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Cover Letter

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The Editor of
Biochemical and Biophysical
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Dear Editor

I am sending the manuscript entitled " GABPα regulates Oct-3/4 expression in mouse embryonic stem cells " by Keita Kinoshita, Hiroki Ura, Tadayuki Akagi, Masayuki Usuda, Hiroshi Koide, and myself, which we would like to submit for publication in Biochemical and Biophysical Research Communications.

In this study, we identified GA-repeat binding protein (GABP) α as a self-renewal-specific gene in ES cells. We also found that expression of GABPα is regulated by STAT3. By RNAi method, we showed that GABPα is essential, but not sufficient, for the self-renewal of ES cells. Furthermore, we found that GABPα regulates the expression of Oct-3/4, a critical factor for ES cell self-renewal, via inhibiting expression of three Oct-3/4 repressors, Cdx-2, Coup-tf1 and GCNF. These results suggest that GABPα is involved in ES cell self-renewal through regulation of Oct-3/4. In spite of great interest in stem cell research, little is known about the molecular mechanism of ES cell self-renewal. Also, this study provides a clue to molecular mechanism of Oct-3/4 expression. Therefore, we believe this paper will attract the general interest of readers of Biochemical and Biophysical Research Communications.

Sincerely yours,

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GABPα regulates Oct-3/4 expression in mouse embryonic stem cells

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ABSTRACT

Embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass of blastocysts, and transcription factors Oct-3/4, Nanog, Sox2, and STAT3, are essential for their self-renewal. In this study, we searched for molecules downstream of STAT3 in ES cells. Using DNA chip analysis, we obtained GA-repeat binding protein (GABP) α. Expression of GABPα was restricted to undifferentiated ES cells and controlled by STAT3. We found that the expression level of Oct-3/4 is reduced by knockdown of GABPα. On the other hand, GABPα-overexpressing ES cells maintained the expression level of Oct-3/4 even in the absence of LIF. Moreover, the induction of Oct-3/4 repressors Cdx-2, Coup-tf1 and GCNF was stimulated by GABPα knockdown. These data suggest that GABPα upregulates the expression of Oct-3/4 via downregulation of Oct-3/4 repressors.

Key words : ES cells, GABPα, Oct-3/4, self-renewal, STAT3, repressor

Introduction

Embryonic stem (ES) cells are derived from the inner cell mass (ICM) of the mammalian blastocyst and possess two major abilities: differentiation into many types of cells, pluripotency; and production of two identical stem-cell daughters upon cell division, self-renewal [1,2]. Several essential transcription factors have been identified, both for the formation of the ICM during mouse preimplantation development and for self-renewal of pluripotent mouse ES cells. These transcription factors include Oct-3/4, Sox2, Nanog, and STAT3 [3-10].

A POU-family transcription factor, Oct-3/4 (encoded by pou5f1), is necessary for the self-renewal of ES cells [3]. Artificial repression of Oct-3/4 induces trophectodermal differentiation, whereas overexpression of Oct-3/4 stimulates differentiation mainly to extraembryonic endoderm, indicating that an appropriate level of Oct-3/4 expression is required for continuous propagation of ES cells [4]. A Sry-related HMG box transcription factor, Sox2, was identified as an Oct-3/4 partner in ES cells [11]. Cooperative action of Oct-3/4 with Sox2 was initially found on the enhancer region of fibroblast growth factor 4 (Fgf4) [11], and subsequently observed on

several other genes, including Utf1, Fbx15, and Nanog [12-14]. Furthermore, it has been reported that Sox2 activity is necessary for the establishment of ES cell lines from ICM [5], suggesting the general importance of this partnership in ES cells. A homeodomain transcription factor, Nanog, is an essential factor in sustaining the pluripotency of mouse ES cells [6,7]. Targeted disruption of Nanog gene in mouse ES cells results in differentiation, primarily along the primitive endoderm lineage, suggesting that Nanog prevents ES cells from endoderm differentiation [6,7].

The pluripotency and self-renewal of mouse ES cells can be maintained by the presence of leukemia inhibitory factor (LIF). LIF stimulation leads to activation of transcription factor STAT3, which plays an important role in the self-renewal of mouse ES cells [8,9]. Previously, we generated ES cells expressing STAT3ER, a fusion protein consisting of STAT3 and the ligand-binding domain of estrogen receptor, and found that their pluripotency can be maintained by activation of STAT3ER with a synthetic estrogen receptor ligand, 4-hydroxytamoxifen (4HT), even in the absence of LIF [10]. Another study showed that forced expression of a dominant-negative mutant of STAT3 causes differentiation of ES cells [10]. Thus, the activation of STAT3 is

essential and sufficient for the self-renewal of mouse ES cells.

To understand how STAT3 is involved in self-renewal, we have used DNA chip analysis to search for downstream targets of STAT3 in mouse ES cells. Here, we report the identification of GA-repeat binding protein (GABP) α as a downstream molecule of STAT3. Furthermore, we show that GABPα is an essential factor in ES cell self-renewal and that GABPα regulates Oct-3/4 expression.

Materials and methods

Cell culture. ES cell lines A3-1 [15] and ZHBTc4 [9] and A3-1 expressing STAT3ER [10] were cultured on gelatin-coated dishes with Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO, USA) containing 15% fetal bovine serum, 2 mM L-glutamine, 1 x non-essential amino acid (Chemicon, CA, USA), 1x nucleosides mix (Chemicon), 40 μ M β̃mercaptoethanol (Sigma), and 0.1 % mouse LIF, produced as conditioned media from human embryonic kidney 293 cells expressing LIF from a transiently transfected plasmid.

Plasmid construction and transfection. The mammalian expression vector pCAG-IP was constructed by inserting the sequence of internal ribosomal entry site (IRES) and the puromycin-resistance gene into the plasmid pCAGGS [16,17]. Plasmids pCAG-wtSTAT3-IP and pCAG-dnSTAT3-IP were constructed by inserting cDNA for wild-type STAT3 and a dominant-negative mutant of STAT3[Y705F] into pCAG-IP, respectively [18]. The cDNAs of Oct-3/4 repressors were amplified by PCR using the following primers: 5'-ATG TAC GTG AGC TAC CTT CTG GAC AA-3' and 5'-TCA CTG

GGT GAC AGT GGA GTT TAA AA-3' for Cdx-2, 5'-ATG GCA ATG GTA GTT AGC AGC TGG CGA GAT-3' and 5'-CTA GGA ACA CTG GAT GGA CAT GTA AGG CCA-3' for Coup-tf1, and 5'-ATG GAG CGG GAC GAA CGG CCA A-3' and 5'-TCA CTC CTT CAC CGT ACT TGT C-3' for GCNF. pSilencer 1.0-U6 (Ambion, Austin, TX, USA) was used as a shRNA expression vector. To generate pSi-puro, the puromycin resistance gene of pPUR (Clontech, Palo Alto, CA, USA) was transferred into pSilencer 1.0-U6. A target sequence for RNA interference of GABPα (5'- GGA GTT CAG CAT GAC TGA T-3') was determined by siRNA Target Finder (Ambion). A 2.8-kb fragment of the mouse Oct-3/4 promoter was cloned by PCR, using 5'-AGA TTT ATT TAT TAT TGT ACG- 3' and 5'-GGG GAA GGT GGG CAC CCC GAG-3', and then inserted into the KpnI and Bgl II sites of pGL2-Basic plasmid (Promega) to obtain pOct-Luc. All constructs were sequenced before transfection. For transfection, ES cells (3×10^5 cells) in a 6-cm dish were transfected with pSi-puro or their derivatives using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA, USA). Two days after transfection, ES cells were treated with 0.5 μg/ml puromycin (Nacalai Tesque, Kyoto, Japan) for several days.

RT-PCR analysis. Total RNAs were isolated from ES cells with Trizol reagent (Invitrogen) and converted to cDNA by Superscript II reverse transcriptase (Invitrogen) with oligo(dT)12–18 primers (Amersham Biosciences). PCR products were subjected to 2.0% agarose gel. Primers used in this study are listed in Table S1.

Luciferase reporter assay. To study the effects of GABPα or Oct-3/4 repressors on the Oct-3/4 promoter, 0.5 μg of pOct-Luc was transfected into ES cells using Lipofectamin 2000, together with 2.5 μg of pSi-puro-GABPα or expression vectors for Oct-3/4 repressors. Two days after transfection, ES cells were harvested and lysed in a cell lysis buffer [20 mM Hepes–NaOH (pH 7.2), 10 mM MgCl2, 1 mM EDTA, 10 mM sodium fluoride, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 20 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 1% Triton X-100, and 10% glycerol]. Luciferase activity was measured using the dual-luciferase assay system (Promega) and a luminescencer AB-2200 (ATTO, Japan).

RESULTS

GABPα is a downstream molecule of STAT3

Based on DNA chip analysis using STAT3ER-expressing ES cells, we identified GA-repeat binding protein (GABP) α as a possible target of STAT3. To confirm this, we performed RT-PCR analysis and found that, in the absence of LIF, the expression level of GABPα is indeed higher in STAT3ER-expressing ES cells cultured with 4HT than without 4HT (Fig. 1A). We also examined the effect of a dominant-negative mutant of STAT3 (dnSTAT3) on GABPα expression. As shown in Fig. 1B, expression of GABPα was detected in control and wild-type STAT3-expressing ES cells, whereas its expression level was strongly reduced by dnSTAT3, suggesting that GABPα is a downstream target of STAT3 in ES cells. Since STAT3 activation is stimulated by LIF, we next compared the expression level of GABPα in the presence and absence of LIF. Strong expression of GABPα mRNA was observed in the presence of LIF. When ES cells were allowed to differentiate by removal of LIF, the level of GABPα mRNA gradually diminished (Fig. 1C). These results indicate that in ES cells, the expression of GABPα is controlled by the LIF/STAT pathway.

We next examined whether GABPα is downstream of Oct-3/4. For this, we used ZHBTc4 ES cells, in which tetracycline stimulation leads to downregulation of Oct-3/4 expression [4]. When ZHBTc4 ES cells were treated with tetracycline in the presence of LIF, we found that the expression level of GABPα was not altered, regardless of the downregulation of Oct-3/4 (Fig. 1D), suggesting that the expression of GABPα is not regulated by Oct-3/4.

GABPα suppresses downregulation of Oct-3/4 upon LIF withdrawal

Since GABPα is expressed in undifferentiated ES cells, we determined if expression of GABPα is sufficient for ES cell self-renewal, as in the case of Nanog [6,7]. ES cells overexpressing GABPα could be cultured in their undifferentiated state by LIF (Fig. 2). On the other hand, when LIF was removed from the culture medium, GABPα-expressing ES cells failed to maintain self-renewal and underwent differentiation, as judged from their morphology (Fig. 2A), as well as induction of several differentiation-associated genes, Fgf-5 (embryonic ectoderm marker), T (mesoderm marker), Gata-4 (primitive endoderm marker) and Hand-1 (trophectoderm marker) (Fig.

2B). These observations indicate that GABPα expression is insufficient for the maintenance of ES cell self-renewal. Interestingly, however, the expression level of Oct-3/4 was maintained by GABPα overexpression even after LIF withdrawal (Fig. 2B). Moreover, the expression levels of Rex-1 and Fgf-4 [11,19], well-known target molecules of Oct-3/4, were also sustained. These results suggest that GABPα plays some role in regulation of Oct-3/4 expression.

GABPα knockdown results in decrease of Oct-3/4 expression, as well as ES cell differentiation

If GABPα regulates the expression of Oct-3/4, loss of GABPα should lead to reduced expression of Oct-3/4. To address this, we knocked down GABPα by RNA interference (RNAi). ES cells were transfected with GABPα shRNA expression vector (pSi-puro-GABPα) and cultured for three days with puromycin. As we expected, the expression levels of Oct-3/4 and its downstream genes were reduced by knockdown of GABPα (Fig. 3A). In addition, the induction of differentiation-associated genes and morphological change of ES cells were observed (Figs. 3A and B). These results

suggest that GABPα is an essential factor for the self-renewal of ES cells, and that GABPα regulates Oct-3/4 expression.

Knockdown of GABPα results in reduced activity of the Oct-3/4 promoter

To confirm the involvement of GABPα in Oct-3/4 expression, we carried out a reporter assay. We cloned the 2.8 kb fragment upstream from the Oct-3/4 gene — which contains a proximal promoter, a proximal enhancer that is activated in embryonic carcinoma cells, and a distal enhancer that is active in ES cells [20] — and inserted this fragment into a reporter plasmid carrying the luciferase gene. ES cells were co-transfected with the reporter plasmid and the GABPα shRNA expression vector and cultured for two days. As shown in Fig. 4A, GABPα knockdown decreased Oct-3/4 promoter activity, suggesting the requirement of GABP α for maintaining the activity of Oct-3/4 promoter.

Expression of Oct-3/4 repressors is induced by GABPα knockdown

Since overexpression of GABPα results in no significant increase in the promoter

activity of Oct-3/4 (data not shown), we hypothesized that GABPα would regulate Oct-3/4 expression through other factors. Previous studies had identified several transcriptional repressors that bind to the Oct-3/4 promoter, including Cdx-2, Coup-tf1, Coup-tf2, and GCNF [21-23]. It was therefore possible that GABPα maintained Oct-3/4 expression through suppression of these repressors. To explore this possibility, we examined the effect of GABPα knockdown on the expression of Oct-3/4 repressors. As shown in Fig. 4B, the expression levels of three Oct-3/4 repressors — Cdx-2, Coup-tf1 and GCNF — were increased by GABPα knockdown, while that of Coup-tf2 was not changed. Furthermore, we confirmed that overexpression of the three repressors in ES cells reduced both the expression level of endogenous Oct-3/4 and the Oct-3/4 promoter activity (Figs. 4A and C). These results suggest that GABPα regulates Oct-3/4 expression through downregulation of Oct-3/4 repressors.

DISCUSSION

In this study, we identified GABPα as a self-renewal-specific gene in ES cells. We showed that GABPα is essential, but not sufficient, for the self-renewal of ES cells. Furthermore, we found that GABPα regulates the expression of Oct-3/4, a critical factor for ES cell self-renewal, by inhibiting three Oct-3/4 repressors, Cdx-2, Coup-tf1, and GCNF. Based on these observations, we hypothesize the following model (Figure S1) : in the presence of LIF, activated STAT3 stimulates the expression of GABPα. GABPα inhibits the expression of Oct-3/4 repressors, and as a result, Oct-3/4 expression is maintained, leading to self-renewal of ES cells. On the other hand, in the absence of LIF, the expression of GABPα is reduced, which triggers the induction of Oct-3/4 repressors. The Oct-3/4 repressors then bind with the Oct-3/4 promoter region to suppress Oct-3/4 expression, resulting in ES cell differentiation.

It is well-establlished that both the STAT3 and Oct-3/4 pathways play a crucial role in the maintenance of self-renewal in mouse ES cells. Our data showed that GABPα is a downstream molecule of the STAT3 pathway, and regulates Oct-3/4 expression. It is likely, thus, that GABPα functions as a connecting molecule between

the two pathways in ES cell self-renewal.

In this study, we showed that GABPα downregulates Cdx-2, Coup-tf1, and GCNF. To examine the molecular mechanism of this regulation, we cloned an upstream region (up to 5kb) of each gene and found that knockdown of GABPα has no effect on the activities of these promoters (data not shown). It is therefore possible that GABPα indirectly regulates the expression of these repressors, although we cannot exclude the possibility that GABPα binds to other regions of the genes.

It had been reported that GABPα-deficient embryos died prior to implantation without formation of blastocyst [24]. This finding suggests that GABPα may be essential for the self-renewal of ES cells. Indeed, by the knockdown method, we demonstrated here that downregulation of GABPα leads to ES cell differentiation. We also tried to establish GABPα-null ES cells by homologous recombination. Although we picked up as many as 600 clones, we were not able to obtain any GABPα-deficient ES cells. On the other hand, we could easily generate GABPα$^{+/-}$ ES cells (14/84 clones). Taken together, these results indicate that GABPα is an essential factor for ES cell self-renewal.

GABP consists of two distinct proteins, GABPα and GABPβ, and there are two distinct genes for GABPβ, GABPβ1 and GABPβ2. GABPα is the only ETS factor that can recruit GABPβs to DNA, and the region downstream of the DNA-binding domain is required for physical interaction with GABPβ. GABPβ does not show any DNA-binding activity, but it dramatically enhances the DNA-binding capacity of GABPα and contains the nuclear localization signal that translocates GABPα to the nucleus [25-28]. When we examined the expression of GABPβ1 and GABPβ2 in ES cells, the expression level of GABPβ1 was constant, either in the presence or absence of LIF, whereas expression of GABPβ2 was restricted to undifferentiated ES cells (data not shown). Considering that overexpression of GABPα maintains Oct-3/4 expression in the absence of LIF (*i.e.,* in the absence of GABPβ2), the GABPα•GABPβ1 complex may be involved in the Oct-3/4 expression in ES cells.

Although previous studies have shown that the Oct-3/4•Sox2 complex, Lrh1, RXRβ, and SF1 upregulate Oct-3/4 expression [22,29-32], the precise regulatory mechanism of Oct-3/4 expression is still unknown. In this study, we identified GABPα as another regulator of the Oct-3/4 expression. We hope that further understanding of

GABPα function will provide a clue to the molecular mechanism of Oct-3/4 expression and ES cell self-renewal.

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Figure Legends

Fig. 1 Expression of GABPα mRNA is restricted to undifferentiated ES cells.

(A) STAT3ER-expressing ES cells were cultured with or without LIF in the presence or absence of 4HT. (B) ES cells were transfected with the empty (control), wtSTAT3, or dnSTAT3 expression vector and cultured for 3 days in the presence of puromycin. (C) ES cells were cultured with or without LIF for the indicated period. (D) ZHBTc4 cells were cultured with or without tetracyclin (Tet). Total RNAs were isolated and subjected to RT-PCR analysis. GAPDH was used as an internal control. Data are representative of three independent experiments.

Fig. 2 GABPα maintains the expression level of Oct-3/4.

(A) Morphology of GABPα-overexpressing ES cells. ES cells were cultured for 8 days with or without LIF. (B) Expression of various marker genes. After 8 days of culture in the presence or absence of LIF, total RNAs were isolated from cells and subjected to RT-PCR analysis. HPRT was used as an internal control. The results represent three independent experiments.

Fig. 3 Effect of GABPα knockdown on ES cells.

(A) Expression of various marker genes. (B) Morphology of ES cells expressing GABPα shRNA. After transfection, ES cells were cultured for 3 days with puromycin in the presence of LIF. The results are representative of three independent experiments.

Fig. 4 GABPα knockdown reduces activity of Oct-3/4 promoter and stimulates the induction of Oct-3/4 repressors.

(A) Effect of GABPα knockdown and expression of Oct-3/4 repressors on activity of Oct-3/4 promoter. Bars represent the mean and standard deviations (*n=3*). (B) Expression of Oct-3/4 repressors in GABPα shRNA-expressing ES cells. (C) Effect of Oct-3/4 repressors on expression of endogenous Oct-3/4. ES cells were transfected with Oct-3/4 repressors and subjected to RT-PCR analysis. Data shown here represent three independent experiments.

Figure1 Kinoshita et.al.

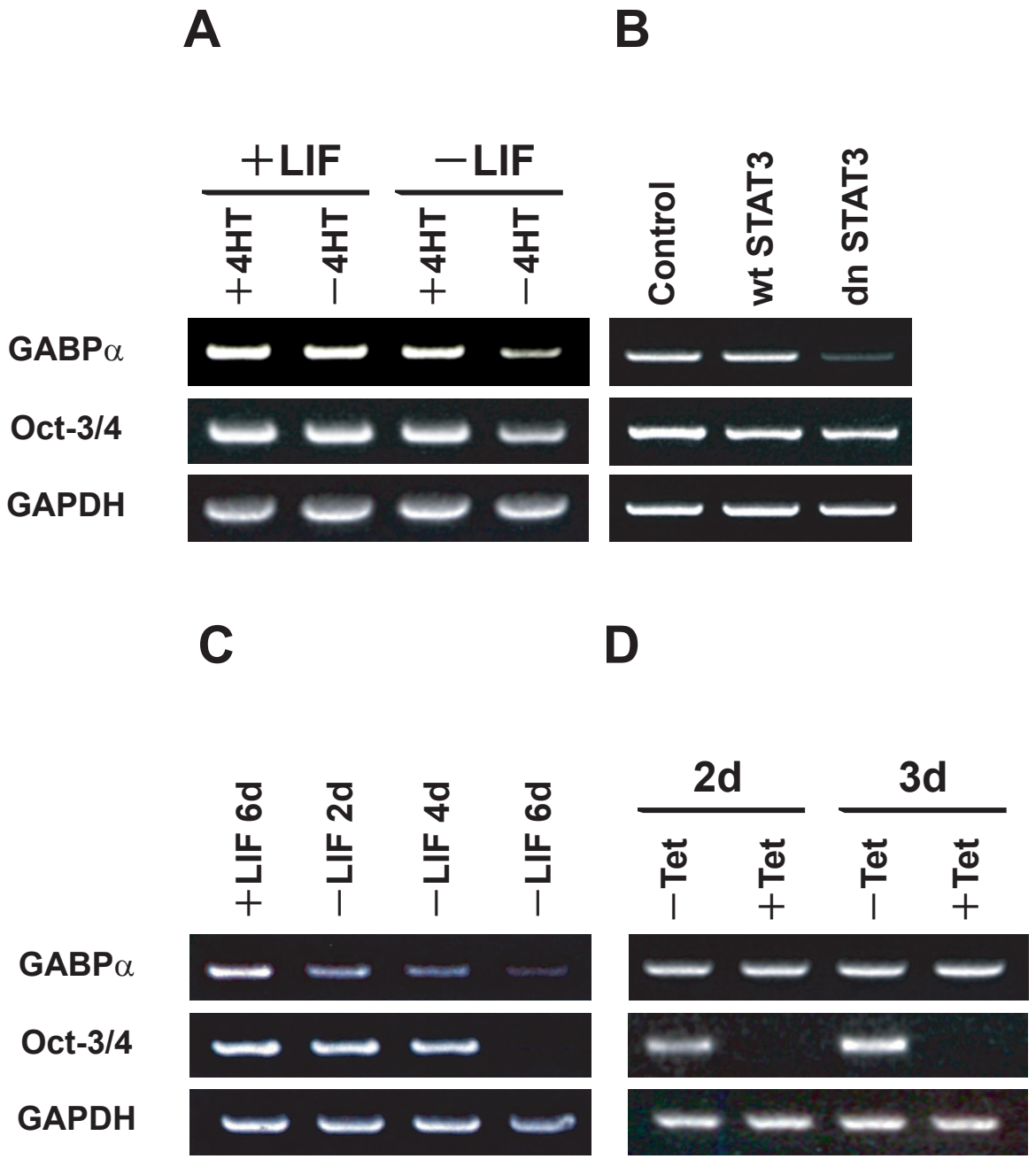


Figure2 Kinoshita et.al.

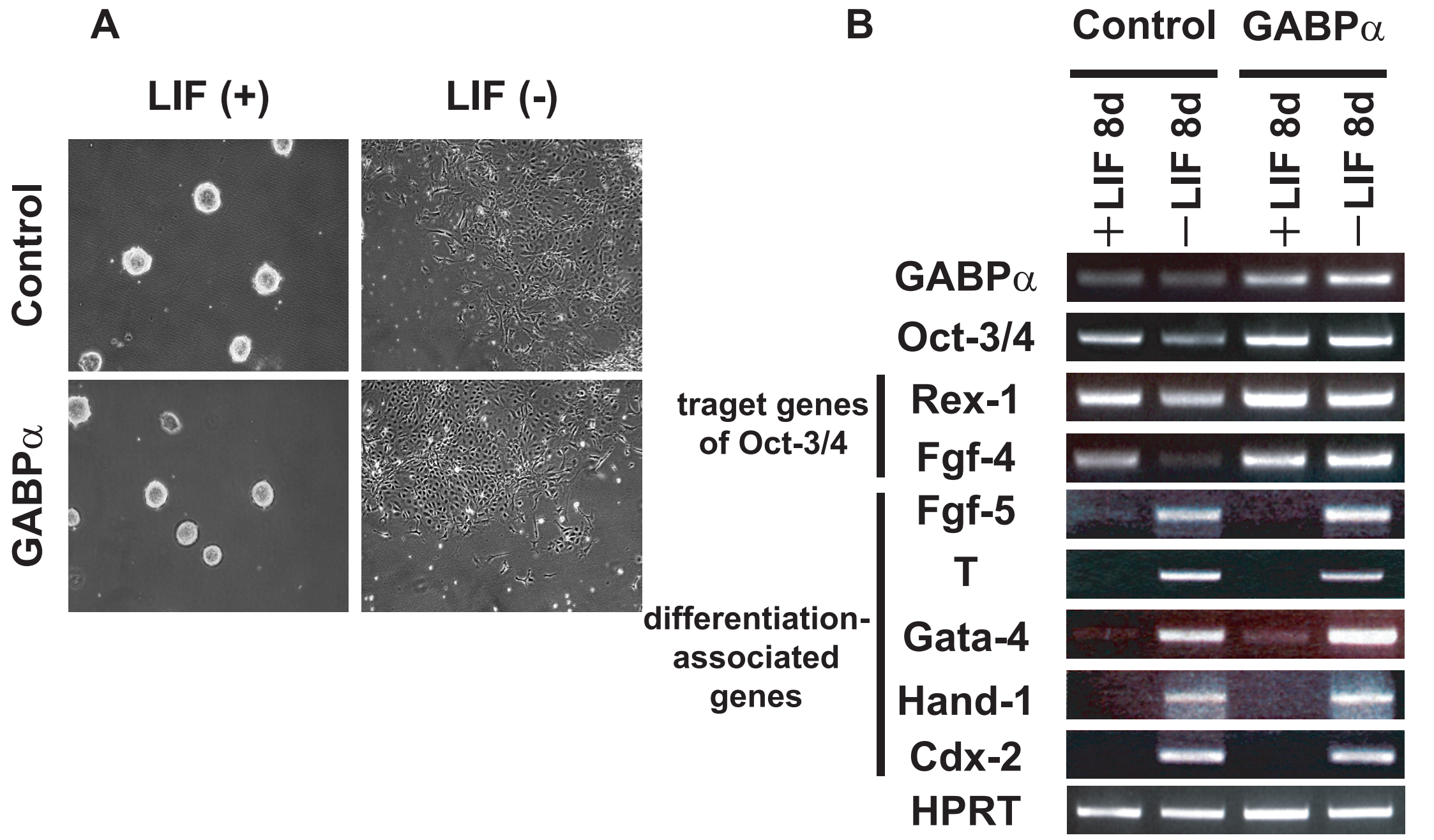
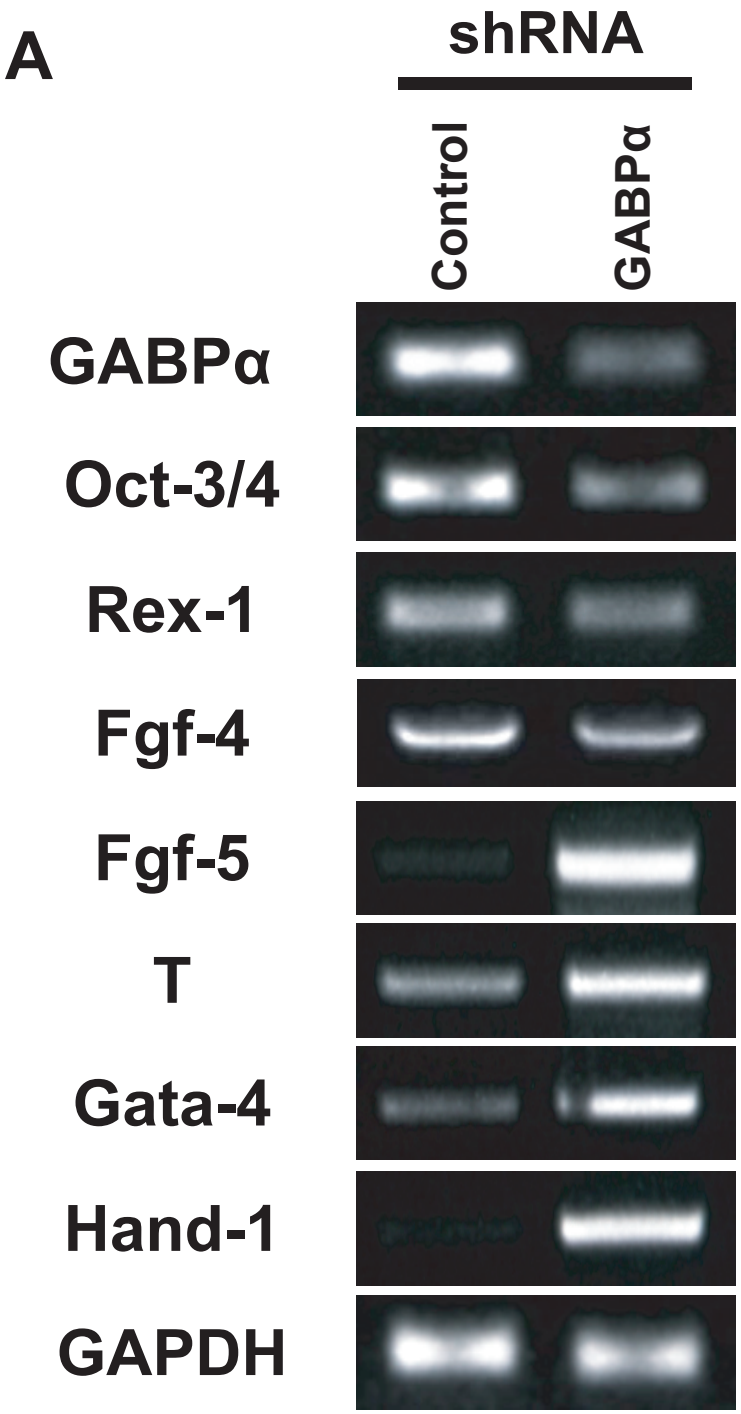


Figure3 Kinoshita et.al.



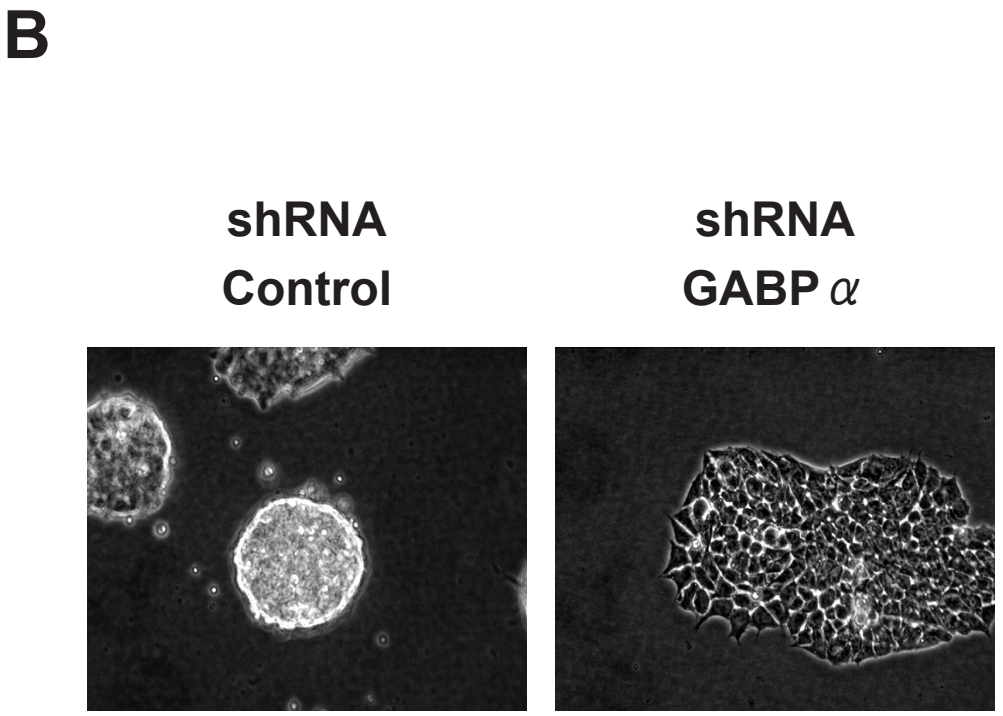
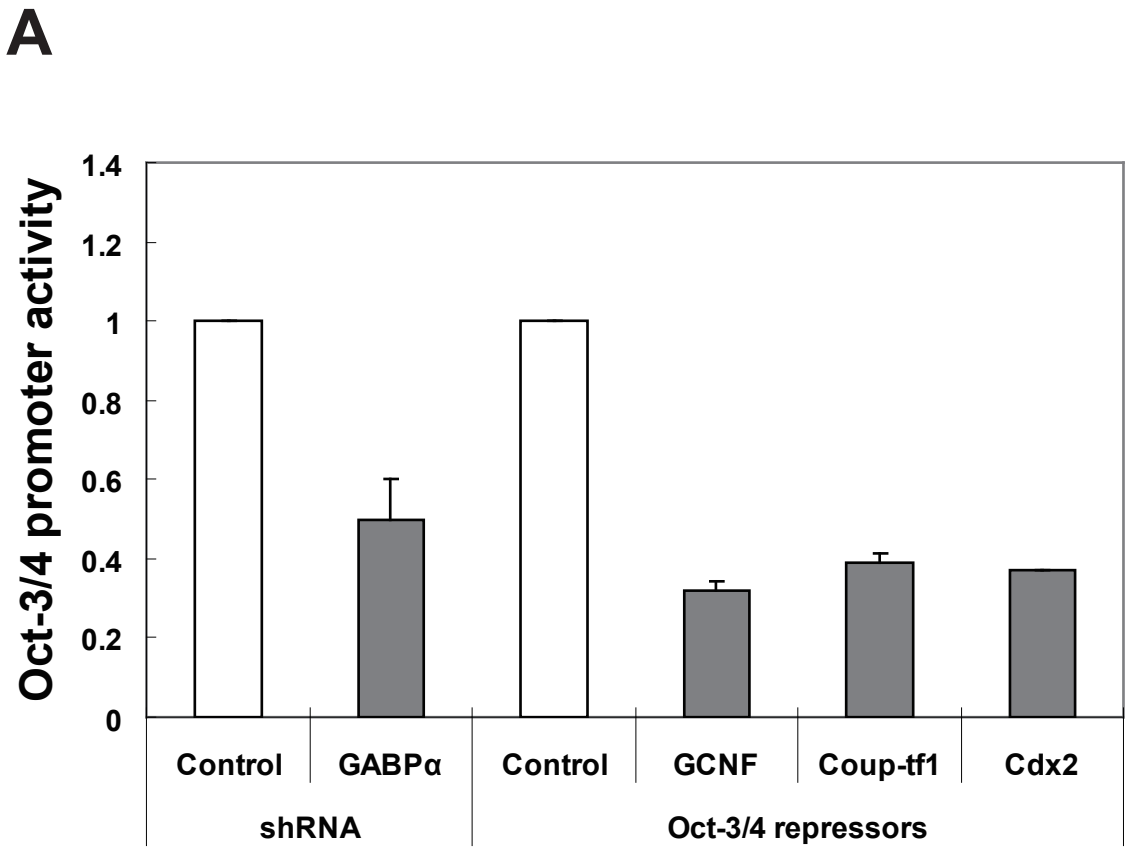
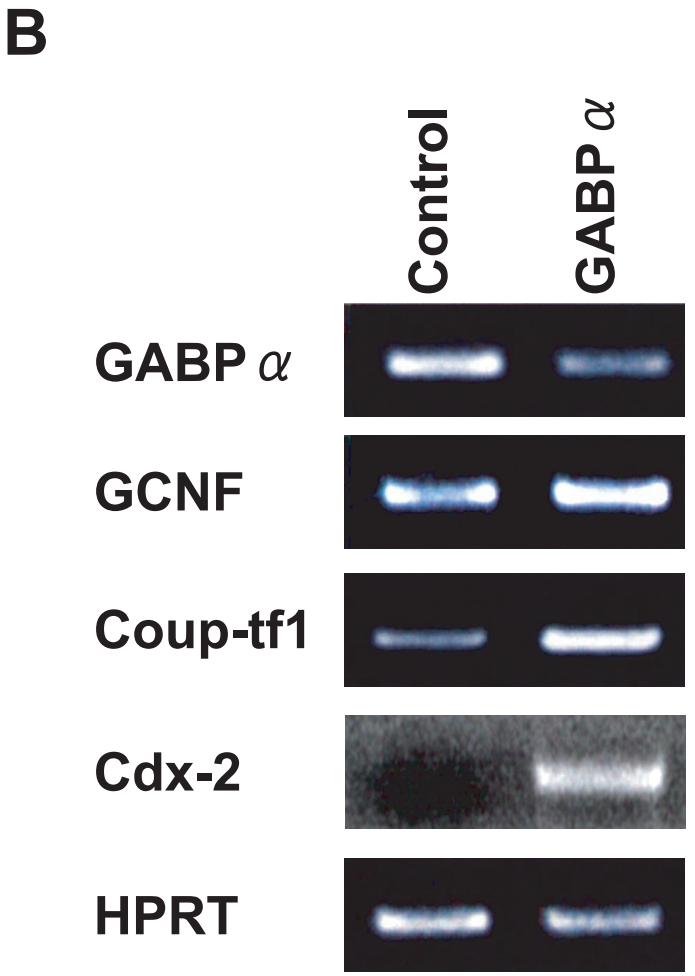
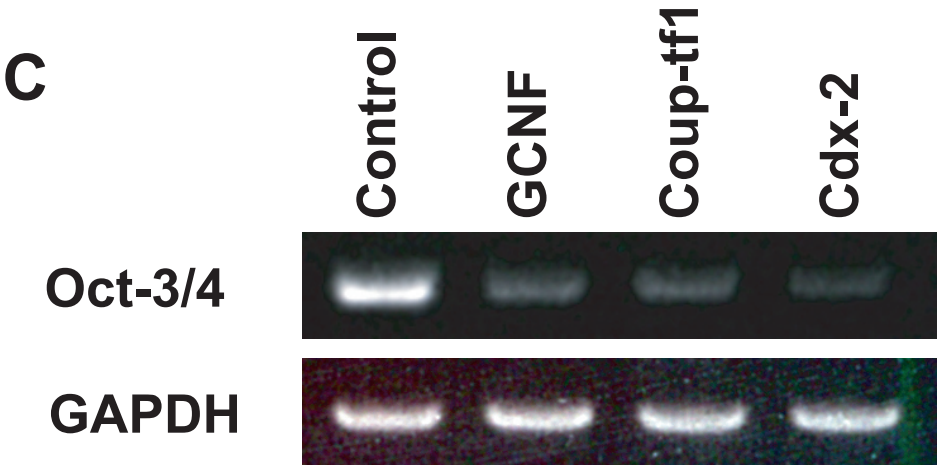


Figure4 Kinoshita et.al.







TableS1 List of RT-PCR primers

	Forward primer	Reverse primer
CDX2	agtgattcctggggcttctt	cccttcctgatttgtggaga
Coup-tf1	agccatcgtgctattcacg	ttctcaccagacacgaggtc
Coup-tf2	gcaagagcttcttcaagcg	gcttctccacttgctcttgg
Fgf-4	ctactgcaacgtgggcatc	accttcatggtaggcgacac
Fgf-5	aaagtcaatggctcccacgaa	cttcagtctgtacttcactgg
GABPalpha	aatcagcctgagttggttgc	catccgtgccagtttcttct
Gata-4	ccgagcaggaatttgaagagg	gcctgtatgtaatgcctgcg
GAPDH	tgatgacatcaagaaggtggtgaag	tccttggaggccatgtaggccat
GCNF	ctgaacaacgaacctgtctc	ttgctctctgaagccctgtt
Hand1	gcctacttgatggacgtgct	gcgccctttaatcctcttct
HPRT	tctcatgccgacccgcagtcc	attcaacttgcgctcatctta
Oct-3/4	gccctgcagaaggagctagaac	gggaatactcaatacttgatct
Rex-1	cgcctgaggaagca	ccttcagcatttcttccctgcctttgc
T	atgccaaagaaagaaacgac	agaggctgtagaacatgatt

FigS1 Kinoshita et.al.

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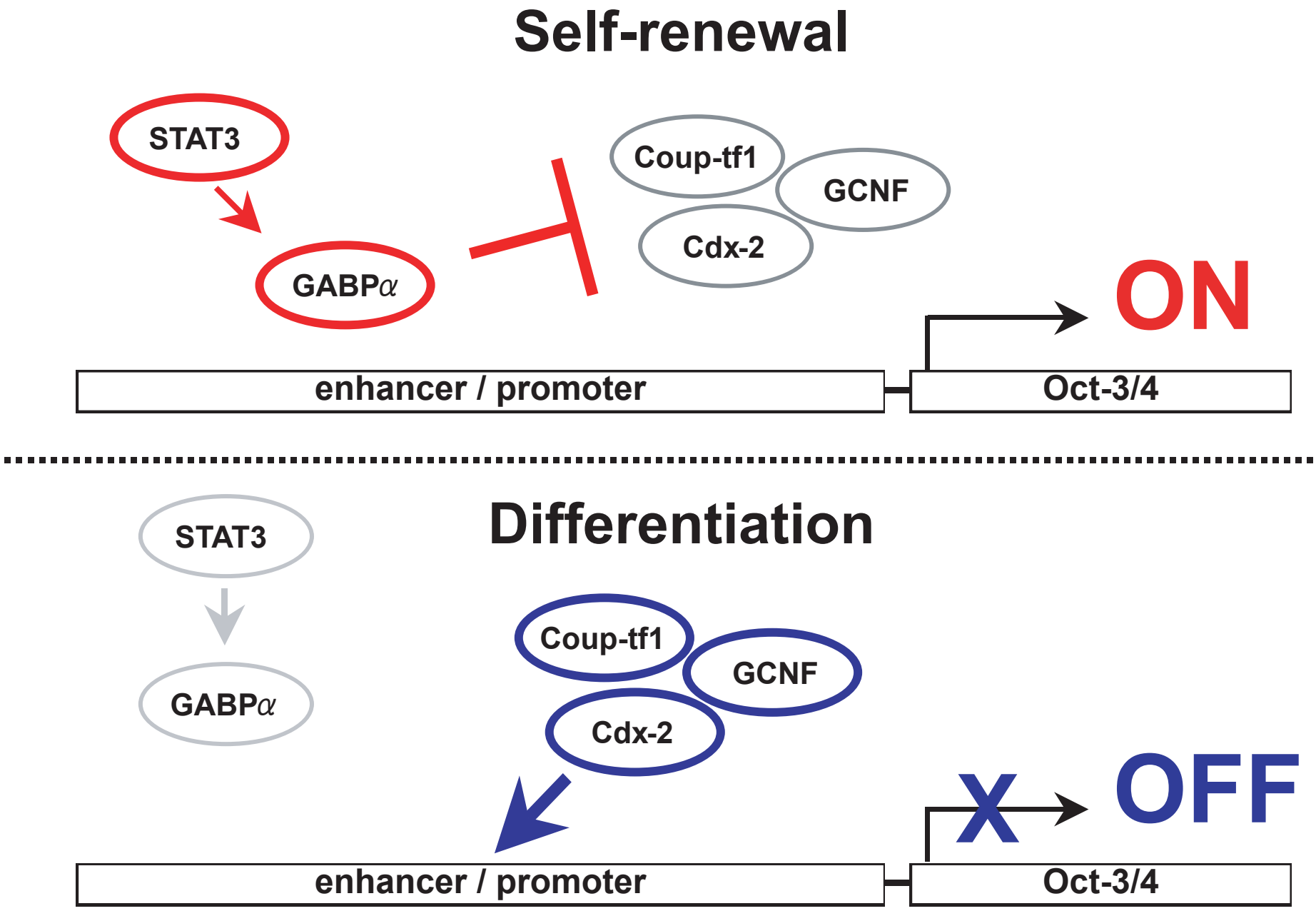

FigS1 legend Kinoshita et.al.

Click here to download Supplementary Material: A model for GABP

Fig. S1 A model for GABPα-mediated maintenance of ES cell self-renewal.