



# AMPK activators contribute to maintain naïve pluripotency in mouse embryonic stem cells

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## ABSTRACT

Pluripotent stem cells retain the property to self-renew and differentiate into all cell types under defined conditions. Among mouse embryonic stem cells (ESCs), which are pluripotent but heterogenous in gene expression and morphology, an ESC population cultured in small molecule inhibitors of two kinases, MAPK/ERK kinase (Mek) and Glycogen synthase kinase 3 (Gsk3), and leukemia inhibitory factor (Lif) (2i/L) is considered to be naïve pluripotent with uniform pluripotent machinery operation. Though the gene regulatory mechanism for the naïve pluripotency has been investigated in recent years, it is still not fully elucidated. Here we show a novel signaling involved in the maintenance of naïve pluripotency. An AMP-activated protein kinase (AMPK) activator, AICAR (5-Aminoimidazole-4-carboxamide-1- $\beta$ -ribose) blocked the differentiation of mouse naïve ESCs in the absence of 2i/L and maintained the naïve state. AICAR with Lif condition induced an almost comparable level of naïve pluripotent gene expression in mouse ESCs. Another AMPK activator, A769662, also showed similar effects. A p38 inhibitor, SB203580, blocked the AMPK activation-elicited naïve state maintenance. On the other hand, p38 activation partially mimicked the maintenance effects of AMPK activators, suggesting that p38 is one of the functional downstream molecules to conduct the AMPK effects. Thus, AMPK pathway should be involved in the molecular circuitry of naïve pluripotency in mouse ESCs. These findings would be a valuable clue to further elucidate the molecular machinery of naïve pluripotency.

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## 1. Introduction

Mouse embryonic stem cells (mESCs) are derived from inner cells mass (ICM) in pre-implantation embryo [1,2]. Culturing in two small molecule inhibitors (2i) of kinases, Mek (MAPK (mitogen-activated protein kinase)/ERK (extracellular signal-regulated kinase) and GSK3 (glycogen synthase kinase 3) and leukemia inhibitory factor (Lif), mESCs can be retained at naïve state with a similar pluripotency as ICM [3,4]. Naïve mESCs display small dome-shaped colonies, uniform pluripotency marker expression with unique gene expressions and epigenetic modifications and has the potential to form all types of somatic cell lineages [5–8]. Lif is the first defined signal to maintain mESCs self-renewal for long-term culture [3,9]. However, mESCs in Lif alone are morphological and transcriptional heterogeneous. Two small molecule inhibitors (2i) of MEK and GSK3 have been reported to sustain robust ES cell self-renewal and suppress differentiation [4]. Combination of Lif and 2i

promotes mESCs in stable naïve state. Upon Lif and 2i withdrawal, naïve ESCs spontaneously exit from the naïve state without an exogenous inducer [5]. Naïve state is defined with specific transcription factors. Essential general pluripotency transcription factors, Oct4 and Sox2, play a central role in the maintenance of pluripotency but are not restricted to naïve state [5]. Naïve mESCs have a specific network of pluripotency transcription factors containing Oct4 and Sox2 together with Nanog, Esrrb, Tfcp2l1, Klf4 and Klf2 that are regulated by Lif and GSK3 pathways [5].

Adenosine monophosphate (AMP)-activated protein kinase (AMPK), a serine/threonine protein kinase, which is activated by increased intracellular AMP or AMP/ATP (adenosine triphosphate) ratio, plays an important role in mediating cellular energy homeostasis. The AMPK pathway participates in many cellular processes such as cytokine production, cell growth, lipid metabolism, protein synthesis and autophagy [10,11]. AMPK signal has been reported to be involved in mouse ESC pluripotency and reprogramming [12–14], but still unclear in naïve pluripotency. Relationship between AMPK and the naïve pluripotency transcription factor network is unknown.

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We have been investigating pluripotent stem cell differentiation [15–19]. During our research, we occasionally found that under an AMPK activator, AICAR (5-Aminoimidazole-4-carboxamide-1- $\beta$ -ribose) treatment, mESCs were resistant to differentiation. Then, we further investigated the phenomenon.

In the present study, we show that AMPK activators, AICAR and A769662, can maintain mESCs in naïve state even in the absence of 2i/L and activates the network of naïve pluripotency transcription factors. Our data offer a new pathway, AMPK signaling, in regulation of naïve pluripotency that provides new insights and clues for the research of molecular mechanisms for naïve pluripotency.

## 2. Materials and methods

### 2.1. Cell culture

Feeder-free mouse embryonic stem cells (mESCs) carrying Rex1 promoter-driven GFPd2 gene (Rex1-GFP cells) were cultured on 0.1% gelatin-coated dishes in basal medium (GMEM (GIBCO) supplemented with 10% Knockout™ Serum Replacement (GIBCO), 1% fetal bovine serum (SAFC Biosciences), 0.1 mM MEM non-essential amino acids (GIBCO), 1 mM sodium pyruvate (SIGMA), 0.1 mM 2-mercaptoethanol (GIBCO), penicillin/streptomycin supplemented with 1000 U/mL Lif (Millipore) and two small molecule inhibitors (2i) PD0325901 1  $\mu$ M (SIGMA) and CHIR99021 3  $\mu$ M (Tocris)) as described previously [4,15]. Cells were passaged every 3 days by dissociation with Accumax (Innovative Cell Technologies) and replated at  $3 \times 10^4$  cells/cm<sup>2</sup> cell density. Medium was changed every 2 days.

### 2.2. Treatment with AMPK activators

AICAR (WAKO) was dissolved in distilled water at 125 mM concentration. A769662 (ADooQ) was dissolved in dimethyl sulfoxide (DMSO) at 100 mM concentration. Dissociated mESCs were plated on gelatin-coated dishes in basal medium supplemented with Lif, 2i, AICAR (final 1–3 mM) or A769662 (final 100  $\mu$ M) with indicated combinations. Cells were passaged every 4–7 days. Medium was changed every 2 days.

### 2.3. Immunostaining

Immunostaining was carried out as described previously [15]. In brief, cells were fixed in 4% paraformaldehyde for 15–20 min, then blocked with 2% non-fat dry milk (BD). Cells were incubated with primary antibodies of indicated dilution (rabbit anti-Nanog antibody (RepoCell, 1:300), rabbit anti-Tfe3 antibody (Sigma, 1:300)), overnight at 4 °C. Secondary antibodies, anti-rabbit IgG antibodies conjugated with Alexa488 or Alexa546 (Invitrogen), were diluted to 1:500, treated for 1 h at room temperature and then washed with phosphate-buffered saline with Tween-20. Nuclei were stained with DAPI.

### 2.4. Western blot analysis

Cells were lysed in Sample buffer solution with 2-mercaptoethanol (ME) (Nacalai Tesque). Cell lysates were run on Blot™ Gel (Invitrogen), followed by electrophoretic transfer onto nitrocellulose membranes. Then they were treated for 30 min in Blocking One (Nacalai Tesque) and incubated overnight at 4 °C with primary antibodies for following targets: phosphorylated-AMPK (Thr172, Cell Signaling (2531S) 1:1000), AMPK (Cell Signaling (2532S), 1:1000), phosphorylated-p38 (Thr180/Tyr182, Cell Signaling (9215S), 1:1000), p38 (Cell Signaling (9212S), 1:1000), phosphorylated-Mapkapk2 (Thr334, Cell Signaling (3007S),

1:1000), Mapkapk2 (Cell Signaling (3042S), 1:1000),  $\beta$ -actin (Sigma (A5441), 1:10000). Secondary antibodies were anti-mouse or rabbit IgG antibodies-conjugated with Horseradish peroxidase (HRP) (Cell Signaling, 1:3000–1:1000). After 2 h of room temperature incubation, Immobilon western chemiluminescent substrate (Millipore) was used for detection. All primary and secondary antibodies were diluted in the Can Get Signal Immunoreaction Enhancer Solution Kit (Toyobo).

### 2.5. Quantitative real-time PCR (qPCR)

Total RNA was isolated using the RNeasy Mini kit (QIAGEN) and cDNA was reverse-transcribed from 1  $\mu$ g of total RNA using SuperScript III (Invitrogen). All qPCR reactions were performed in triplicate of each sample obtained from at least 3 independent cultures in SYBR Green Master Mix (Applied Biosystems). An endogenous control GAPDH was used to normalize expression of mouse cells. Primer sequences: GAPDH-forward (F): TGT GTC CGT CGT GGA TCT GA; GAPDH-reverse (R): TTG CTG TTG AAG TCG CAG GAG; Rex1-F: TCT TCT CTC AAT AGA GTG AGT GTG C; Rex1-R: GCT TTC TTC TGT GTG CAG GA Oct3/4-F: GGA CAT GAA AGC CCT GCA GAA; Oct3/4-R: GAC AGA TGG TGG TCT GGC TGA A; Nanog-F: GAA TTC TGG GAA CGC CTC ATC; Nanog-R: CCT TGT CAG CCT CAG GAC TTG; Sox2-F: AAC CGA TGC ACC GCT ACG A; Sox2-R: TGC TGC GTA GGA CAT GCT G; Klf4-F: AGA CCA GAT GCA GTC ACA AGT C; Klf4-R: CCC AGT CAC AGT GGT AAG GTT T; Klf2-F: CTA AAG GCG CAT CTG CGT A; Klf2-R: TAG TGG CGG GTA AGC TCG T; Esrrb-F: AGA GAC CCT CTC CTA TTC CCA C; Esrrb-R: TCT CCA TGG GTT CCA GAA ATG G; Tfcp2l1-F: CCA GGA AAA GGC TAG AAC CCA T; Tfcp2l1-R: AGG TTC TCA GGT CTC CAG ATC A.

### 2.6. Imaging analysis

Images were photographed with inverted microscope (Olympus IX71) equipped with U-RFL-T (Olympus), or with LSM700 inverted confocal microscope (Zeiss) equipped with 405 nm, 488 nm, and 556 nm lasers using  $\times 10$  or  $\times 20$  objective.

### 2.7. Statistical analysis

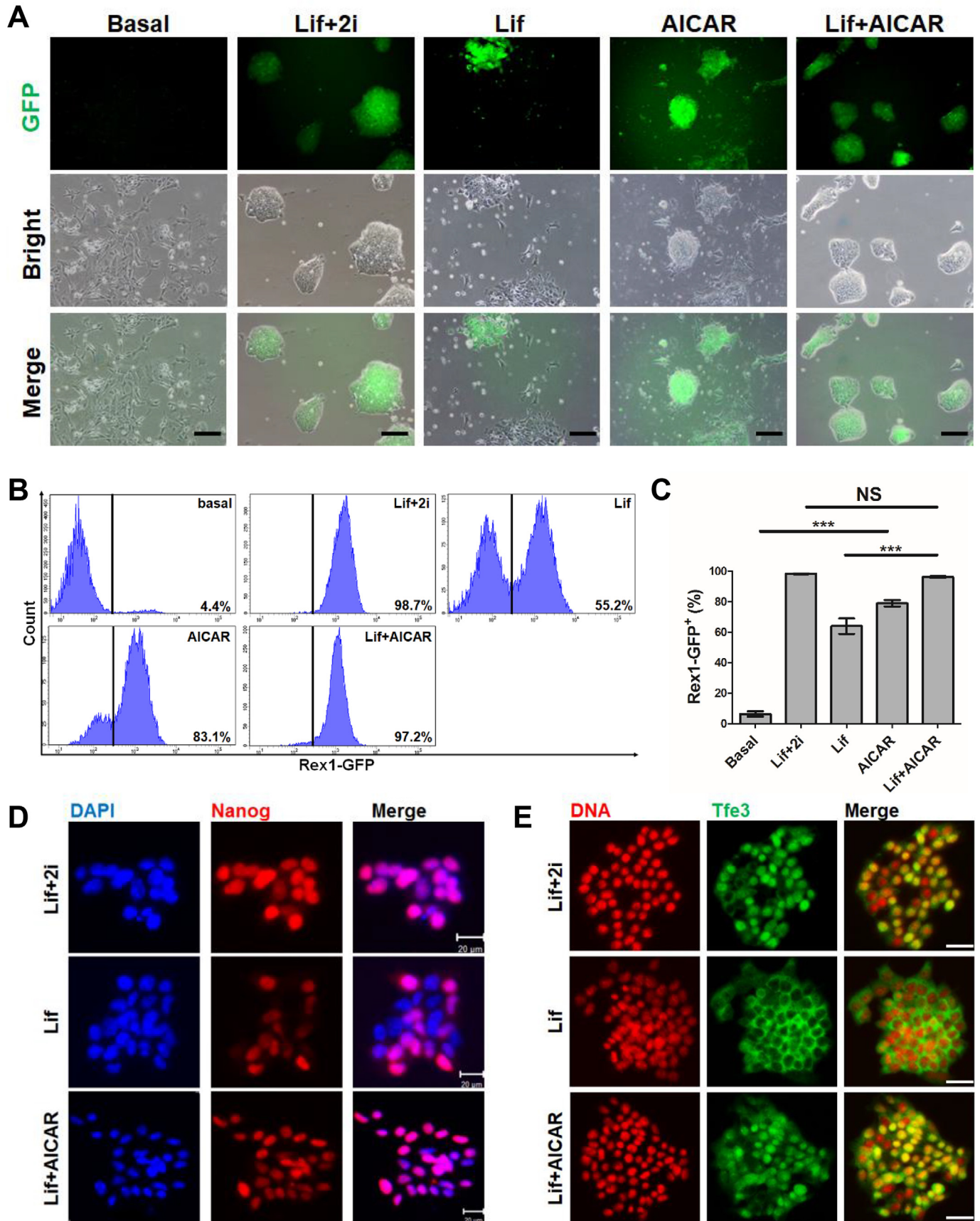
All experiments were performed at least 3 times independently. Statistical analysis of data was performed by ANOVA followed by Tukey's multiple comparison.  $p < 0.05$  was considered significant. Error bars indicate mean  $\pm$  SD.

## 3. Result

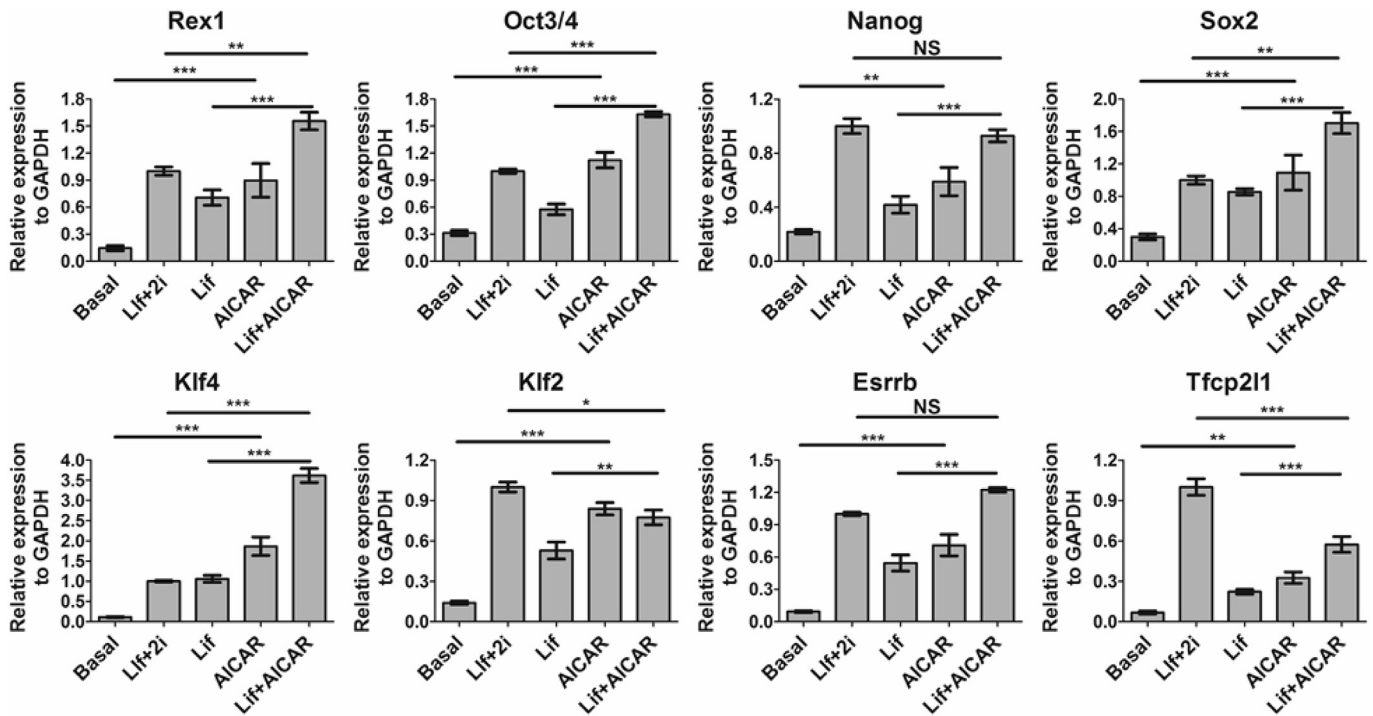
### 3.1. AICAR maintains naïve morphology and protein expression

For live cell monitoring of naïve pluripotency, we used a mESC line, Rex1-GFP cells, in which expression of destabilized GFP protein with a half-life of 2 h (h) is driven by endogenous Rex1 promoter [20,21].

To evaluate whether AICAR possesses an ability to maintain naïve pluripotency, we cultured mESC with combinations of Lif, 2i, and AICAR in basal maintenance medium for 4 passages (Fig. 1). Cells completely lost Rex1-GFP expression and differentiated in basal medium alone. Lif+2i condition uniformly induced Rex1-GFP-positive (Rex1-GFP<sup>+</sup>) dome-shaped naïve cell colonies. Lif alone was not sufficient to maintain naïve cell colonies with the appearance of heterogeneous cell morphology. AICAR alone showed not complete but distinct appearance of Rex1-GFP<sup>+</sup> naïve cell-like colonies. Lif + AICAR was almost complete for uniform naïve cell colony appearance as well as Lif+2i (Fig. 1A). In order to quantify the naïve-specific marker Rex1-positive cells after 4 passages, we



**Fig. 1. AICAR maintains naïve state of mESCs.** (A) Colony morphology. Rex1-GFP2 cells were cultured in basal medium, Lif+2i, Lif alone, AICAR alone, and Lif + AICAR condition, respectively after 4 passages. Scale bars: 200  $\mu$ m. (B and C) FACS analysis and quantitative evaluation for Rex1-GFP<sup>+</sup> cell appearance after 4 passages. Percentages of the



**Fig. 2. AICAR activates naïve pluripotent factor expression in mESCs.** qPCR showing mRNA expression of naïve markers. GAPDH was used as control. Mean  $\pm$  SD of three independent experiments are shown. Results of Lif+2i were set as 1 (\* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001, NS: not significant).

used flow cytometry to analyze the expression of Rex1. Whereas in basal medium alone, almost all of cells became Rex1-GFP-negative (negative control), Lif+2i showed almost complete naïve maintenance with more than 95% ( $98.3\% \pm 0.46\%$ ) of Rex1-GFP<sup>+</sup> cells (positive control). AICAR alone induced significantly more Rex1-GFP<sup>+</sup> cells ( $78.9\% \pm 3.6\%$ ) than basal medium alone. Surprisingly, Lif + AICAR showed similar Rex1-GFP<sup>+</sup> cell maintenance ( $96.2\% \pm 0.92\%$ ) to those in Lif+2i condition (Fig. 1B and C). Since Rex1 is a specific naïve marker and is downregulated upon withdrawal of 2i within 24 h from the onset of differentiation [22,23], AICAR has the potential to antagonize the exit from naïve state.

We evaluated other features of naïve ESCs with immunostaining of colonies. Nanog staining showed highly homogeneous nuclear distribution in Lif + AICAR as well as Lif+2i condition compared to Lif alone (Fig. 1D). A bHLH transcription factor, Tfe3, is reported that its nuclear exclusion normally relates to loss of naïve state pluripotency [24]. Whereas clear cytoplasmic localization of Tfe3 was observed in Lif alone condition, nuclear localization was mainly observed in Lif+2i and Lif + AICAR (Fig. 1E). All these results indicate that AICAR can contribute to maintain naïve state of mESCs even in the absence of Lif and 2i.

### 3.2. AICAR activates the network of naïve pluripotency

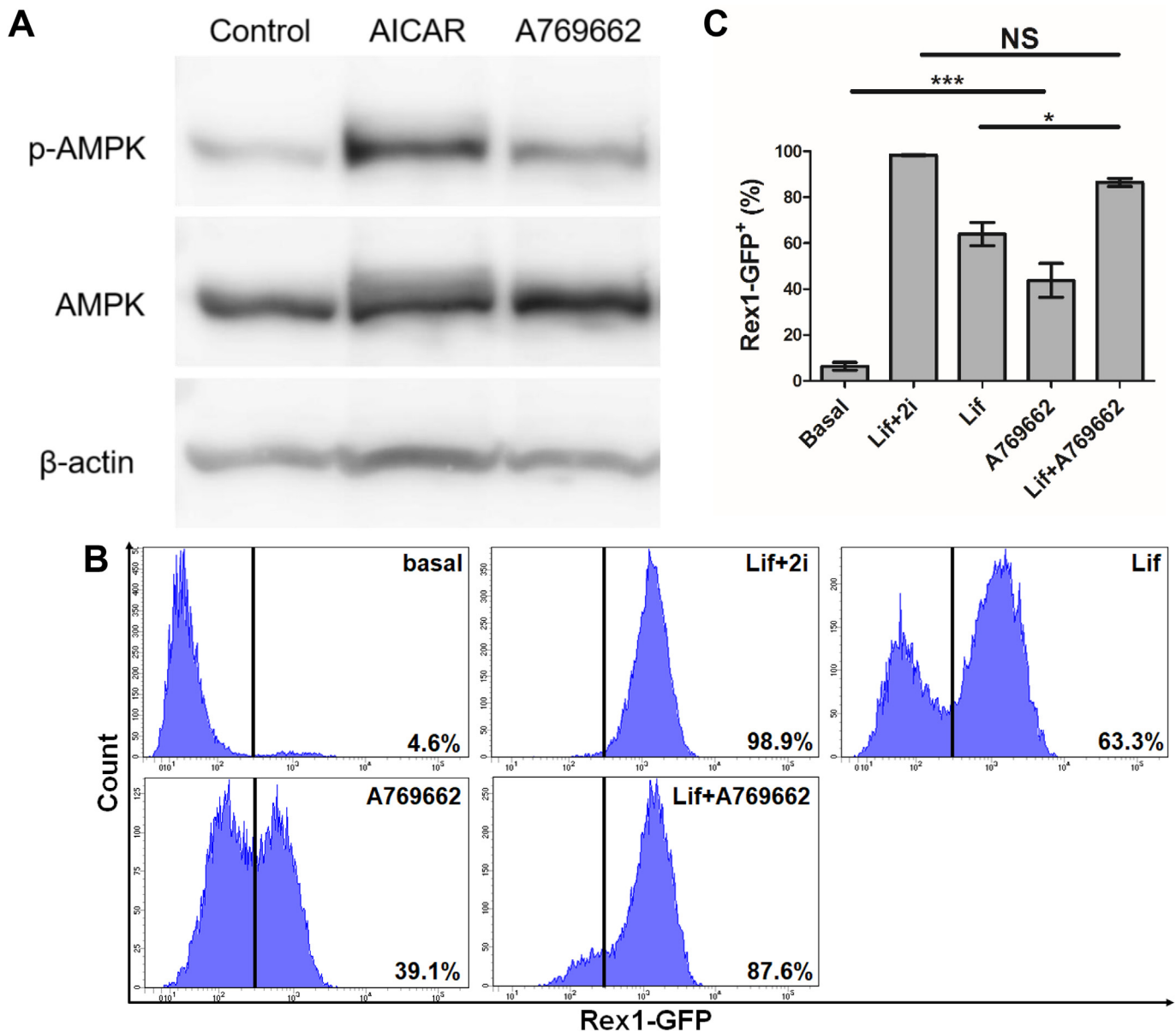
Next we examined mRNA expressions of molecular network for naïve pluripotency by qPCR. With AICAR alone treatment, various naïve pluripotent genes, Rex1, Oct4, Nanog, Sox2, Klf4, Klf2, Esrrb, and Tfcp2l1, were significantly higher than basal condition (Fig. 2). In Lif + AICAR condition, all these genes except Klf2 and Tfcp2l1 were comparable or significantly higher than those in Lif+2i

condition. Tfcp2l1, which plays a central role in Lif/Stat3 axis and is activated by Gsk3i [25,26], was at approximately 30% in AICAR alone and at 50% in Lif + AICAR, respectively, compared to that in Lif+2i. Instead, Klf4 expression in Lif + AICAR condition was significantly higher than that in Lif+2i. Thus, AICAR can broadly induce gene expression of core molecular network for naïve pluripotency to comparable level of Lif+2i. Strong induction in Klf4 with AICAR may suggest that Klf4 should be a direct downstream of AICAR.

### 3.3. Another AMPK activator A769662 also maintains naïve state

To evaluate whether the maintenance of naïve pluripotency with AICAR is the effect of AMPK activation or other AICAR effects, we tested another AMPK activator, A769662 [27]. Whereas AICAR activates AMPK by mimicking AMP and through acting on AMPK- $\alpha$  subunit, A769662 activates AMPK through acting on AMPK- $\beta$  subunit, indicating that those two activators have distinct molecular mechanisms. Western blot analysis for phosphorylated AMPK and total AMPK in mESCs after AMPK activator treatments confirmed that AICAR and A769662 actually activated AMPK pathway (Fig. 3A). Then we tested the effects of A769662 on the maintenance of naïve pluripotency. We cultured naïve mESCs with combinations of Lif, 2i, and A769662 in basal medium for 4 passages. A769662 alone sustained approximately 40% of Rex1<sup>+</sup> cells which was significantly higher than basal medium. Lif + A769662 maintained more than 80% of Rex1<sup>+</sup> cells which is comparable level with Lif+2i (Fig. 3B and C). Taken together A769662 also had the effect on maintenance of naïve mESCs.

GFP<sup>+</sup> population ( $n = 3$ ; \*\*\* $p$  < 0.001, NS: not significant) are indicated. (D) Fluorescent immunostaining for Nanog expression in cells of culturing in Lif, Lif + AICAR and Lif+2i. Five hundred cells were plated and stained for Nanog after 4days. Scale bars: 20  $\mu$ m. (E) Fluorescent immunostaining for Tfe3 expression in maintained mESCs with Lif, Lif + AICAR and Lif+2i. Scale bars: 20  $\mu$ m.

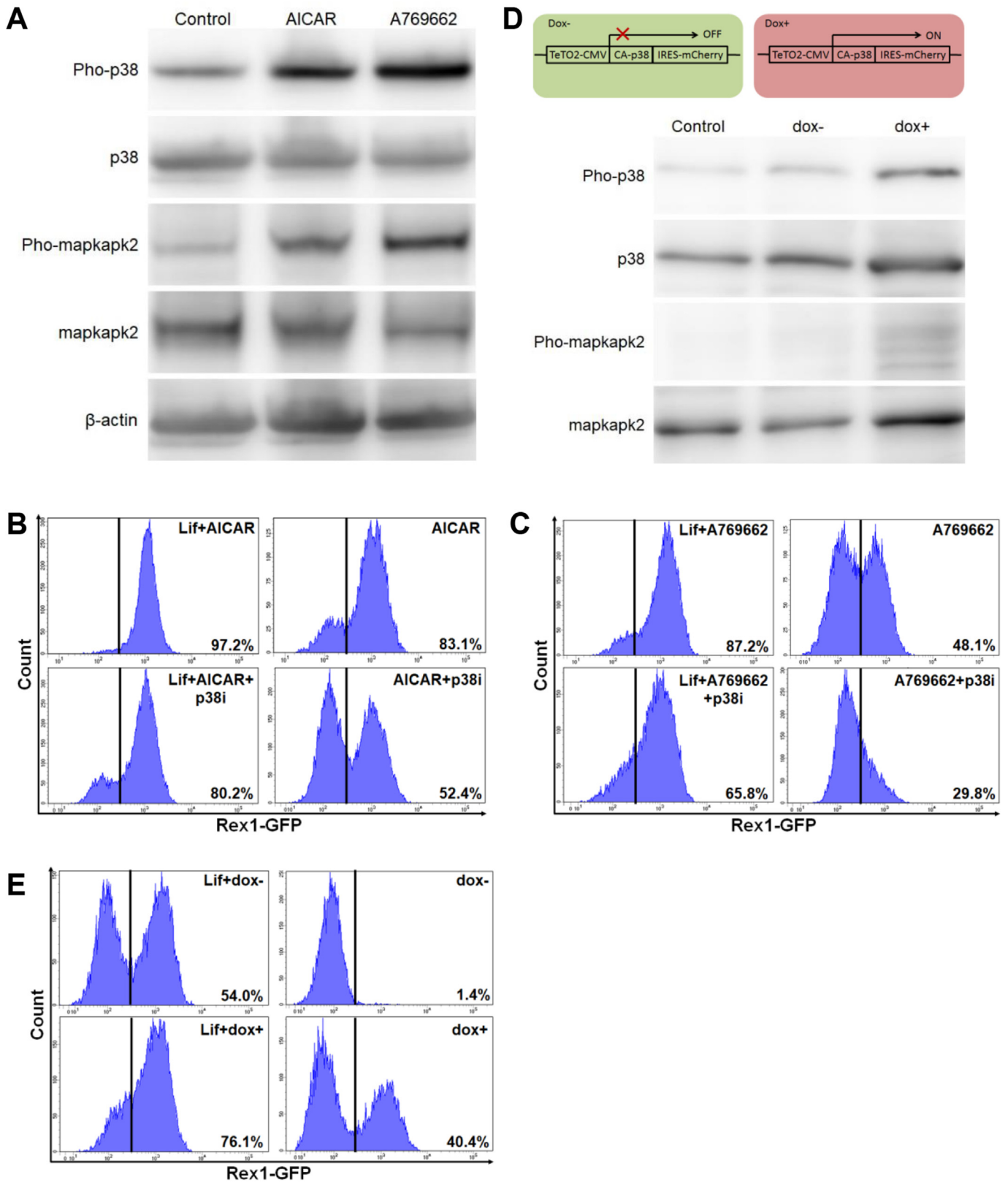


**Fig. 3.** AMPK activators maintain naïve state of mESCs. (A) AMPK activation. Western blot analysis shows protein expression of AMPK $\alpha$  and phosphorylated AMPK $\alpha$  (Thr172) by AMPK activators. (B and C) FACS analysis and quantitative evaluation for Rex1-GFP<sup>+</sup> cell appearance. Rex1-GFP<sup>+</sup> cells were cultured in basal medium with Lif + A769662 and A769662 after 4 passages. Percentages of the GFP<sup>+</sup> population (n = 3; \*p < 0.05, \*\*\*p < 0.001, NS: not significant) are indicated.

#### 3.4. p38 is a downstream of AMPK for maintenance of naïve pluripotency

AMPK has many downstream signaling pathways involved in broad biological phenomena including metabolism, cell growth, autophagy and so on [11,28,29]. Among them, p38 is reported to be activated by AMPK pathway [29] and also involved in reprogramming of somatic cells to pluripotent stem cells [30]. Then, we speculated that p38 should be a potential downstream involved in the AMPK-elicited maintenance of naïve pluripotency. We confirmed that AICAR or A769662 can activate p38 with western blot for phosphorylation of p38 as well as that of a p38 target molecule, mapkapk2 (Fig. 4A). Then, we examined the effects of a p38 inhibitor, SB203580 on AMPK activators-induced naïve state maintenance. Flow cytometry analysis for Rex1-GFP revealed that SB203580 reduced Rex1-GFP<sup>+</sup> cells under basal medium with

AICAR or A769662 treatment (Fig. 4B and C). We further confirmed a gain-of-function effect of p38. We generated a mESC line carrying tetracycline-inducible (Tet-ON) constitutive active form of p38 (CA-p38) [30]. A p38 cDNA containing D176A and F327S mutations [31] was inserted into a piggyBac (PB) vector carrying rtTA expression coupled to mCherry [32]. We introduced the Tet-On CA-p38 with mCherry gene into Rex1-GFP<sup>+</sup> cell line. Dox treatment (Dox+) actually induced p38 activation, confirmed by phosphorylation of p38 and mapkapk2 (Fig. 4D). p38 activation with Dox alone was able to induce Rex1-GFP<sup>+</sup> cell appearance. p38 activation with Lif further enhanced the effect of Lif alone on Rex1-GFP<sup>+</sup> cell maintenance (Fig. 4E), though effect of p38 activation on the maintenance of naïve pluripotency was weaker than that of AICAR or A769662. All these results suggest that p38 is one of the functional downstream molecules conducting the AMPK effect on the maintenance of naïve pluripotency.



**Fig. 4. p38 is a functional downstream of AMPK.** (A) p38 activation with AMPK activators. Western blot analysis shows protein expression of p38, phosphorylated p38 (Thr180/Tyr182), a unique p38 downstream target mapkpk2 and phosphorylated mapkpk2 (Thr334). Note that p38 is activated commonly with AMPK activators. (B and C) FACS analysis for Rex1-GFP<sup>+</sup> cell appearance with a p38 inhibitor. p38i: SB203580 (10  $\mu$ M). (D) Tetracycline-inducible (Tet-ON) constitutive active form of p38 (CA-p38) expression marked with mCherry. Western blot confirms dox-inducible activation of p38 pathway with CA-p38 expression. Doxycycline (1  $\mu$ g/ml) treatment. (E) FACS analysis for Rex1-GFP<sup>+</sup> cell appearance after p38 activation.

#### 4. Discussion

Here we showed that AMPK activators contribute to maintain naïve pluripotency in mESCs. AMPK activators are capable of activating the molecular network of naïve pluripotency in mESCs. p38 is revealed to be one of the possible downstream of AMPK signaling for the maintenance of naïve pluripotency.

Two distinct AMPK activators showed similar effects on the maintenance of naïve pluripotency. AICAR is an AMP analog that can mimic the endogenous AMP inducing activation of AMPK pathway [28]. A769662 directly activates AMPK pathway by mimicking both effects of allosteric activation and inhibition of dephosphorylation which is different from AICAR [27]. Both of compounds similarly induced AMPK phosphorylation and p38 activation, leading to the similar maintenance effects of naïve pluripotency, indicating that AMPK pathway is involved in the maintenance of naïve pluripotency. Nevertheless, relationship between AMPK and the pluripotency transcription factor network is still unclear. AICAR induced expression of naïve markers Rex1, Nanog, Klf4, Klf2, Esrrb and Tfcp2l1 (Fig. 3). Especially, Klf4, which is a direct target of Lif/Stat3 axis and has been reported that forced expression lead to mESCs self-renewal [33], was upregulated significantly by AICAR, suggesting that one of the targets of AICAR for the maintenance of naïve state is Klf4 signaling. On the other hand, Tfcp2l1, another target of Lif/Stat3, was not sufficiently upregulated as much as Klf4, suggesting that AMPK does not simply mediate Lif/Stat3 signaling. Klf2, which is directly repressed by Tcf3 thereby activated by GSK3i [5], was not upregulated as obviously as Klf4. Esrrb which is also a pivotal target of the Gsk3/Tcf3 axis [34] was induced not significantly as Klf4, suggesting that the effect of AICAR in naïve maintenance is not mediated by Gsk3/Tcf3 pathway. Thus, activation of naïve pluripotency by AMPK activators should be mediated by a new signaling independent on Lif and Gsk3.

AMPK pathway is one of the central regulators for controlling cell polarity, migration, cytoskeletal dynamics and metabolism [11]. AMPK was shown to play an important role in the activation of the p38 pathway in the ischemic heart [29] and activation of p38 was reported to induce the expression of some pluripotent genes for promoting somatic cells reprogramming [30]. p38 inhibitor blocked the AICAR-elicited maintenance of naïve state and p38 activation partially induced naïve maintenance (Fig. 4), suggesting that p38 pathway is at least one of the functional downstream of AMPK. Since the AMPK-mTOR pathway was reported to be involved in reprogramming mouse somatic cells to induced pluripotent stem cells (iPSCs) through autophagy [14], it is assumed that mTOR might be the downstream of AMPK in the modulation of naïve pluripotency. Meanwhile, AMPK activators results in increasing NAD<sup>+</sup> levels and the NAD<sup>+</sup>/NADH ratio which leads to Sirt1 activation [35]. Sirt1 was reported to deacetylate Oct4, which promoted the maintenance of naïve pluripotency [36]. Molecular mechanism of AMPK pathway for the maintenance of naïve pluripotency including these molecules should be further elucidated.

Collectively, this study offers a new molecular pathway that contributes to the maintenance of naïve pluripotency in mouse ESCs, providing us new insights and clues for elucidation of the molecular machinery for naïve stem cell status.

#### Conflicts of interest

The authors declare no conflict of interest.

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