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Kat6b modulates Oct4 and Nanog binding to chromatin in embryonic stem cells and is required for efficient neural differentiation

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Abstract

Chromatin remodeling is fundamental for the dynamical changes in transcriptional programs that occur during development and stem cell differentiation. The histone acetyltransferase Kat6b is relevant for neurogenesis in mouse embryos and mutations of this gene cause intellectual disability in humans. However, the molecular mechanisms involved in Kat6b mutant phenotype and the role of this chromatin modifier in embryonic stem (ES) cells remain elusive. In this work, we show that Kat6b is expressed in ES cells and is repressed during differentiation. Moreover, we found that this gene is regulated by the pluripotency transcription factors (TFs) Nanog and Oct4. To study the functional relevance of Kat6b in ES cells, we generated a Kat6b knockout ES cell line (K6b-/-) using CRISPR/Cas9. Fluorescence correlation spectroscopy analyses suggest a more compact chromatin organization in K6b-/- cells and impaired interactions of Oct4 and Nanog with chromatin. Remarkably, K6b-/- cells showed a reduced efficiency to differentiate to neural lineage. These results reveal a role of Kat6b as a modulator of chromatin plasticity, its impact on chromatin-TFs interactions and its influence on cell fate decisions during neural development.

Keywords: Pluripotency transcription factors, Neural progenitors, Super-enhancer, Fluorescence Correlation Spectroscopy, CRISPR/Cas9.

Abbreviations

Transcription factors (TFs), histone acetyltransferase (HAT), Embryonic stem (ES), pluripotent stem (PS), heterochromatin protein 1 (HP1).

Introduction

Embryonic development requires a precise spatiotemporal coordination of gene expression that includes modifications in the nuclear concentration of relevant transcription factors (TFs), changes in the chromatin organization and a redistribution of TFs among different targets [1–3]. Chromatin remodeling is also fundamental for the maintenance of cellular identity and the preservation of stable and inheritable gene expression programs. Consequently, mutations in many chromatin modifiers are associated to diverse developmental pathologies [4–6].

Kat6b is a transcriptional coactivator with histone acetyltransferase (HAT) activity important for the establishment and self-renewal of mouse neural stem cells [7]. Kat6b is expressed in the inner cell mass (ICM) of mouse blastocysts [8], in the ventricular zone of the cerebral cortex during neurogenesis [8] and in neural stem cells of adult mice [7]. Remarkably, mutations in Kat6b are detected in Say-Barber/Biesecker/Young-Simpson (SSBYS) and Genitopatellar (GPS) syndromes, both associated with intellectual disabilities [9–11]. These observations suggest that Kat6b could be involved in the regulation of neural development. However, the molecular mechanisms underlying the mutant Kat6b phenotype remain elusive.

Embryonic stem (ES) cells constitute an attractive model to study differentiation and associated pathologies since they recapitulate many aspects of embryo development [12–15]. The transcriptional programs that preserve pluripotency and self-renewal of ES cells are regulated by the master TFs Oct4, Sox2 and Nanog that act together with specific chromatin remodelers inducing the expression of pluripotency genes and repressing those involved in differentiation [16,17].

In this work, we studied Kat6b gene regulation and explored the effects of its knockout on self-renewal and neural differentiation of ES cells. The combination of different methodologies

including traditional gene expression analysis, CRISPR/Cas9 technology and fluorescence correlation spectroscopy allowed us to determine that Kat6b impacts on chromatin structure and TFs dynamics in ES cells.

Results

Kat6b expression in ES cells depends on Oct4 and Nanog and decreases during differentiation

We studied Kat6b expression in naïve pluripotent ES cells and after inducing differentiation through non-directed and directed protocols. Fig. 1 shows that Kat6b is expressed in undifferentiated W4 ES cells as assessed by RT-qPCR. Additionally, Kat6b mRNA levels decreased when the cells were induced to differentiate by removing LIF and 2i for 2 and 4 days (Fig. 1A), by using the hanging drop protocol up to day 11 (Fig. 1B) or by following a protocol directed to neural progenitor cells (Fig. 1C). In line with these results, analyses of available RNA-seq and microarray data showed that Kat6b gene is downregulated in many directed and non-directed differentiation protocols performed in pluripotent stem (PS) cell lines (Table S1).

The Kat6b expression pattern found in PS cells led us to explore if this gene could be modulated by pluripotency TFs. We first analyzed genome wide data available from ChIP-seq experiments and found Oct4, Sox2 and Nanog bound to Kat6b gene regulatory region in ES cells (Fig. 1D and Table S2), suggesting a possible regulatory role. Interestingly, this region was enriched in Med1 and H3K4me3, both markers of active enhancers, and defined as super-enhancer [18] (Fig. 1D). These structures are large clusters of enhancers usually associated to key genes that define cell identity. These observations suggest that Oct4 and/or Nanog may regulate Kat6b expression. To test this hypothesis, we used a shRNA approach to interfere with the expression of Oct4 and Nanog in ES cells. As shown in Fig. 1E, Kat6b mRNA levels decreased when Oct4, Nanog or both TFs were downregulated, indicating that these TFs could be involved in Kat6b gene induction.

Kat6b knockout ES cells preserve self-renewal and present altered morphology

Based on the observed relation between Kat6b and pluripotency TFs Oct4 and Nanog in the previous section, we studied Kat6b relevance in self-renewal and pluripotency of ES cells. With this purpose, we generated a Kat6b knockout (KO) ES cell line (K6b-/-) using CRISPR/Cas9 technology directing Cas9 to the first coding exon of the gene (Fig. S1A and Supplemental Methods). We sequenced the target region in the genomic DNA of the obtained clones, selected a single clone with mutations that generated a premature stop codon in both alleles (Fig. S1B) and verified the absence of Kat6b by Western blot and immunofluorescence (Fig. 2A).

We next analyzed the expression of pluripotency markers in K6b-/- and wild type (WT) ES cells and found that Kat6b KO did not affect mRNA levels of all the evaluated markers (Fig. 2B). Additionally, the alkaline phosphatase activity was similar for the two ES cell lines (Fig. 2C). Moreover, similarly to WT cells, K6b-/- cells did not present significant levels of early differentiation markers when cultured in ES medium (Fig. 2B). Finally, Ki67 proliferation marker staining and MTT assay showed that the cell cycle and viability were not affected by Kat6b KO (Fig. 2D). Altogether, these results suggest that Kat6b is not required for ES cells self-renewal.

Despite having similar mRNA levels of pluripotency TFs, K6b-/- ES colonies presented a less-compact morphology in comparison to WT cells (Fig. 2E). Moreover, quantitative analysis of DAPI-stained cells showed an increase of the middle-plane area of K6b-/- ES cells nuclei (Fig. 2F) suggesting a possible role of Kat6b that impacts in ES cell morphology.

Kat6b knockout promotes a compact chromatin structure and impairs interactions of Oct4 and Nanog with chromatin targets

Heterochromatin protein 1 (HP1) plays key roles in the assembly and maintenance of heterochromatin and participates in the regulation of gene expression [19–22]. Previous works showed that the effective mobility of HP1 within the nuclear space of ES cells depends on chromatin compaction, being faster for loosely-compacted chromatin [23–25]. Therefore, we studied HP1α distribution and dynamics in the nucleus of WT and K6b-/- ES cells to test if Kat6b KO affects chromatin plasticity.

We acquired images of ES cells transfected with a vector encoding HP1α-GFP. This protein presented a heterogeneous distribution with bright *foci* (Fig. S2) described in the literature as highly condensed chromatin domains [26–29]. WT and K6b-/- cells did not show differences in *foci* sizes and nuclear densities (Fig. S2).

We next ran single point fluorescence correlation spectroscopy (FCS) measurements in low-intensity regions of these cells to determine HP1α dynamics in relatively more open chromatin domains. Fig. S3 shows that, similarly to other chromatin-interacting proteins [1,30], the autocorrelation data of HP1α-GFP in ES cells could be interpreted with a model that includes the diffusion of the protein and its interactions with targets in two distinct temporal windows (Supplemental Information, Eqn S1). FCS analyses revealed differences of HP1α dynamics in K6b-/- and WT ES cells (Fig. 3C,D). Specifically, the lifetime of HP1α-chromatin long-lived interactions in KO cells was higher than WT cells suggesting a more condensed chromatin structure. These experiments performed in other three independently generated Kat6b KO clones showed results also consistent with a more compact chromatin landscape in KO cells respect to WT (Fig S4).

It is now widely accepted that the modulation of gene expression depends on how TF distributes within the nuclear space and the time-scale of the interactions with chromatin targets [31]. Considering the modifications in HP1α-GFP dynamics and therefore in chromatin

organization, we speculated that the interactions between chromatin and pluripotency transcription factors could also be altered in K6b-/- ES cells. To test this hypothesis, we performed single point FCS measurements in WT and K6b-/- ES cells expressing the fusion proteins Oct4-GFP or Nanog-GFP.

Fig. 3C shows that the lifetime of long-lived and short-lived interactions of Oct4-GFP with chromatin decreased in KO cells suggesting that the modifications of the chromatin structure in K6b-/- cells cause a reduction of the affinity of Oct4 on DNA targets. In addition, Nanog-GFP in K6b-/- cells presented impaired short-lived interactions and a smaller long-lived bound fraction suggesting that the more compact chromatin landscape in KO cells reduces the exposition of Nanog targets. Altogether, FCS analyses suggest that Kat6b promotes a looser chromatin structure facilitating Oct4 and Nanog interactions with chromatin.

Kat6b knockout preserves pluripotency of ES cells

To analyze the effect of Kat6b KO on pluripotency, we studied the capability of K6b-/- ES cells to differentiate. We first conducted two non-directed *in vitro* differentiation protocols and analyzed by RT-qPCR the expression of pluripotency TFs and specific differentiation markers. K6b-/- cells successfully downregulated pluripotency markers and differentiated to endoderm, mesoderm and ectoderm as observed for WT cells (Fig. 4A,B). Moreover, WT and K6b-/- cells injected into nude mice gave rise to teratomas displaying structures representative of the three germ layers (Fig. 4C). As a whole, these results demonstrate that K6b -/- cells are pluripotent, both *in vitro* and *in vivo*.

Kat6b knockout reduces the efficiency of ES differentiation towards neural progenitor cells

We next investigated Kat6b relevance on neural differentiation conducting a protocol directed to neural progenitors.

The overall morphology of K6b-/- and WT ES cells was different at the end of the protocol since KO cells were flatter (Fig. 5A). Although the pluripotency markers Nanog and Oct4 were downregulated in a similar manner, we observed that Kat6b KO affected the expression of some differentiation markers at day 6 of the protocol. Interestingly, mRNA levels of the mesoderm marker Brachyury were higher in K6b-/- cells, while the levels of the neural progenitor marker Sox1 were lower (Fig. 5B), suggesting a reduced efficiency in neural progenitor differentiation.

Additionally, the number of cells from day 4 of the differentiation protocol was higher in the KO condition (Fig. 5A). In agreement with this observation, MTT viability assay indicated that Kat6b KO cells showed an increased viability at days 4 and 6 of the differentiation protocol to neural progenitor (Fig. 5C).

Altogether, these results indicate that K6b-/- ES cells display higher viability, lower expression of Sox1 and a parallel increase in Brachyury expression during neural progenitor differentiation suggesting that K6b-/- ES cells present an impaired capability to differentiate towards neural progenitor.

Discussion

Chromatin remodeling during PS cell differentiation affects the interactions of transcription-related proteins and chromatin defining specific transcriptional programs that determine cell identity [32,33]. In this context, HATs play key roles as epigenetic regulators of gene expression [34–36]. These enzymes catalyze the acetylation of specific lysine residues of histones tails, disrupting interactions between nucleosomes and weakening histone-DNA interactions [37]. This loose chromatin structure is generally associated to a high transcriptional activity since DNA is more accessible to transcription factors and other transcription-related proteins. For example, it was shown that acetylation of H3K56 plays a critical role in the interactions of Oct4 with DNA targets, essential for ES cells pluripotency [38]. Additionally, it was reported that the histone acetylation of Nanog promoter by Myst2, a member of the Myst family, is required for Oct4 binding and the consequent Nanog gene induction in ES cells [39]. Moreover, Myst1 induces Nanog and Sox2 transcription by recruitment of a subunit of a histone methyltransferase complex, with the consequent H3K4 trimethylation [40,41]. Although Kat6b targets are not completely known yet, it is speculated that this Myst family member mediates H3K9 and H3K23 acetylation [40]. Since many different HAT complexes associate to active gene promoters, it is proposed that they are involved in transcriptional control [40].

Kat6b is a chromatin modifier relevant for the establishment and self-renewal of mouse neural stem cells [7]. In contrast to most HATs that are usually ubiquitously expressed, Kat6b presents an atypical gene expression pattern in development [8] and adult tissues [7]. In this work, we studied the expression and relevance of this chromatin remodeler in ES cells to unravel its role in ground state pluripotency and during neural differentiation.

We found that Kat6b is expressed in ES cells and that its expression decreases upon triggering differentiation through various protocols. Genome wide studies of PS cell lines subjected to both directed and non-directed differentiation protocols also showed a similar Kat6b gene expression behavior [42–47] suggesting that Kat6b downregulation occurs at the exit of the undifferentiated state regardless of cell fate.

We showed that Kat6b expression is modulated by Oct4 and/or Nanog; we cannot rule out that Oct4 modulates Kat6b expression through Nanog since Oct4 silencing also repressed Nanog expression as previously found in our experimental system [48,49]. Supporting this statement, microarray analysis of RNAi-mediated silencing in ES cells showed that silencing of Nanog but not Oct4 affects Kat6b expression [17].

Nanog is one of the first components of the pluripotency core to be silenced when cells exit the naive undifferentiated state and is a specific marker of the ground state of pluripotency [50,51]. We found that the expression profile of Kat6b during differentiation was similar to that of Nanog independently of the differentiation protocol performed. Moreover, it has been reported that the expression of Nanog correlates with the expression of Kat6b in ES cells [52]. These results suggest that Nanog regulates Kat6b expression. Furthermore, ChIP-seq studies assigned a super-enhancer with high levels of Oct4, Sox2 and Nanog bound to the regulatory regions of Kat6b [18]. Super-enhancers are usually associated to key cell identity genes suggesting that Kat6b may have some relevant function in ES cells.

We explored the role of Kat6b using a knockout ES cell generated with the CRISPR/Cas9 technique. Although these cells preserved pluripotency as assessed *in vitro* and *in vivo* by teratoma formation, their morphology was different in comparison to WT cells. FCS measurements showed longer HP1-chromatin interactions in K6b-/- cells, suggesting that this chromatin remodeler induces a more open chromatin structure. Further studies are required to reveal whether these changes are a consequence of a modification in H3 histone acetylation or

they also involve acetylation of other histone residues and/or acetylation of other proteins like HP1 itself.

In the same direction, the disruption of Oct4 and Nanog interactions with chromatin found in this work probably impacts on the expression output and also suggests a more compact chromatin structure in the Kat6b knockout. Since Kat6b is reported to interact with TFs such as RUNX2 which is involved in osteoblast differentiation [53], it would be interesting to explore if the impaired TF-DNA dynamics found in the knockout ES cell line is a consequence of Kat6b acetylation of these pluripotency TFs. Although there are no reports related to Kat6b acetylation of TFs, various HATs acetylate diverse TFs , such as C/EBPα, FOXO1 and p53, modulating their activity. [40]. Moreover, the activity of pluripotency TFs is also reported to be regulated by acetylation. For example, maintenance of naïve pluripotency is dependent on a deacetylated Oct4. During naïve to primed transition, the activity of the deacetylase SirT1 is reduced, Oct4 becomes hyper-acetylated and upregulates the primed pluripotency gene network [54]. Moreover, Sox2 deacetylation by Sirt1 is required for cell reprogramming [55] and its acetylation by p300/CBP enhances its nuclear export and its ubiquitin-mediated proteosomal degradation [56]. The identification of Kat6b targets is essential to a deep comprehension of its function.

Furthermore, when we studied the role of Kat6b in neural differentiation, we found differences between KO and WT cells which suggest that this chromatin remodeler is also involved in the response to specific differentiation signals. Our data showed a lower expression of the neural progenitor marker Sox1 and an increase in the mesoderm marker Brachyury in K6b-/- cells at day 6 of neural differentiation. These results suggest that K6b-/- cells present a lower efficiency of differentiation towards the neural lineage and are probably also differentiating towards other cell types, possibly mesoderm derivatives. The existence of contaminating cells has been previously observed when following this differentiation protocol; these cells were described as undifferentiated stem cells that remain pluripotent and/or non-neural flat cells [57].

The cellular identity of the latter has not been specified and was defined only by their morphology and the absence of neural progenitor markers. Besides, Thomson *et al.* [14] found cells expressing either Brachyury or Sox1 during the differentiation of ES cells to mesendoderm indicating the existence of at least two cell populations. These evidences suggest that the upregulation of Brachyury could be the result of an increase in the number of non-neural contaminating cells expressing this mesoderm marker in the KO cells.

Additionally, we found more viable cells after day 4 of the neural differentiation protocol in K6b-/- cells. This protocol was reported to result on a large amount of dead cells up to day 3 and many of the surviving cells differentiate to neural progenitors from day 4 [57–59]. Since the balance between cell death and proliferation determines the number of cells at a specific time of the differentiation process, it is possible that Kat6b could be involved either in apoptosis induction or in proliferation inhibition of specific cells that were committed to non-neural lineages.

Vast evidence supports the involvement of histone acetyltransferases with a Myst domain in both, cell proliferation and apoptosis. Mutations in Enok, a HAT of the Myst family in *Drosophila*, produce an arrest in the proliferation of neuroblasts, generating defects in the brain formation of the fly [60]. Furthermore, studies in *S. cerevisiae* showed that mutations in Sas3, homolog of Myst3, and Gcn5, another HAT, affect the proliferation when arresting cells in the G2/M phase of the cell cycle [61]. In mouse fibroblasts, Myst3 acetylates p53 in response to DNA damage and modulates its activity generating an arrest in the cell cycle [62]. Finally, it was reported that Kat6b silencing produced a decrease in PI3K/AKT-mediated cell proliferation in prostate cancer cells [63].

Kat6b is not the only chromatin modifier that has been associated to neural disorders. Genome-wide studies and human exome sequencing found mutations in different chromatin remodelers [4,5] in samples of patients with neurobiological diseases or syndromes such as Rett

[64,65], Rubinstein-Taybi [66,67], Coffin-Siris [68], Nicolaides-Baraitser [69], Brachydactyly mental retardation [70] and Weaver [71]. All these genetic disorders are characterized by defects in the development of the nervous system and intellectual disability. This correlation suggests that a highly controlled epigenetic regulation is necessary during neural development to achieve precise control of gene expression in time and space.

In summary, our results suggest that Kat6b modulates the organization of chromatin and its interactions with pluripotency TFs impacting on neural differentiation of mouse ES cells**.** More generally, this work contributes to understand how epigenetic marks modulate gene expression in ES cells, a piece of the puzzle to unravel the molecular mechanisms involved in cell fate decisions.

Materials and Methods

Full methods are available on Supplemental Information

Cell culture, culture conditions and differentiation

W4 ES cell line was provided by the Rockefeller University Core Facility. R1 (ATCC) and W4 ES cells were cultured in ES medium consisting of DMEM, 2 mM Glutamax, 100 mM MEM NEAA, 0,1 mM 2-mercaptoethanol, 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco), supplemented with 15% FBS (Gibco), 1000 U/ml LIF (Millipore), 1 μM PD0325901 (Tocris) and 3 μM CHIR99021 (Tocris). Cells were cultured on 0,1% gelatin coated dishes at 37°C in a 5% CO_2 (v/v) incubator and passaged every three days using TrypLE (Gibco). All the differentiation protocols were performed as previously described [48,72]. Cells were routinely assessed for mycoplasm contamination by genomic DNA extraction and PCR analysis [73].

Gene expression analysis

Gene expression was analyzed by RT-qPCR, immunofluorescence and/or western blot as previously described [74]. Primers and antibodies used are listed in Supplemental Methods.

Downregulation of Nanog and Oct4 by shRNA approach

R1 ESCs cultured in standard medium on gelatin coated p60 dishes were transfected with 3 μg pLKO.1-puro derived vectors (Sigma), expressing shRNA targeting Nanog (SHCLND-XM_132755), Oct4 (SHCLND-NM_013633) or eGFP (SHC005), which was used as control vector. Transfection, selection and mRNA expression analysis were carried out as previously described [48].

Teratoma formation and histological analysis

Teratoma formation and histological analysis was performed as previously described [72] with minor modifications. Briefly, 100 ml of the cell suspension (10^6 cells) were subcutaneously injected into the dorsal flank of 6–8-week-old male nude (*nu/nu*) mice. Tumors were surgically dissected from the mice when their diameter was of approximately 1 cm. These procedures were conducted following local ethical guidelines.

Viability test

Viability was evaluated by MTT assay according to the manufacturer's instructions. Briefly, ES cells were incubated in culture medium with 5 mg/ml MTT for 30 minutes, washed in PBS and incubated in 200 µl of isopropanol for 15 minutes. The samples were transferred to a 96-well plate with transparent flat base (Nunc) and the absorbance was measured at 570 nm on an Optima Fluostar plate reader.

Sample preparation for fluorescence correlation spectroscopy measurements

For FCS measurements, ES cells were grown onto coated coverslips treated with 100 µg/ml PDL (Sigma-Aldrich) and 20 µg/ml Laminin (Invitrogen). Cells were transfected with plasmids pEGFP-C1-HP1α, pCS2-Oct4-EGFP or EGFP-Nanog, kindly provided by Dr. Nicolás Plachta. Single-point FCS measurements were performed after 48 hs of transfection. Data collection and analysis are described in Supplemental Methods.

Statistical analysis

Experimental results are expressed as mean ± standard error of the mean (SEM) of at least three replicates, considered as independent experiments performed at different times and having a different cell passage. Statistical significance among groups was analyzed using either paired Student's t-test or randomized block design (RBD) ANOVA. In some experiments, significance between groups was analyzed by lineal mixed models (LMM) due to imbalance of

data. Residuals fitted normal distribution and homogeneity of variance. Otherwise, the data were transformed (log) to meet both assumptions. For experiments showed in Fig1, comparisons between means were assessed using the Tukey test. For experiments showed in Fig2F and FigS2, differences between groups were evaluated using the Mann-Whitney *U* test. Differences were regarded as significant at $p \leq 0.05$. Statistical analysis was performed using Infostat Software [75].

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Figure Legends

Fig. 1. Kat6B is repressed when ES cells exit pluripotency and its expression is regulated by Nanog and Oct4

RNA was extracted from ES cells and the expression of Kat6b and the pluripotency markers Nanog and Oct4 were measured by RT-qPCR at the indicated time points. Gene expression was normalized to Gapdh and referred to the undifferentiated state (day 0). Experimental results were expressed as mean ± SEM. W4 ES cells were induced to differentiate (A) in the absence of LIF and 2i, (B) by the Hanging drop differentiation protocol and (C) by a neural progenitor differentiation protocol. Neural marker Nestin was measured to validate differentiation. (D) ChIP-seq binding profiles (reads per million per base pair) for Oct4, Sox2, Nanog, Mediator coactivator (Med1) and H3K4me3 at the Kat6b locus in ES cells. Data from ChIP-seq experiments was obtained from Whyte et. al. [18] and analyzed with UCSC Genome Browser software. (E) R1 ES cells were transfected with pLKO.1-puro derived vectors targeting transcription factors (shOct4, shNanog or both), or eGFP (shGFP, control), as indicated under each bar. Transfected cells were selected with puromycin and RNA was extracted after 48h. Diagram of the experimental design (upper panel) and mRNA levels of Nanog, Oct4 and Kat6B measured by RT-qPCR, normalized to Gapdh expression and referred to the control condition (lower panel). Statistical analysis was performed using randomized block design (RBD) ANOVA. Significant differences were assessed using the Tukey test. Asterisks indicate significant differences (*$p<0.05$,**$p<0.01$).

Fig. 2. Kat6b knockout ES cells preserve self-renewal but present altered morphology

(A) CRISPR-mediated Kat6B knockout. Representative immunoblot (upper panel) and immunostaining (lower panel) showing Kat6b depletion in W4 K6b-/- ES cells compared to W4

parental cells, performed with a Kat6b antibody that recognizes the C-terminal domain. GAPDH was used as loading control for Western blot experiments. Nuclei were visualized by DAPI staining (scale bar: 20 μm). (B) RT-qPCR analysis of pluripotency (gray) and early differentiation (green) markers in WT and K6b-/- ES cells. Gene expression was normalized to Gapdh and presented as mean ± SEM. Statistical analysis was assessed by lineal mixed models (LMM) with a randomized block design (RBD). No significant differences were observed between WT and K6b-/- ES cells (C) Alkaline Phosphatase staining of both cell lines cultured ES medium (scale bar: 200 μm). (D) Cell-cycle distribution of WT and K6b-/- after propidium iodide staining and flow cytometric analysis (left panel). MTT cell viability assay. Bars represent mean ± SEM from three independent experiments (middle panel). Ki67 proliferation marker immunostaining. Bars represent mean ± SEM of intensity quantification from three independent experiments (right panel). Statistical analysis was performed using a paired Student's t-test. Representative images (lower panel, scale bar: 20 μm). (E) Representative brightfield microscopy images of both ES cell lines cultured in ES medium (scale bar: 100 μm) and enlargement of the images to show the morphology of a colony. (F) Nuclei were stained with DAPI and their middle-plane areas were quantified. Bars represent mean ± SEM of 350-360 cells. Differences between groups were evaluated using the Mann-Whitney *U* test. Asterisks indicate significant differences (***p<0.001) between WT and K6b-/- cells.

Fig. 3. Oct4, Nanog and HP1 dynamics is altered in Kat6b knockout ES cells

(A) Representative confocal images of WT cells transfected with vectors encoding for Oct4-GFP, Nanog-GFP or HP1α-GFP. (B) Mean normalized autocorrelation function (ACF) obtained in single point FCS experiments ran in WT (blue) and K6b-/- (orange) cells expressing Oct4-GFP, Nanog-GFP or HP1α-GFP. (C) The experimental ACFs were fitted with Eqn S1 to obtain the long-lived and short-lived characteristic times of the interactions of the proteins with

chromatin (left) and the fractions of free, long-lived bound and short-lived bound proteins (pie charts, right). Experimental results were expressed as mean ± SEM. Statistical analysis was performed by lineal mixed models (LMM). Asterisks indicate significant differences (*p<0.05, **p<0.01, ***p<0.001) between WT and K6b-/- cells.

Fig. 4. Kat6b knockout ES cells are pluripotent *in vitro* and *in vivo*

WT and K6b-/- ES cells were induced to differentiate (A) in the absence of LIF and 2i or (B) by the Hanging drop protocol. The expression of the indicated genes was analyzed by RT-qPCR and normalized to Gapdh expression at days 0, 2 and 4 for the LIF/2i withdrawal protocol, and at day 0 and day 11 for the Hanging drop protocol. Results are plotted in log2 scale and presented as mean ± SEM. Statistical analysis was performed using randomized block design (RBD) ANOVA. No significant differences were observed between WT and K6b-/- ES cells. (C) Pluripotency *in vivo* was analyzed by teratoma formation. Diagram of the experimental design (left) and representative images of histological analysis from tumors generated from WT and K6b-/- ES cells (right). Images of sections stained with hematoxylin and eosin show representative structures from the three germ layers (white arrows). Scale bar: 50 μm.

Fig. 5. Kat6b knockout ES cells have diminished efficiency to differentiate to neural progenitors

WT and K6b-/- ES cells were subjected to the neural progenitor differentiation protocol. (A) Bright-field images of cells at days 0, 1, 2, 4 and 6 of the protocol (scale bar: 100 μm). Whereas WT cells presented the expected phenotype at days 4 and 6, K6-/- cell line showed higher number of cells and a flatter morphology. (B) Expression of pluripotency markers (Nanog and Oct4), neural markers (Nestin, Blbp and Sox1) and mesodermal marker Brachyury was analyzed by RT-qPCR at the indicated times after the induction of differentiation. Values were

normalized to Gapdh expression and referred to the control condition (day 0). Results are plotted in log2 scale and presented as mean ± SEM. (C) Cell viability was analyzed by MTT assay at days 0, 2, 4 and 6 of the neural differentiation protocol in both cell lines. Statistical analysis was performed using randomized block design (RBD) ANOVA. Asterisks indicate significant differences between WT and K6b-/- cells (*$p<0.05$).

Highlights

- Chromatin remodeling is key during development and stem cell differentiation
- Kat6b expression is regulated by Nanog and Oct4 in pluripotent stem cells
- Kat6b knockout impacts in HP1, Oct4 and Nanog dynamics
- Kat6b knockout embryonic stem cells show reduced neural differentiation efficiency
- Kat6b is a modulator of chromatin plasticity that influences cell fate decisions

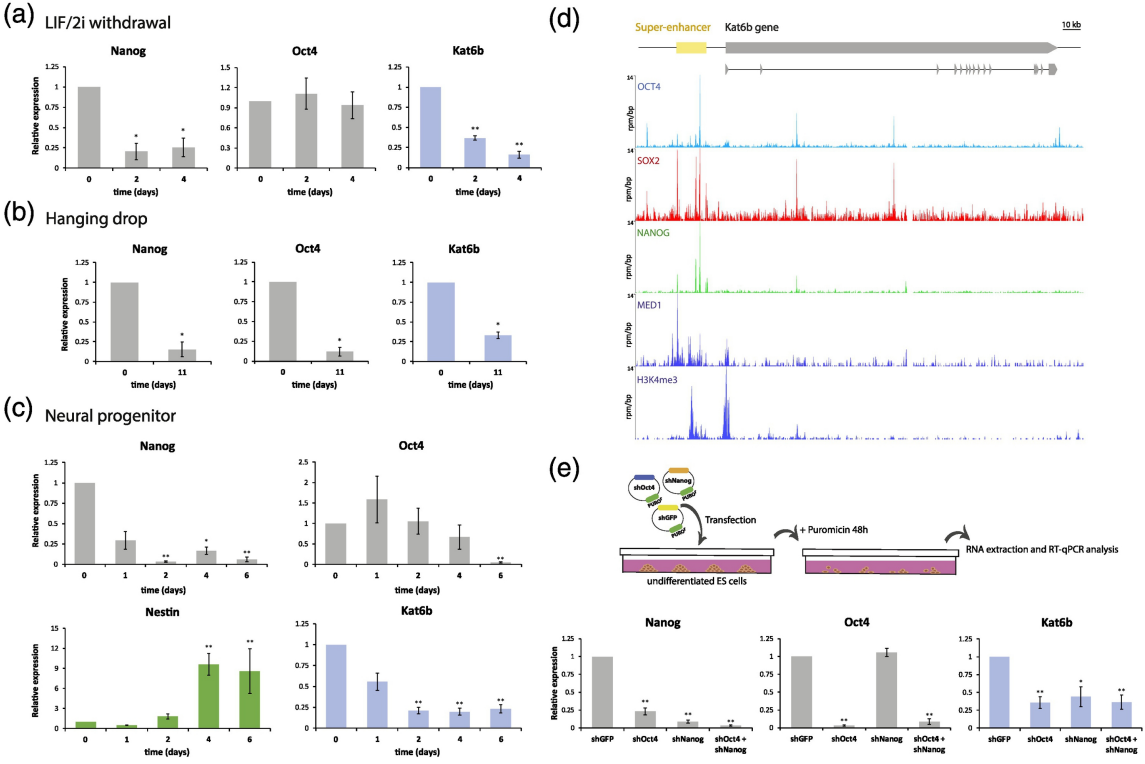
(a)
LIF/2i withdrawal
Nanog
Oct4
Kat6b
Relative expression
time (days)
(b)
Hanging drop
Nanog
Oct4
Kat6b
Relative expression
time (days)
(c)
Neural progenitor
Nanog
Oct4
Nestin
Kat6b
Relative expression
time (days)
(d)
Super-enhancer
Kat6b gene
10 kb
OCT4
SOX2
NANOG
MED1
H3K4me3
rpm/bp
(e)
shOct4
shNanog
shGFP
PGK
Transfection
+ Puromicin 48h
RNA extraction and RT-qPCR analysis
undifferentiated ES cells
Nanog
Oct4
Kat6b
Relative expression
shGFP
shOct4
shNanog
shOct4 + shNanog

Figure 1

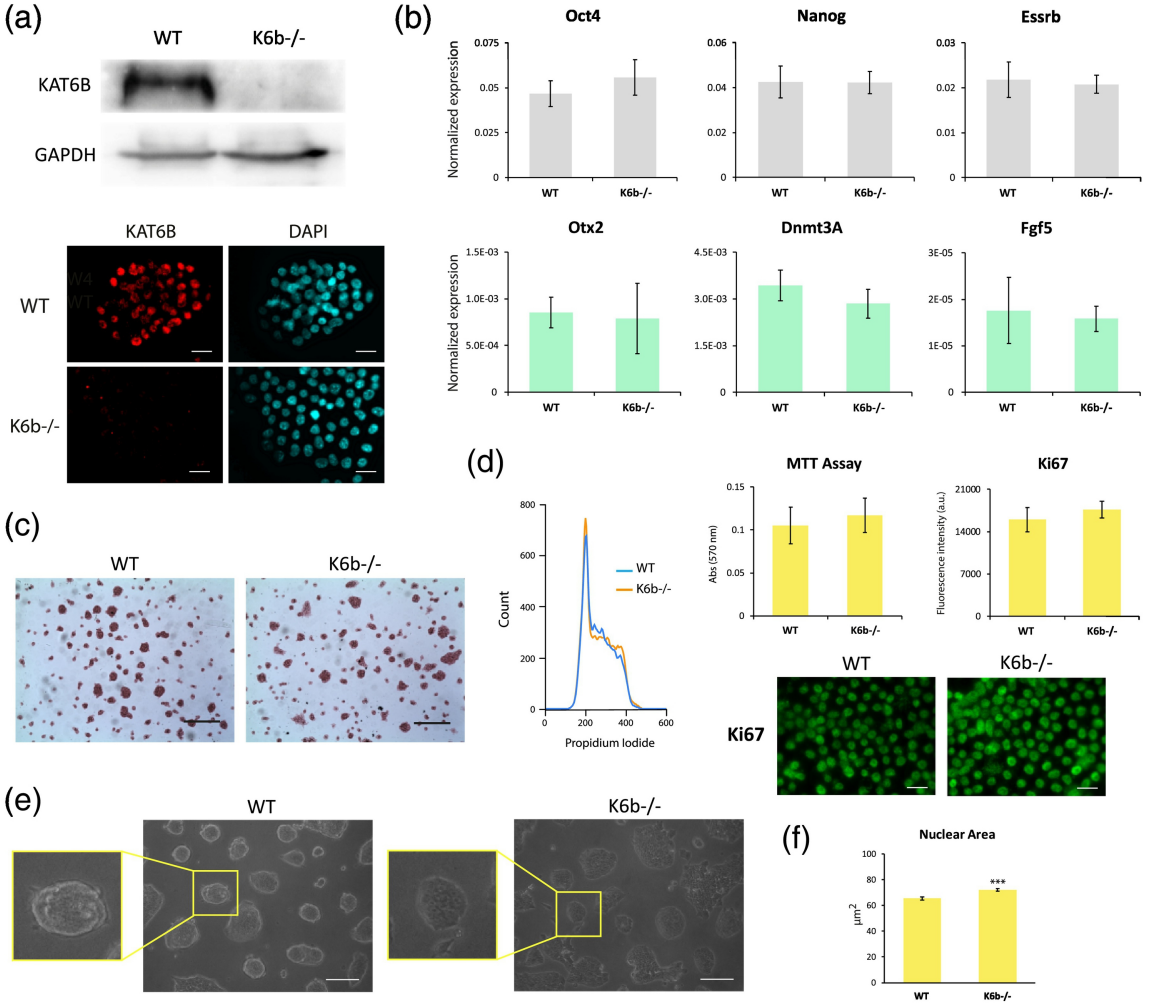
(a)
WT
K6b-/-
KAT6B
GAPDH
KAT6B
DAPI
WT
K6b-/-
(b)
Oct4
Nanog
Essrb
Otx2
Dnmt3A
Fgf5
Normalized expression
WT
K6b-/-
(c)
WT
K6b-/-
(d)
Count
Propidium Iodide
WT
K6b-/-
MTT Assay
Abs (570 nm)
Ki67
Fluorescence intensity (a.u.)
WT
K6b-/-
Ki67
(e)
WT
K6b-/-
(f)
Nuclear Area
µm2
***
WT
K6b-/-

Figure 2

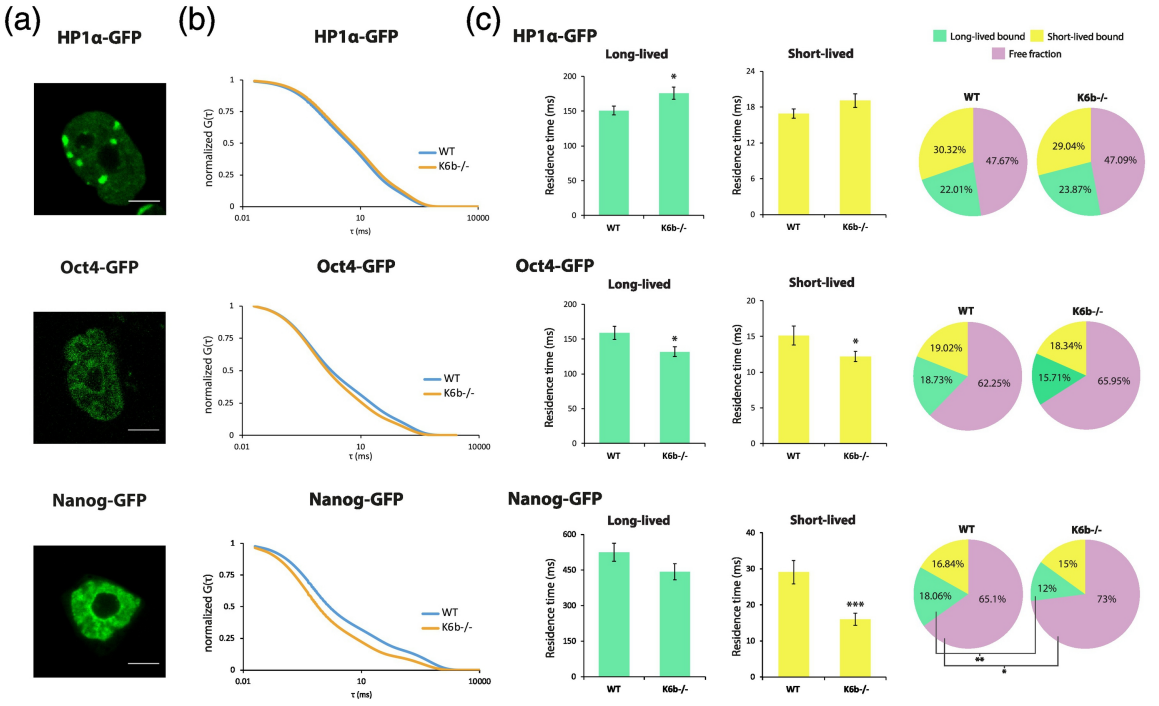
(a)
HP1α-GFP
Oct4-GFP
Nanog-GFP
(b)
HP1α-GFP
normalized G(τ)
τ (ms)
WT
K6b-/-
Oct4-GFP
Nanog-GFP
(c)
HP1α-GFP
Long-lived
Short-lived
Residence time (ms)
Long-lived bound
Short-lived bound
Free fraction
30.32%
47.67%
22.01%
29.04%
47.09%
23.87%
Oct4-GFP
19.02%
62.25%
18.73%
18.34%
65.95%
15.71%
Nanog-GFP
16.84%
65.1%
18.06%
15%
73%
12%

Figure 3

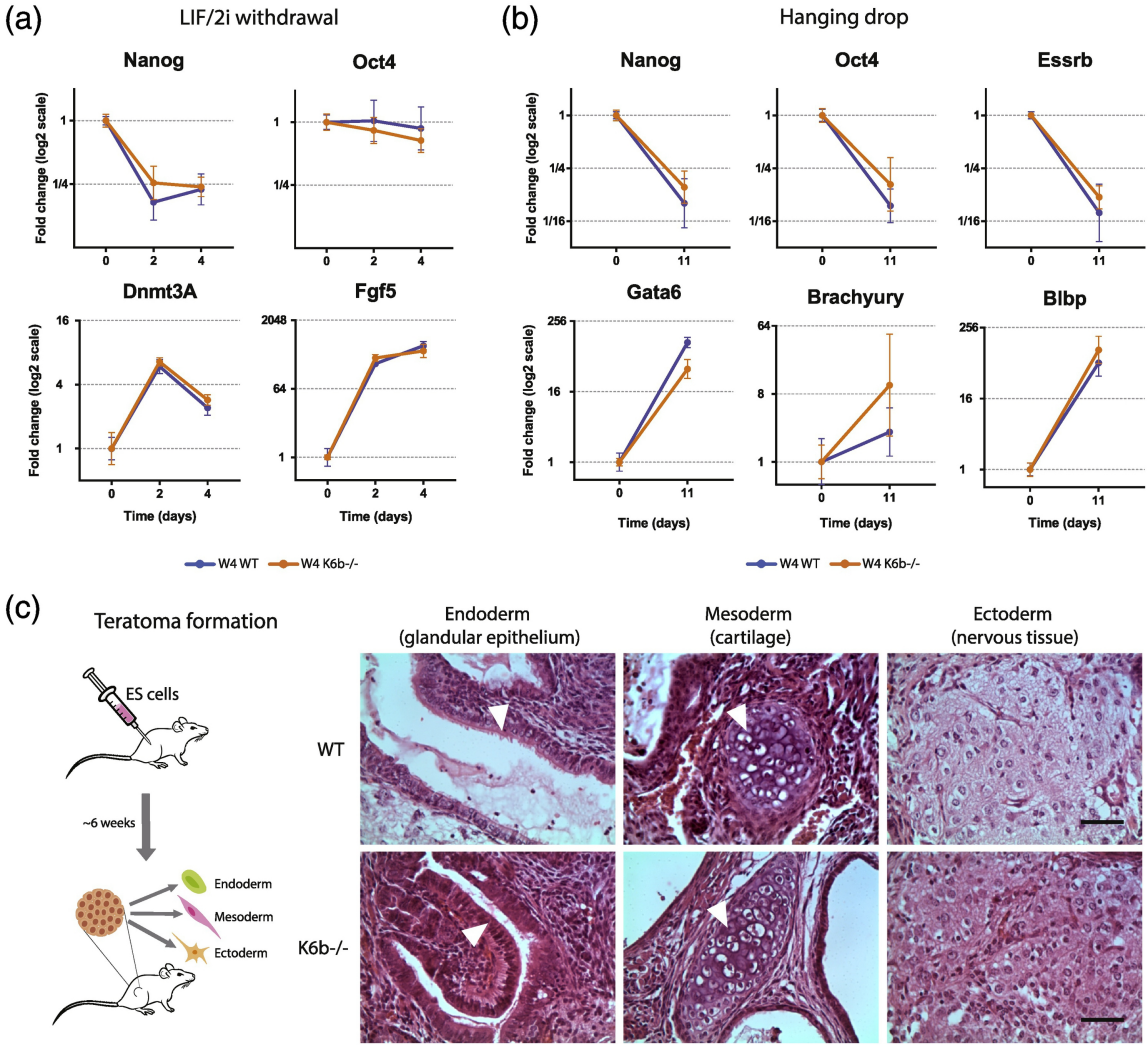
(a)
LIF/2i withdrawal
Nanog
Oct4
Dnmt3A
Fgf5
Fold change (log2 scale)
Time (days)
W4 WT
W4 K6b-/-
(b)
Hanging drop
Nanog
Oct4
Essrb
Gata6
Brachyury
Blbp
Fold change (log2 scale)
Time (days)
W4 WT
W4 K6b-/-
(c)
Teratoma formation
ES cells
~6 weeks
Endoderm
Mesoderm
Ectoderm
Endoderm (glandular epithelium)
Mesoderm (cartilage)
Ectoderm (nervous tissue)
WT
K6b-/-

Figure 4

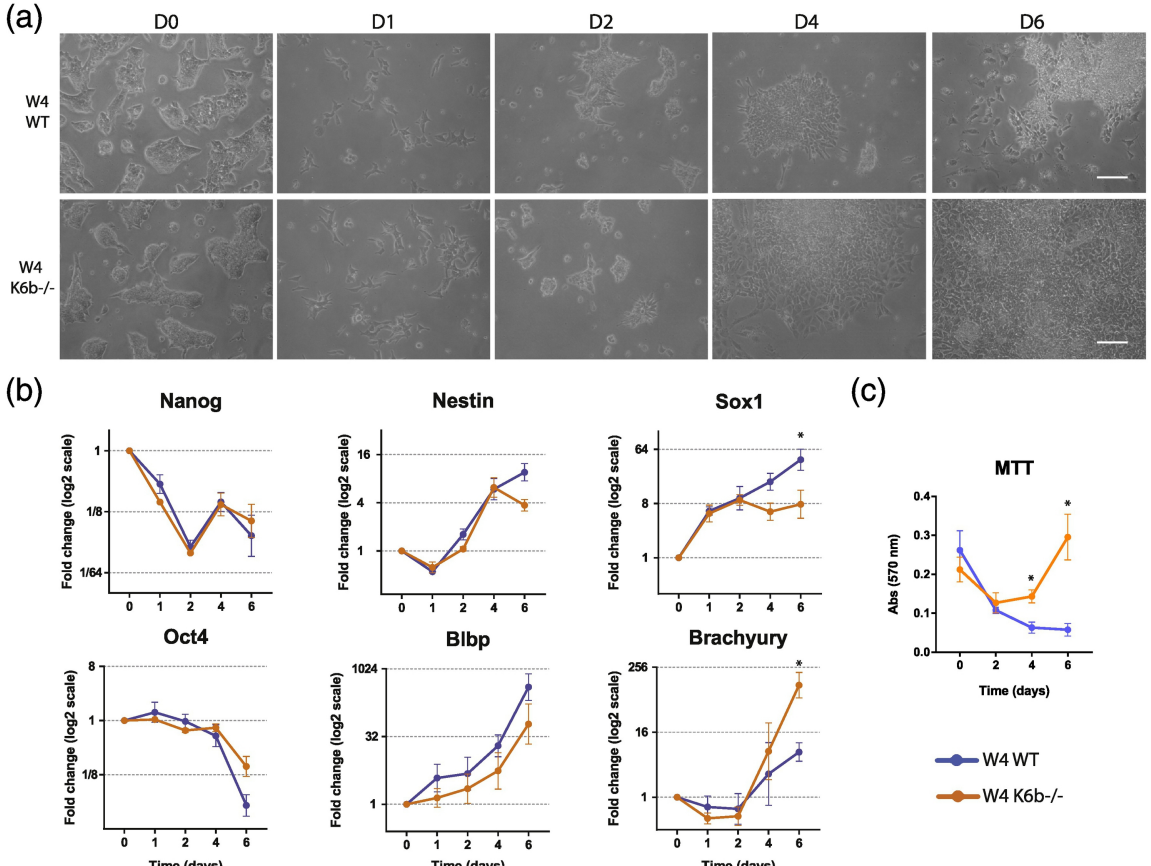
(a)
D0
D1
D2
D4
D6
W4 WT
W4 K6b-/-
(b)
Nanog
Nestin
Sox1
Oct4
Blbp
Brachyury
Fold change (log2 scale)
Time (days)
(c)
MTT
Abs (570 nm)
Time (days)
W4 WT
W4 K6b-/-

Figure 5