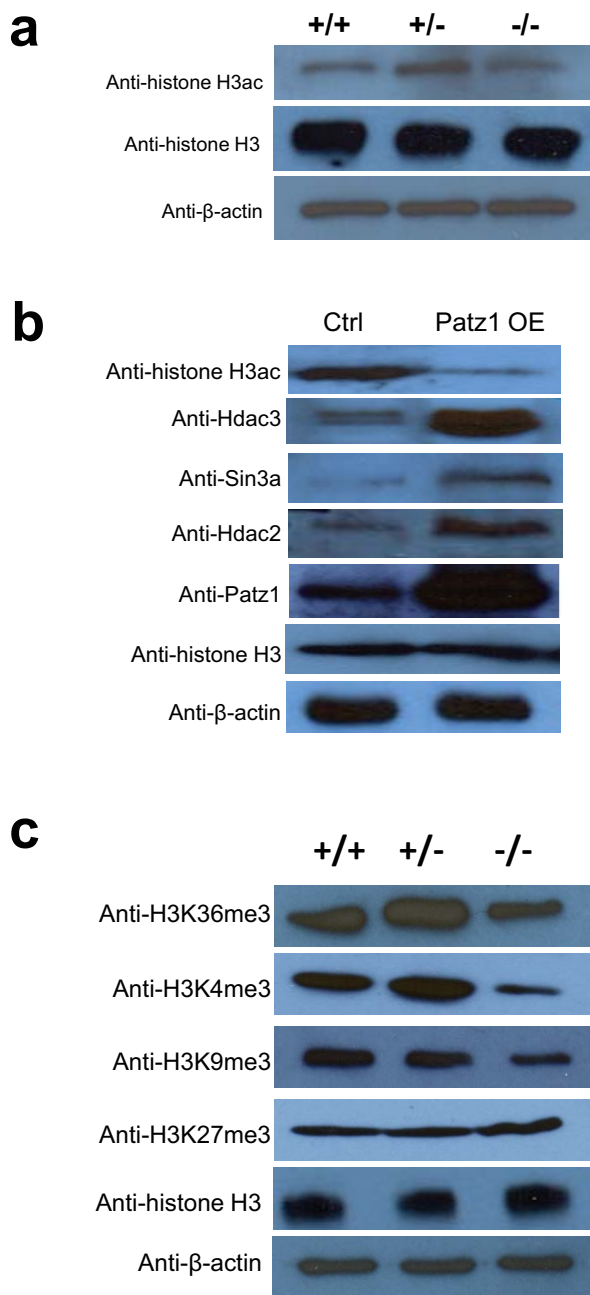


Figure 6 | Level of Patz1 modulates chromatin modifications in MEFs.

(a) H3 acetylation level was increased in *Patz1*^{+/-} MEFs. (b) H3 acetylation was repressed by Patz1 overexpression. H3Ac was significantly repressed in Patz1 OE MEFs. Histone deacetylase related proteins, including Sin3a, Hdac2 and Hdac3 were down-regulated in *Patz1*^{+/-} MEFs. (c) Up-regulated H3K4me2, H3K4me3, H3K36me3 levels and repressed H3K9me3 level in heterozygous knockout MEFs. Histone H3 and β-actin served as loading control. Cropped blots were used here. The uncropped blot image was available in Supplementary Figure 7.

increased while the repressive histone marks were decreased in *Patz1*^{+/-} compared to WT cells. HP1α, well-known for its role in heterochromatin formation, was also decreased in *Patz1*^{+/-} MEFs. Notably, the level of H3K4me2 was significantly upregulated in *Patz1*^{+/-} MEFs and reduced in Patz1 overexpressed MEFs (data not shown). Acquisition of H3K4me2 at the pluripotency genes occurs at the initial stage of reprogramming that primes the gene for activation later on, which is a prerequisite for pluripotency induction³⁷. Thus, it appears that heterozygous loss of *Patz1* promotes a

globally more open chromatin state accessible for activation of pluripotency transcriptional network, thus enhancing reprogramming.

Discussion

We previously demonstrated that Patz1 is an essential pluripotency regulator²⁹. It is therefore intricate to find it to inhibit pluripotency reacquisition. In this study, we revealed the first evidence for the involvement of Patz1 in reprogramming process (Fig. 7): (i) overexpression of Patz1 inhibits the acquisition of pluripotency, while interference or heterozygous loss of Patz1 enhances iPSC generation; (ii) knockout of *Patz1* hinders the reprogramming process by inducing cellular senescence. This suggests that a critical control of Patz1 dosage is essential for the generation of iPSCs.

Patz1 has emerged as a transcription factor that has been implicated in various cellular or developmental processes, such as transcription regulation, carcinogenesis, spermatogenesis, thymocyte development, neurological process, pluripotency maintenance, cellular senescence and apoptosis. The involvement of PATZ1 in tumor suppression or carcinogenesis has been a debating issue for decades. Patz1 is previously known as MAZR (Myc-associated zinc finger related protein), which can activates *c-Myc* promoter¹⁶. But subsequent studies reported the implications of Patz1 in tumor suppression. Here we showed that Patz1 possibly acts as a repressor of *c-Myc* in MEFs. *c-Myc* protein level was the highest in *Patz1*^{-/-} MEFs. Overexpression of Patz1 significantly repressed *c-Myc*, but induces p53 and p16. Previous studies have found that *c-Myc* and p53 negatively regulates each other in tumorigenesis, cell differentiation and cell apoptosis etc.^{33–36}. p53 is known to negatively regulate *c-Myc* transcription through a mechanism that involves histone deacetylation³⁴. Given that Patz1 is an interacting partner of Ncor1 and Sirt1 and modulates histone acetylation level^{18,26}, it is therefore suggested that ectopic Patz1 could act via repression of *c-Myc*, possibly via cooperation with p53, to inhibit reprogramming efficiency.

A more recent finding revealed that the tumor suppressor role of PATZ1 is p53-dependent²⁵. In the presence of p53, PATZ1 interacts with p53 to enhance its functions in tumor suppression; in the absence of p53, PATZ1 is more likely to enhance cell survival and proliferation. Previous studies have proposed some mechanisms by which p53 inhibits reprogramming, such as inducing cell cycle arrest and apoptosis, inhibiting mesenchymal to epithelial transition^{5,38,39}. Here we show that the level of Patz1 is also critical for reprogramming process. In the absence of Patz1, p53/p16 axis is activated, and the cells undergo cellular senescence. When only one of *Patz1* alleles is disrupted, *Ink4a/Arf* locus is repressed, whereby preventing the cells from senescence induction. Overexpression of Patz1, however, robustly activates p53 and p16, thereby inhibiting cell proliferation in MEFs. Given that p53 is a known inhibitor of reprogramming, we therefore speculate that excessive expression of Patz1 may act through p53/p16 to induce cell senescence, and thus inhibiting reprogramming process. The observation of higher proliferation rate in *Patz1*^{+/-} MEFs indicates that there are other pathways involved. It could not be ruled out that when Patz1 level is reduced in *Patz1*^{+/-} MEFs, the anti-proliferative effects of p53 could no longer be stabilized. This may somewhat indicates that the role of Patz1 relies on its expression levels and possibly is p53-dependent as well.

We also propose that the restriction of iPSC generation in *Patz1*^{-/-} MEFs may be associated with cellular senescence. We have shown that knockout of *Patz1* up-regulates senescence effectors p16, p21 and p53, inducing cellular senescence in MEFs. Patz1 may be involved in cellular senescence by regulating epigenetic status of *Ink4a/Arf* locus. *Ink4a/Arf* locus needs to be repressed for the transition from somatic cell to pluripotent state^{3–5}. Jmjd3 for example, which possesses tumor suppressor character, inhibits the reprogramming process by demethylation of H3K27me3 at *Ink4a/Arf*⁴⁰. Similarly, haploinsufficient loss of *Patz1* may accelerate the reprogramming kinetics by pre-repressing of *Ink4a/Arf* locus. As seen

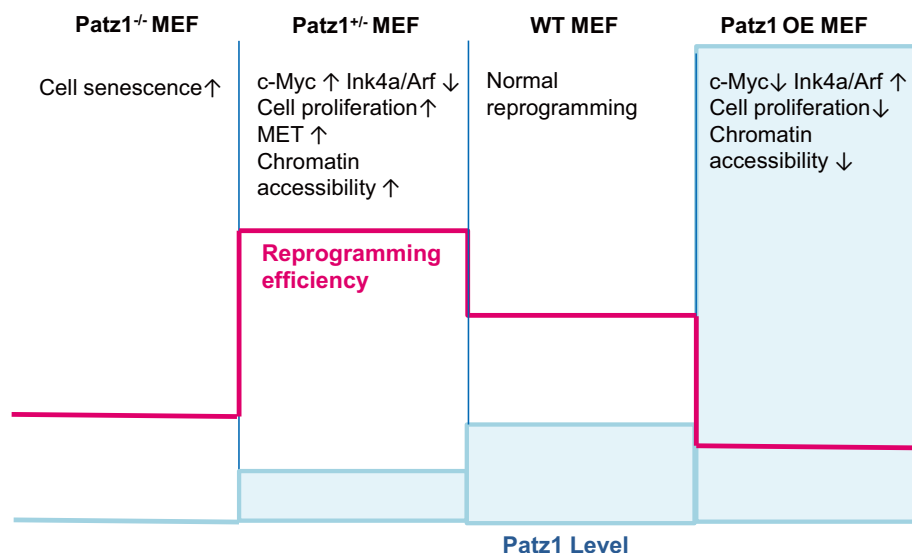


Figure 7 | Proposed role of Patz1 during somatic cell reprogramming. Briefly, overexpression of Patz1 creates a condensed chromatin which represses the reprogramming process; Patz1 overexpression also represses c-Myc and induces cell senescence to inhibit reprogramming. Heterozygous knockout of *Patz1* can promote MET, activate c-Myc, overcome *Ink4a/Arf* barrier to surpass senescence and also create an open, hyperdynamic chromatin structure accessible for pluripotency gene reactivation, thus enhancing cellular reprogramming. Patz1^{-/-} MEFs undergo cell senescence and are hard to be reprogrammed.

from ChIP results, H3K27me3 was obviously induced and H3K4me3 level was significantly reduced in Patz1^{+/-} MEFs. Interestingly, it is also found that Patz1 binds to *Ink4a* locus in wt MEFs, but not in Patz1^{+/-} MEFs (Supplementary Figure 5). It seems that Patz1 is involved in the recruitment of epigenetic regulators to this locus, thus modulating *Ink4a/Arf* activity.

Since many MET inducing genes were up-regulated while EMT inducing related genes were down-regulated in Patz1^{+/-} and Patz1^{-/-} MEFs, we propose a novel function of Patz1 in MET inhibition. MET, a reversed process to EMT, is one of the key cellular events during early stage of reprogramming process⁴¹. Factors that promote MET, including Klf4 and E-cad can enhance pluripotency acquisition; while factors that drive EMT or prevent MET, such as TGF- β and some mesenchymal markers, impede the reprogramming at the initial stage^{41,42}. Interestingly our Patz1 ChIP-seq results revealed that many MET and EMT associated genes are bound by Patz1 in ES cells (Supplementary Fig. 6a). Thus Patz1 may help to activate the epithelial program and suppress mesenchymal genes to overcome the EMT epigenetic barrier of fibroblasts. Since Klf4 is one of the major effector to drive MET during reprogramming process, we checked Klf4 expression level in Patz1^{+/+}, Patz1^{+/-} and Patz1^{-/-} MEFs. Klf4 levels seemed to be similar in those MEFs (Supplementary Fig. 6b). Hence we reckon that Patz1 may act independently from Klf4, perhaps directly regulating MET related genes or their regulators.

Somatic cells utilize a large number of epigenetic regulations to prevent the expression of unwanted genes. Pluripotent cells however possess a rather open chromatin configuration which allows transcriptional programs to switch rapidly upon induction of differentiation. How to overcome the epigenetic barriers of somatic cells in reprogramming remains a key question. In this report we provide some evidence that heterozygous loss of *Patz1* enhances reprogramming possibly through promoting a more active chromatin state ready for reprogramming onset. One possible way for Patz1 modulating chromatin status is via histone acetylation. Previous studies have reported the interaction of Patz1 and NCoR complex and Sirt1^{18,26}. Our results show that Patz1 is possibly a supreme factor regulating histone acetylation, not only by participating directly to recruit HDAC complexes, but also as a regulator of HDAC related

genes. The acetylation of histones serves as a marker for active or open chromatin, and its level is higher in plastic pluripotent chromatin. In view of previous finding that inhibitors of HDAC activity significantly improve the reprogramming efficiency¹¹, we propose that the inhibitory effect of Patz1 OE could be attributed to the repressed histone acetylation level, which hinders the chromatin resetting for efficient reprogramming. This is consistent with the general notion that Patz1 is a transcriptional repressor. Moreover, the repression of Patz1 OE on histone acetylation could be one of the possible mechanism by which Patz1 OE inhibits expression of lineage genes during differentiation, as histone acetylation are increased during differentiation, leading to rises in gene expression from all germ layers.

In summary, through the exploration of Patz1's roles in somatic reprogramming, we provide new evidence that the level of Patz1 is closely related to cell cycle regulation, chromatin conformation and MET, thus modulating reprogramming process. We believe our results provide a good model to study how genetic factors crosstalk with epigenetic factors in reprogramming process. We propose that the functions of Patz1 are cellular context- and dosage- dependent, largely because it may interact with different proteins to exert specific roles in different types of cells. It would be of great significance and interest to illustrate more underlying mechanisms in future studies. Nevertheless, this study is among a few studies in which different dosages of pluripotency factors play diverse roles in reprogramming process.

Methods

Cell culture. MEFs were cultured with medium containing Glasgow Minimum Essential Medium (GMEM; Invitrogen), 15% ES cell qualified fetal bovine serum (Invitrogen), 0.055 mM β -mercaptoethanol (Sigma), 100 mM sodium pyruvate (FBS) (Invitrogen) and 0.1 mM MEM nonessential amino acid (NEAA) (Invitrogen). iPSCs were maintained with KSR medium consisting of DMEM, 15% KSR (Invitrogen), 2 mM L-Glutamine, 1 mM sodium pyruvate, 1000 units/ml of LIF, 1% P/S, 0.055 mM β -mercaptoethanol and 0.1 mM MEM. All the cells were cultured in 37°C with 5% CO₂ incubator.

Plasmid construction. For retrovirus packaging plasmids used for Patz1 overexpression, full-length Patz1 cDNA was amplified by PCR and ligated into MunI and NotI restriction sites of pMX plasmid (Addgene). The primers were: forward: 5'-ATATACAATTGATGGAGCGGGTCAACGACGCTTC; reverse: 5'-TAATAGCGGCCGCTCACTTCCTCCCTCAGGCCCATG



For Patz1 RNAi, Oligonucleotides targeted for knocking down Patz1 were cloned into pSuper.retro.puro (Oligoengine)²⁹. The sequence was as follows: CTGGAGATGCACACCATCA.

Retrovirus packaging, infection and iPSC induction. The concentrated viruses were prepared as previously described⁴³. *Pou5f1*-GFP MEFs were seeded onto a gelatin-coated 24 well plate at 50–70% confluency while Patz1^{+/+}, Patz1^{+/-} and Patz1^{-/-} MEFs with a number of $\times 10^5$ 6 h before infection. 10 μ l of each concentrated retrovirus, supplemented with 8 μ g/ml polybrene (Sigma), were added to the MEF cells. After 12–16 hrs of incubation, the infected MEFs were maintained in fresh medium for protein or RNA extraction.

For iPSC induction, retrovirus infected MEFs were then passaged onto the inactivated feeder layer 2 days post infection (dpi) and cultured with KSR medium from 5 dpi. KSR medium was replaced every day and appearance of GFP⁺ colonies was counted till the end of the experiment. Alkaline phosphatase staining assay was performed using Alkaline Phosphatase Detection Kit (Millipore) according to manufacturer's instructions and results were captured with camera.

EB formation assay. iPSCs were dissociated as per passaging and re-suspended with LIF withdrawal medium in Ultra-Low Attachment Surface culture plates (Corning). After culturing for 4 days in suspension, embryoid bodies were transferred to gelatin-coated coverslips placed in 24-well plates. The adherent EBs were cultured with EB medium (DMEM containing 15% KSR, 2 mM Glutamine, 0.1 mM MEM) for other 10 days. Immunostaining was then performed with antibodies for specific lineage markers. Images were captured under a confocal microscope (Olympus FV1000) at 60 \times magnification.

Immunostaining. iPSCs or differentiation culture were fixed with 4% paraformaldehyde in PBS. After permeabilization in 1% Triton X-100/PBS for 20 min, immunostaining were performed using the following primary antibodies: anti-Oct4 (Santa Cruz), anti-Nanog (Santa Cruz), anti-SSEA1 (Millipore), anti-Gata4 (Santa Cruz), anti-SMA (Abcam), anti-Nestin (R&D). Secondary antibodies used were Alexa Fluor 488/546 anti-mouse IgM, and Alexa Fluor 488/546 anti-mouse or anti-rabbit IgG (Invitrogen). DAPI (Vector Laboratories) was used for staining the nuclei.

RNA extraction, reverse transcription and quantitative real-time PCR. Total RNAs were isolated using TRIzol Reagent (Invitrogen) followed by purification with RNeasy Mini Kit (Qiagen) according to the provided protocol. RNA was dissolved in diethyl pyrocarbonate (DEPC)-treated water (Ambion). The Superscript III First-Strand Synthesis System with oligo (dT) primer (Invitrogen) was used to convert messenger RNA (mRNA) to cDNA. The cDNA was diluted 20 times with nuclease-free water for quantitative real-time PCR, which was performed with CFX96TM Real-Time System (BioRad) and SYBR Green Master Mix (BioRad). Relative expression levels of target genes from sample cDNA were normalized against β -actin levels and reflected as a fold change compared to control.

Chromatin Immunoprecipitation (ChIP). The protocol for ChIP was performed as previous described⁴⁴. Briefly, MEFs were cross-linked with 1% formaldehyde for 10 min followed by neutralization with 0.125 M glycine at room temperature. Chromatin (around 500 bp after sonication) were immunoprecipitated with Protein G magnetic Dynabeads (Invitrogen) coated with anti-Patz1 antibody (Santa Cruz) or anti-H3K4me3 antibody (Abcam) or anti-H3K27me3 antibody (Abcam). Precipitated DNA was dissolved in 80 μ l nuclease free water for quantitative real-time PCR.

Western blotting. The primary antibodies that were used: anti-Patz1 antibody (Santa Cruz), anti-Hdac3 (Santa Cruz), anti-mSin3a (Santa Cruz), anti- β actin (Santa Cruz), anti-H3ac (Abcam), anti-Hdac2 (Santa Cruz), anti-H3K4me3 (Abcam), anti-H3K36me3 (Abcam), anti-H3K9me3 (Abcam), anti-H3K27me3 (Abcam), anti-p53 (BD Science) and anti-Myc (Santa Cruz).

Gene expression microarray analysis. Microarray assay and analysis were conducted as previously described⁴³. Genes were selected according to the fold change (>2.0 or <0.6) in Patz1^{+/+} or Patz1^{-/-} MEFs respectively. Cluster 3.0 was utilized to perform hierarchical clustering on these selected genes. Data was adjusted to center genes and array by mean, before being clustered with the Euclidean distance similarity metric and Average linkage clustering method. This gave 4 clusters of genes that are differently expressed after visualized using Java Treeview. GO analysis (GATHER) were performed with each cluster of genes and enriched GO terms ($p < 0.05$) were selected and classified into groups accordingly.

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Author contributions

Conceived and designed the experiments: Q.W. Performed the experiments: H.M., J.R.O.,

B.C.P.T. and Z.G. Analyzed the data: H.M. and Q.W. Contributed reagents/materials/analysis tools: B.F., Y.H.L., M.F., H.L. and Q.W. Wrote the paper: H.M., M.F., H.L. and Q.W. All authors reviewed the manuscript.

Additional information

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