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Cover Letter

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The Editor of Biochemical and Biophysical Research Communications

Dear Editor

I am sending the manuscript entitled "β-catenin up-regulates Nanog expression through interaction with Oct-3/4 in embryonic stem cells" by Yukinari Takao, Takashi Yokota and myself, which we would like to submit for publication in Biochemical and Biophysical Research Communications.

In this paper, we examined the possible involvement of β-catenin in the self-renewal of embryonic stem (ES) cells. We found that β-catenin is regulated by LIF and that activated mutant of β-catenin can support the long-term proliferation of ES cells even in the absence of LIF. We identified the 10th armadillo repeat of β-catenin as a responsible region for β-catenin-mediated maintenance of ES cells. Furthermore, we demonstrated that β-catenin enhances the transcriptional activity of Oct-3/4 through association, which leads to β-catenin-mediated up-regulation of Nanog. These results indicate that β-catenin is involved in the LIF-dependent self-renewal of ES cells through cooperative up-regulation of Nanog with 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 paper shows for the first time that a well-known oncoprotein, β-catenin, can interact with Oct-3/4, a critical factor for ES cell self-renewal and pluripotency. Therefore, we believe this paper will attract the interest of general readers of Biochemical and Biophysical Research Communications.

Sincerely yours,

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* Manuscript

β-catenin up-regulates Nanog expression through interaction with Oct-3/4 in embryonic stem cells

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Abstract

It is well known that mouse embryonic stem (ES) cells can be maintained by the presence of leukemia inhibitory factor (LIF). Recent studies have revealed that Wnt also exhibits activity similar to LIF. The molecular mechanism behind the maintenance of ES cells by these factors, however, is not fully understood. In this study, we found that LIF enhances level of nuclear β-catenin, a component of the Wnt signaling pathway. Expression of an activated mutant of β-catenin led to the long-term proliferation of ES cells, even in the absence of LIF. Furthermore, it was found that β-catenin up-regulates Nanog in an Oct-3/4-dependent manner and that β-catenin physically associates with Oct-3/4. These results suggest that up-regulating Nanog through interaction with Oct-3/4 involves β-catenin in the LIF- and Wnt-mediated maintenance of ES cell self-renewal.

Keywords : β-catenin; Oct-3/4; Nanog; embryonic stem cells; self-renewal

Introduction

Embryonic stem (ES) cells, derived from the inner cell mass of the blastocyst stage embryo, are capable of self-renewal and of differentiation into any type of cell in the body. However, the molecular mechanisms behind the self-renewal and differentiation in ES cells are not yet fully understood. In the case of mouse ES cells, the self-renewal of ES cells can be maintained in the presence of leukemia inhibitory factor (LIF). LIF stimulation leads to activation of transcription factor STAT3, which plays a critical role in the self-renewal of ES cells [1,2].

In addition to STAT3, three transcription factors — Oct-3/4, Sox2, and Nanog — are key regulators in the self-renewal of ES cells. Disruption of Oct-3/4 results in the differentiation of ES cells into trophoectoderm [3]. Sox2 forms a complex with Oct-3/4 to stimulate the induction of several Oct-3/4 downstream molecules [4]. Nanog was identified as a factor whose expression allows ES cells to self-renew in the absence of LIF, and its disruption induces differentiation of ES cells into extraembryonic endoderm lineages [5,6]. The finding that these three factors co-occupy a substantial portion of their target genes suggests that they work collaboratively to maintain ES cell self-renewal [7]. Recent studies have also demonstrated that the Oct-3/4•Sox2 complex regulates Nanog expression through

binding to the promoter region of Nanog [8,9].

Unlike mouse ES cells, LIF stimulation cannot support the self-renewal of human ES cells [10], suggesting that in primate ES cells, a pathway other than the LIF signaling pathway is involved in their self-renewal. Interestingly, Sato *et al.* have demonstrated that activation of the Wnt pathway by a glycogen synthase kinase (GSK)-3-specific pharmacological inhibitor, Bio, results in the maintenance of self-renewal in both mouse and human ES cells [11]. Moreover, the conditioned medium from mouse fibroblasts expressing Wnt can maintain the pluripotency of mouse ES cells [12-14]. These observations suggest the involvement of the Wnt pathway in ES cell self-renewal and raised the possibility that β-catenin, a component of the Wnt signaling pathway, might play some role in the LIF-dependent self-renewal of ES cells, as in the case of adenomatous polyposis coli (APC) [15]. In the present study, we explored this possibility, and found that β-catenin is involved in the maintenance of ES cells by up-regulating Nanog through binding with Oct-3/4.

Materials and methods

Plasmid construction and transfection

Plasmids pCAGIP-myc-β-catenin and pCAGIP-myc-Oct-3/4 were constructed by inserting cDNAs of myc-tagged mouse β-catenin and mouse Oct-3/4 into the mammalian expression vector pCAG-IP [16], which carries the sequence of the internal ribosomal entry site (IRES) and the puromycin resistance gene. Truncated mutants — Arm N-6 (1-390 aa of β-catenin), Arm N-8 (1-473 aa), Arm N-9 (1-519 aa), Arm N-10 (1-582 aa) and Arm N-12 (1-694 aa) — were constructed from β-catenin[S33A] by PCR. All mutants were tagged with myc epitope and inserted into pCAG-IP. Plasmid pGEX-β-catenin was constructed by inserting β-catenin cDNA into pGEX-2T (GE Healthcare Bio-Sciences, Piscataway, NJ, USA). Reporter plasmids, pGL3-Nanog promoter(WT) and -Nanog promoter(Oct^{mut}), which contains no mutation (*i.e.* the wild type) and mutations in the Oct-3/4 binding element, respectively, were constructed by a similar method described earlier [8]. pOct-Luc2 was produced by inserting the following oligonucleotides into pTAL-Luc (Clontech, Palo Alto, CA, USA): 5'-CGCGTATTTGCATATTTGCATATTTGCATATTTGCATATTTGCATA-3' and 5'-GATCTATGCAAATATGCAAATATGCAAATATGCAAATATGCAAATA-3'. All constructions were sequenced before use.

Cell culture and transfection

ES cell lines, A3-1 [17] and ZHBTc4 [3], were cultured in gelatin-coated dishes in the absence of feeder cells, as described previously [16,18]. ES cells were transfected with pCAG-IP and its derivatives using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA, USA). Two days after transfection, ES cells were treated with 1 μg/ml puromycin (Nacalai Tesque, Kyoto, Japan) for several days and then subjected to experiments.

Preparation of nuclear extract and Western blot analysis

To prepare nuclear extracts, ES cells were harvested and incubated in 10 mM Hepes (pH 7.8), 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, and an inhibitor mixture (5 μg/ml pepstatin A, 5 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM sodium fluoride, 0.1 mM sodium orthovanadate and 25 mM β-glycerophosphate) for 15 min at 4°C. Nonidet P-40 was then added at a final concentration of 0.6% (v/v). Samples were mixed vigorously and centrifuged. Pelleted nuclei were resuspended in 50 mM Hepes (pH 7.8), 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1% (v/v) glycerol, and the inhibitor mixture, sonicated, and centrifuged for to save supernatant. To prepare total

cell lysates, ES cells were harvested and lysed in buffer A [20 mM Hepes (pH7.2), 10 mM $MgCl_2$, 1 mM EDTA, the inhibitor mixture, 1% (v/v) Triton X-100, and 10% (v/v) glycerol]. A portion of lysate (50 μg of protein) was subjected to SDS-PAGE (8% gel) for Western blotting with antibodies against β-catenin (Cell Signaling, Beverly, MA, USA), lamin B and myc epitope (9E10) (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

RT-PCR analysis

Total RNA (1 μg) was converted to cDNA with Superscript II reverse transcriptase (Invitogen) and oligo(dT)12-18 primers (GE Healthcare Bio-Sciences). PCR was performed with the following primers: 5'-CTCGAGATGGCTACTCAAGCTGACC-3' and 5'-GATGGGATCTGCATGCCCTC-3' for β-catenin. Primers for Oct-3/4, Rex-1, Nanog, Fgf5, GATA4, and brachyury were described previously [3,16,18].

Pull-down assay

Using pGEX-2T and pGEX-β-catenin, glutathione S-transferase (GST) and GST-fused β-catenin (GST-β-catenin) were produced in *Escherichia coli*. Harvested

bacteria were suspended in buffer A containing 1 mM DTT, sonicated, and centrifuged to prepare lysate. A portion of lysate (300 μg of protein) was incubated with glutathione Sepharose (GE Healthcare Bio-Sciences) for 2 h at 4°C, which was then incubated overnight at 4°C with total cell lysate (300 μg of protein) prepared from HEK293 cells or HEK293 cells expressing myc-tagged Oct-3/4. After washing, proteins were eluted from glutathione Sepharose by boiling in the SDS-sample buffer.

Results

The amount of β-catenin protein in nucleus is reduced upon LIF removal

If β-catenin acts downstream of LIF for the self-renewal of ES cells, β-catenin should be regulated by LIF. To find out, we examined whether the expression level of β-catenin is regulated by LIF. ES cells were cultured in the presence or absence of LIF, and the expression levels of β-catenin were compared. When we examined the total amounts of β-catenin mRNA and protein, no significant difference was observed between the presence and absence of LIF (Figure 1a), suggesting that LIF does not regulate β-catenin at the transcriptional level. Since β-catenin translocates from cytosol into the nucleus to regulate gene expression, we next compared the protein level of β-catenin in the nucleus by preparing nuclear extracts from ES cells (Figure 1b). Three days after LIF removal, we observed a reduction in nuclear β-catenin. These data suggest that β-catenin could be involved in the LIF-mediated maintenance of ES cell self-renewal.

Expression of self-renewal markers in ES cells is maintained by overexpression of activated mutant of β-catenin even in the absence of LIF

To examine the effect of activation of β-catenin on the undifferentiated state of

ES cells, we ectopically expressed an activated mutant of β-catenin, β-catenin[S33A, T41A, S45A] in ES cells. After transfection, ES cells were selected by puromycin in the presence of LIF. When transfected with the empty vector or wild-type β-catenin, ES cells failed to form compact colonies in the absence of LIF and showed differentiated morphology (Figure 2a). In contrast, ES cells expressing the β-catenin mutant formed compact colonies.

Maintenance of the undifferentiated state by the β-catenin mutant was also confirmed by expression of three transcription factors specific for undifferentiated ES cells, Oct-3/4, Rex-1 and Nanog (Figure 2b). On the other hand, when we examined the expression levels of differentiation-associated markers (Figure 2b), activated β-catenin inhibited the induction of GATA4, while it partially suppressed Fgf5 induction. Brachyury shows a high expression level in ES cells expressing the β-catenin mutant, probably because it is a direct target of β-catenin [19]. Taken together, these data suggest that β-catenin can maintain undifferentiated ES cells, though not completely.

Next, we investigated whether β-catenin allows long-term proliferation in ES cells in the absence of LIF, and found that the activated mutant-expressing ES cells could be cultured for as long as one month in the absence of LIF (Figure 2c). When we counted the number of viable cells, however, the growth rate in the absence of LIF

was found to be lower than that in its presence. The long-term proliferation is not due to transformation of ES cells by the β-catenin mutant, since we confirmed their ability to differentiate by removing β-catenin mutant with Cre-lox recombination system after one month culture without LIF (Figure S1).

Identification of the region in β-catenin that is required for maintenance of undifferentiated ES cells

Since β-catenin are composed of multiple motifs called armadillo repeats, we wished to determine which armadillo repeat of β-catenin is required for the maintenance of ES cells. For this purpose, five kinds of truncated β-catenin mutants were prepared (Figure 3a). When these mutants were expressed in ES cells, the Arm N-10 and Arm N-12 mutants could maintain the undifferentiated phenotype in the absence of LIF, as judged from morphology and expression of self-renewal markers (Figures 3b and c). On the other hand, ES cells expressing the other mutants underwent differentiation. We conclude therefore that the region around the tenth armadillo repeat is required for β-catenin to maintain ES cells.

β-catenin requires Oct-3/4 for up-regulation of Nanog

Our data suggest that β-catenin up-regulates both Oct-3/4 and Nanog, critical transcription factors for ES cell self-renewal (Figure 2b). We noticed, however, that β-catenin and Nanog are down-regulated three days after LIF removal, while Oct-3/4 requires six days for its down-regulation (Figure 1), suggesting that Nanog might be a direct target of β-catenin. First, we examined the effect of β-catenin on the activity of Nanog promoter (-322/+50) by luciferase assay. This promoter region has been shown to include the regulatory elements essential for pluripotential state-specific expression of Nanog [8]. In the presence of LIF, expression of the activated β-catenin mutant increased promoter activity, when compared with the control (Figure 1a). When LIF was removed from the medium, the mutant-expressing ES cells still retained significant promoter activity. Since the effect of β-catenin on the Nanog promoter activity corresponded well with that on Nanog mRNA expression (Figure 2b), it seems that the promoter region (-322/+50) contains a β-catenin-responsive element.

Recent studies have reported that Nanog expression is regulated by Oct-3/4 in ES cells, and that the promoter region (-322/+50) contains one Oct-3/4•Sox2 binding element [8,9]. To examine whether the Oct-3/4 binding element is involved in the β-catenin-mediated Nanog up-regulation, we introduced mutations into the binding sequence of Oct-3/4. When the mutant reporter plasmid was introduced in β-catenin

mutant-expressing ES cells, the mutations resulted in decreased transcriptional activity of β-catenin, both in the presence and absence of LIF (Figure 4a), suggesting that existence of the Oct-3/4 binding element on the Nanog promoter is indispensable for the β-catenin-dependent up-regulation of Nanog.

To clarify the requirement for Oct-3/4, we used ZHBTc4 cells, where Oct-3/4 expression is down-regulated by tetracycline stimulation [3]. When ZHBTc4 cells expressing the β-catenin mutant were cultured in the absence or presence of tetracycline, the β-catenin mutant failed to maintain the expression of Nanog in its presence (*i.e.*, in the absence of Oct-3/4) (Figure 4b). These results suggest that Oct-3/4 is essential for Nanog up-regulation by β-catenin.

To explore β-catenin has some functional association with Oct-3/4, we examined the effect of β-catenin on transcriptional activity of Oct-3/4. When we introduced a reporter plasmid carrying five tandem repeats of the Oct-3/4 binding sequence into β-catenin mutant-expressing ES cells and control cells, we found that the β-catenin mutant enhances the transcriptional activity of Oct-3/4 in ES cells (Figure 4c), suggesting that β-catenin could physically interact with Oct-3/4. We therefore performed a pull-down assay. After mixing cell lysates containing glutathione S-transferase(GST)-fused β-catenin and myc-tagged Oct-3/4, GST-β-catenin was pulled

down, and the co-precipitation of myc-Oct-3/4 was examined. As shown in Figure 4d, co-precipitation of myc-Oct-3/4 was detected in the case of GST-β-catenin, but not in the case of GST alone. By Western blotting, we confirmed that the protein levels of GST and GST-β-catenin in each sample are equal (data not shown). These data suggest that β-catenin forms a complex with Oct-3/4.

Discussion

In this study, we found that the amount of nuclear β-catenin decreased when ES cells were differentiated by LIF removal. Expression of the activated mutant of β-catenin maintained the expression level of Nanog, as well as the long-term proliferation of ES cells, even in the absence of LIF. Furthermore, we demonstrated that β-catenin-mediated up-regulation of Nanog requires Oct-3/4, and that β-catenin interacts with Oct-3/4 *in vitro*. Taken together, our findings suggest that β-catenin is involved in the LIF-dependent self-renewal of ES cells, probably through cooperative up-regulation of Nanog with Oct-3/4.

In general, β-catenin stability is negatively regulated through its phosphorylation by GSK-3β. The pathway from LIF stimulation to the inactivation of GSK-3β through the phosphoinositide 3-kinase (PI3K)/Akt pathway has been described in ES cells [20]. However, neither stimulation with LIF or short-term treatment with a PI3K inhibitor, LY294002, showed any effect on the stability of β-catenin, although long-term treatment (around five days) of LY294002 did reduce β-catenin phosphorylation. In addition, expression of activated Akt showed no effect on localization and activity of β-catenin [21]. Consequently, it does not seem that LIF regulates the stability of β-catenin directly through activation of the PI3K/Akt pathway

in ES cells.

Ogawa *et al.* have demonstrated that expression of the activated mutant of β-catenin alone is not sufficient, but requires the presence of LIF for the maintenance of ES cell self-renewal [13]. We observed, however, that a neutralizing antibody against LIF gave no effect on the β-catenin-mediated maintenance of ES cells (data not shown), suggesting that the β-catenin maintains ES cells in a LIF-independent manner. The reason for the discrepancy between their data and ours is not clear, but may be due to the difference of cell line or expression level of the mutant protein, since we found that the activated β-catenin mutant alone could not fully maintain the undifferentiated state of another ES cell line, E14TG2a (data not shown). We also noticed that, although we can culture β-catenin-expressing ES cells for a long period in the absence of LIF, the maintenance of the undifferentiated state by β-catenin is not complete, as judged from the expression levels of differentiation-associated markers. It can be concluded, therefore, that β-catenin promotes the maintenance of ES cell self-renewal, although activation of β-catenin is not sufficient for maintaining complete self-renewal of ES cells.

The involvement of β-catenin has been reported in the self-renewal of several stem cells [22]. Furthermore, several studies, including ours, suggest that β-catenin

plays an important role in the self-renewal of ES cells [12,13]. Establishment of β-catenin-null ES cells, however, has also been reported [23]. Since we observed that γ-catenin, a protein highly homologous to β-catenin, can partially maintain the undifferentiated state of ES cells (Figure S2), it is possible that γ-catenin compensates for the loss of β-catenin.

The mechanism how β-catenin is involved in ES cell self-renewal is still unclear. It is reported that activation of β-catenin leads to induction of STAT3 mRNA in ES cells [12]. Involvement of c-myc, a direct target molecule of β-catenin, in the self-renewal of ES cells has been demonstrated [24]. These observations suggest that β-catenin up-regulates STAT3 and c-myc to promote the self-renewal of ES cells. In the present study, we found another β-catenin-mediated pathway that would lead to the maintenance of ES cell self-renewal: up-regulation of Nanog through interaction with Oct-3/4. It is well established that β-catenin co-activates the TCF family proteins. Thus, our present findings raise the intriguing possibility that β-catenin may be a co-activator of Oct-3/4. To act as a co-activator of TCFs, β-catenin binds to several chromatin remodeling factors, such as Brg1 and CBP/p300 [25]. Interestingly, β-catenin-binding regions of Brg1 and CBP/p300 encompass the tenth armadillo repeat fragment, which as we demonstrated is critical in the β-catenin-mediated maintenance

of undifferentiated ES cells. It is possible therefore that the Oct-3/4•β-catenin complex requires Brg1 and/or CBP/p300 for up-regulation of Nanog. It is also possible that Oct-3/4 binds with β-catenin through this armadillo repeat. We are currently investigating both possibilities.

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Figure Legends

Figure 1 LIF stimulates the accumulation of nuclear β-catenin. (a) Immunoblot analysis (left panel) and RT-PCR analysis (right panel) of β-catenin in ES cells. ES cells were cultured in the absence of LIF for the indicated period. Harvested cells were subjected to immunoblot analysis with anti-β-catenin antibody and RT-PCR analysis. Lamin B and GAPDH were used as internal controls. (b) Immunoblot analysis of nuclear β-catenin. After culture in the absence of LIF for the indicated period, ES cells were harvested, from which nuclear extract was prepared as described in Materials and methods. The results represent three independent experiments.

Figure 2 Activated β-catenin can maintain undifferentiated ES cells. (a) Morphology of ES cells. (b) RT-PCR analysis of marker genes specific for self-renewing or differentiated ES cells. ES cells were transfected with either pCAG-IP (control), pCAGIP-myc-β-catenin (β-cat), or pCAGIP-myc-β-catenin[S33A,T41A,S45A] (mβ-cat). After selection with puromycin, transfectants were cultured in the presence or absence of LIF for the indicated period. (c) Proliferation curve of ES cells expressing β-catenin[S33A,S41A,S45A] in the

presence or absence of LIF. Data shown here represent three independent experiments.

Figure 3 Deletion analysis of β-catenin. (a) Five deletion mutants of β-catenin[S33A] used in this study. (b) Morphology of ES cells expressing truncated mutants of β-catenin. (c) Comparison of expression of self-renewal markers among deletion mutants. After transfection with truncated mutants, ES cells were selected with puromycin and cultured in the presence or absence of LIF for the indicated period. Representative data of three independent experiments are shown.

Figure 4 β-catenin up-regulates Nanog through binding with Oct-3/4. (a) Effect of β-catenin on activity of Nanog promoter. Two days after transfection with pGL3-Nanog promoter (WT) or pGL3-Nanog promoter (Oct^{mut}), cells were harvested and subjected to luciferase assay. Luciferase activities were evaluated with a luciferase assay system (Promega). The luciferase activity obtained in control ES cells, which were transfected with pGL3-Nanog promoter (WT) and cultured in the presence of LIF, was set to 1.0. Bars represent the mean and standard errors ($n=3$). (b) Down-regulation of Nanog in ZHBTc4 cells expressing β-catenin mutant upon tetracycline treatment. ZHBTc4 cells transfected with β-catenin[S33A,S41A,S45A]

were selected with puromycin and cultured for three days in the presence or absence of LIF and/or tetracycline (Tet). The results represent three independent experiments. (c) β-catenin enhances the transcriptional activity of Oct-3/4. ES cells expressing β-catenin[S33A,S41A,S45A] were transfected with pOct-Luc2 and cultured for two days in the absence of LIF. Bars represent the mean and standard errors (*n=3*). (d) Co-precipitation of Oct-3/4 with β-catenin. Lysate containing GST-β-catenin was mixed with glutathione Sepharose, then incubated overnight with lysate containing myc-Oct-3/4. After washing, samples were eluted from Sepharose and subjected to immunoblotting with anti-myc antibody. Results are representative of three independent experiments.

Figure 1

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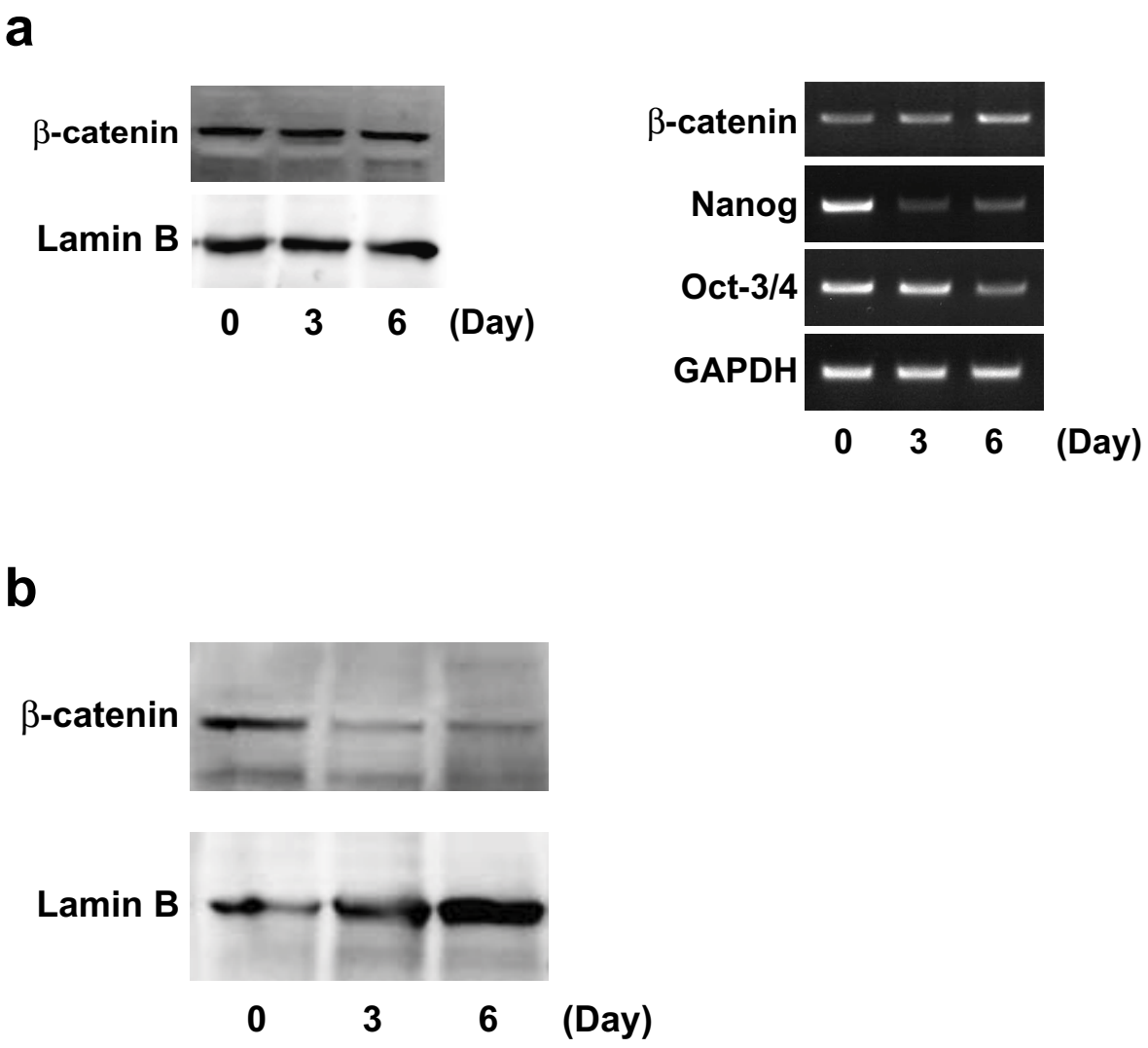

Figure 2

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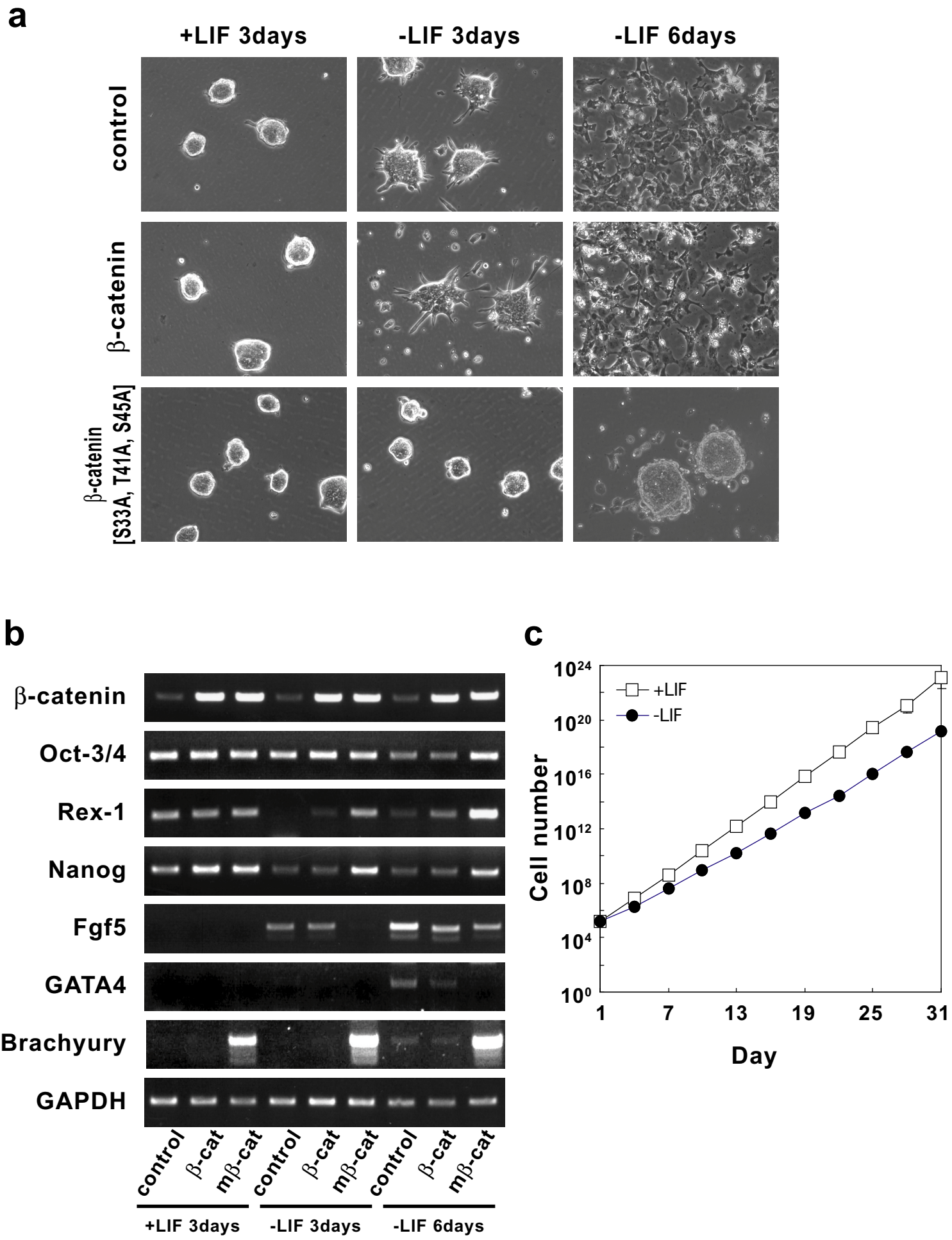

Figure 3

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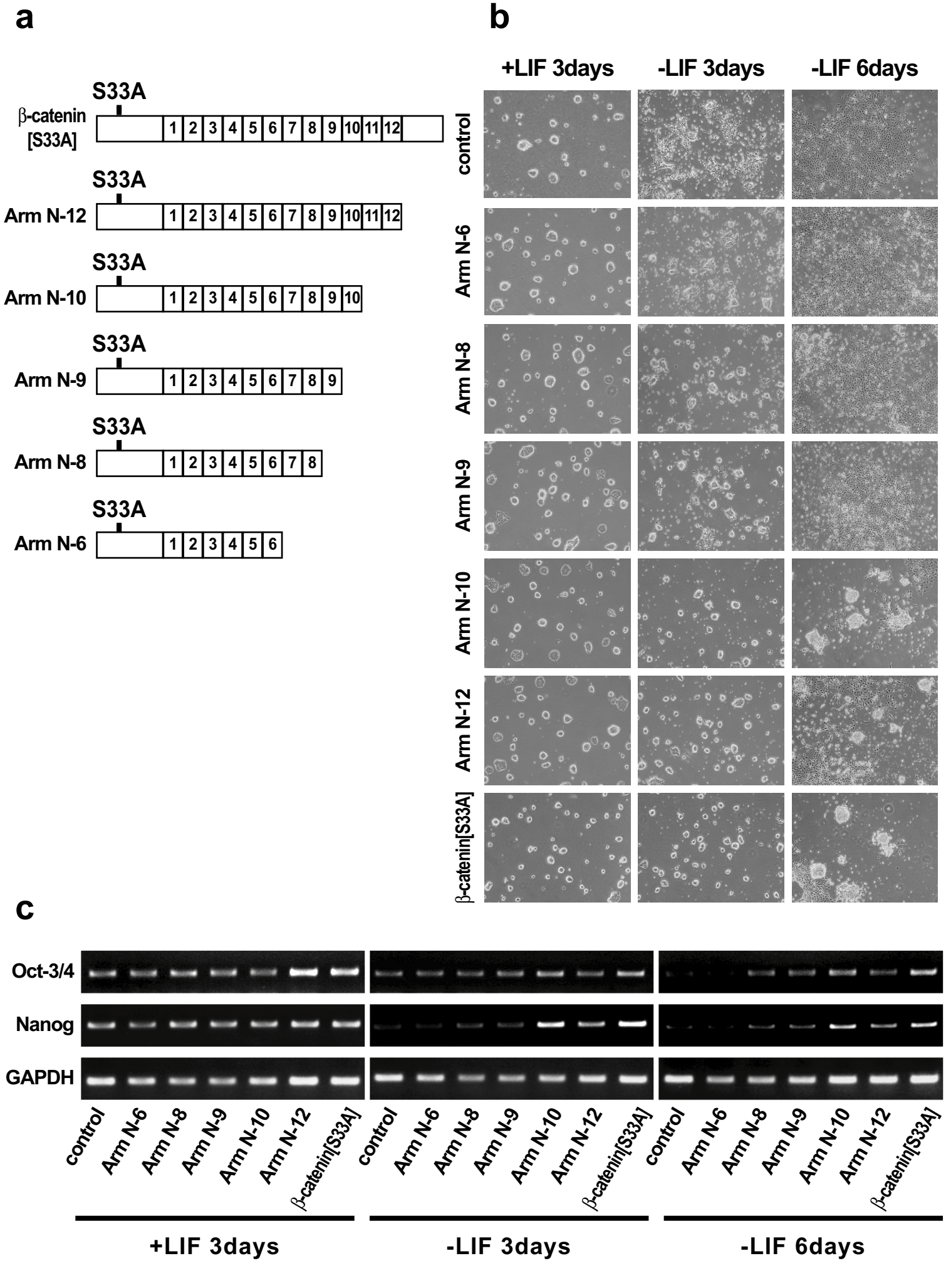

Figure 4

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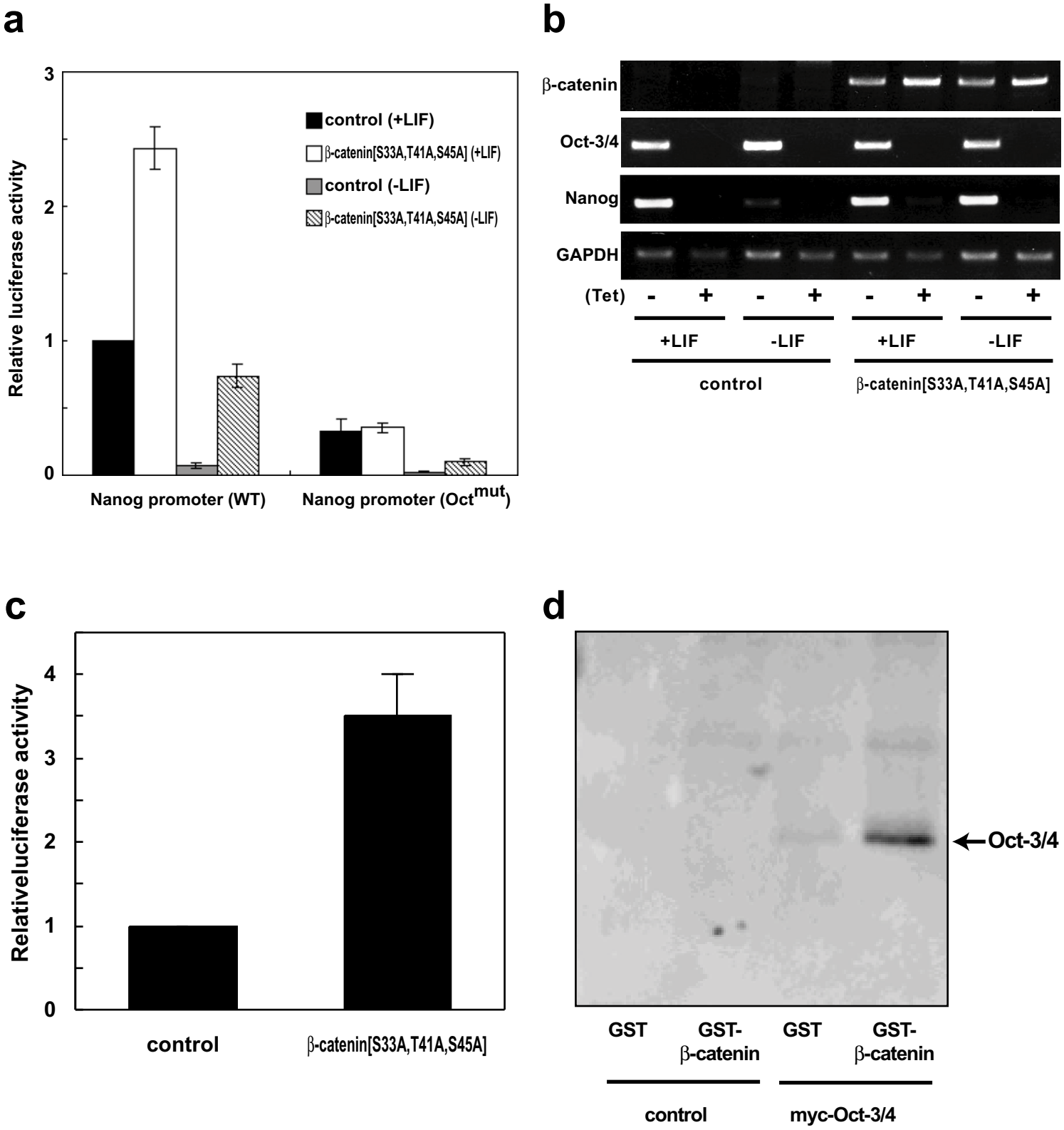

Supplementary figure legend
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Figure S1 Long-term proliferation of ES cells expressing activated β-catenin. (a) Strategy for removal of the β-catenin mutant by Cre-lox. (b) Morphology of Cre-reverted ES cells. (c) Expression of marker genes in Cre-reverted ES cells. After transfection with pCAL-β-catenin[S33A,T41A,S45A]-IPLIG, puromycin-resistant ES cells were cultured for one month in the absence of LIF. ES cells were then transfected with Cre recombinase, and GFP-positive cells were sorted to be cultured in the presence or absence of LIF for the indicated period. Data shown here represent three independent experiments.

Figure S2 γ-catenin can partially maintain the undifferentiated state of ES cells. ES cells were transfected with either pCAG-IP (control), pCAGIP-γ-catenin, or pCAGIP-γ-catenin[S24A], and puromycin-resistant transfectants were cultured in the presence or absence of LIF for the indicated period. The results represent three independent experiments.

Supplementary figure 1

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Fugure S1

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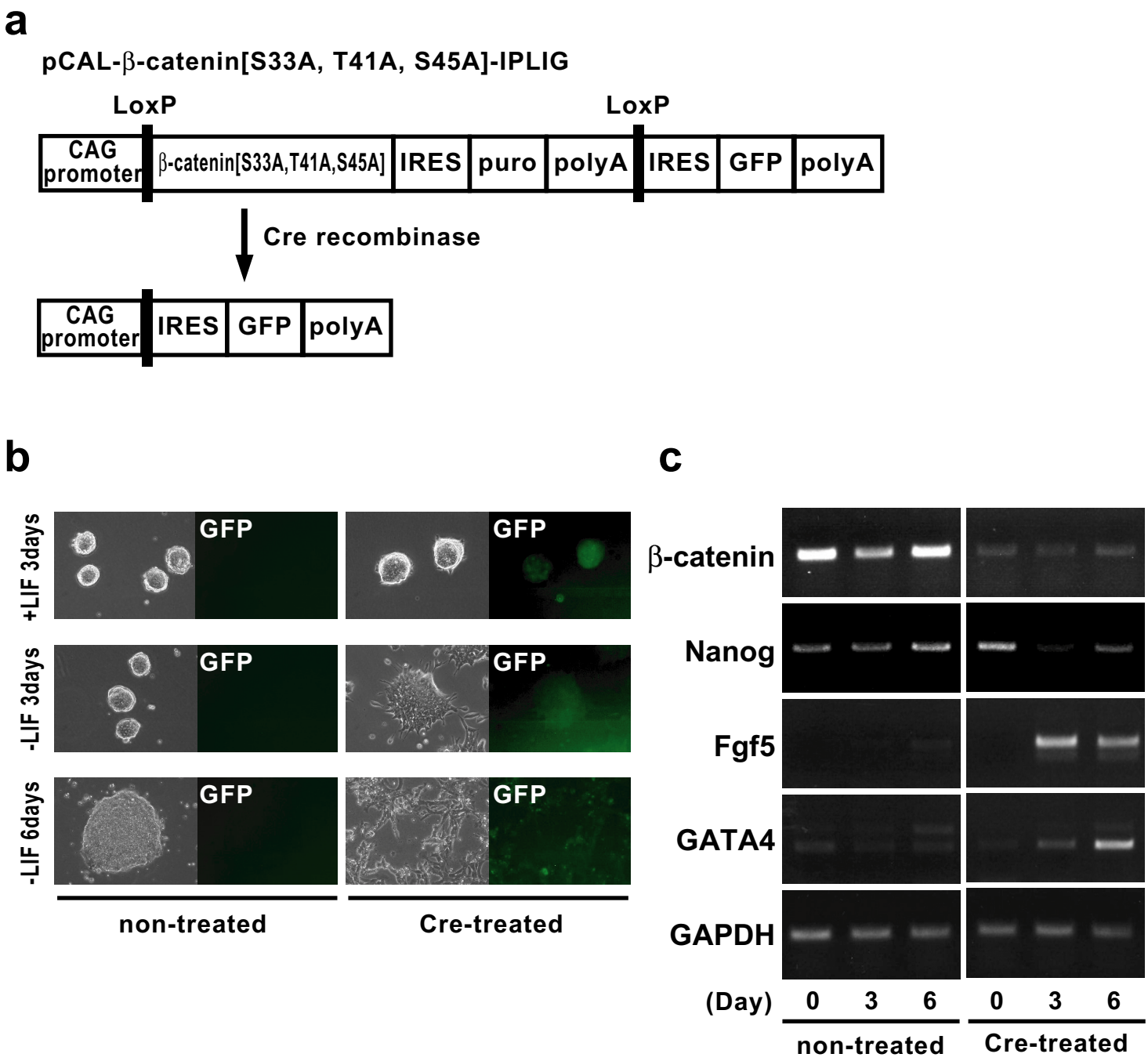

Supplementary figure 2

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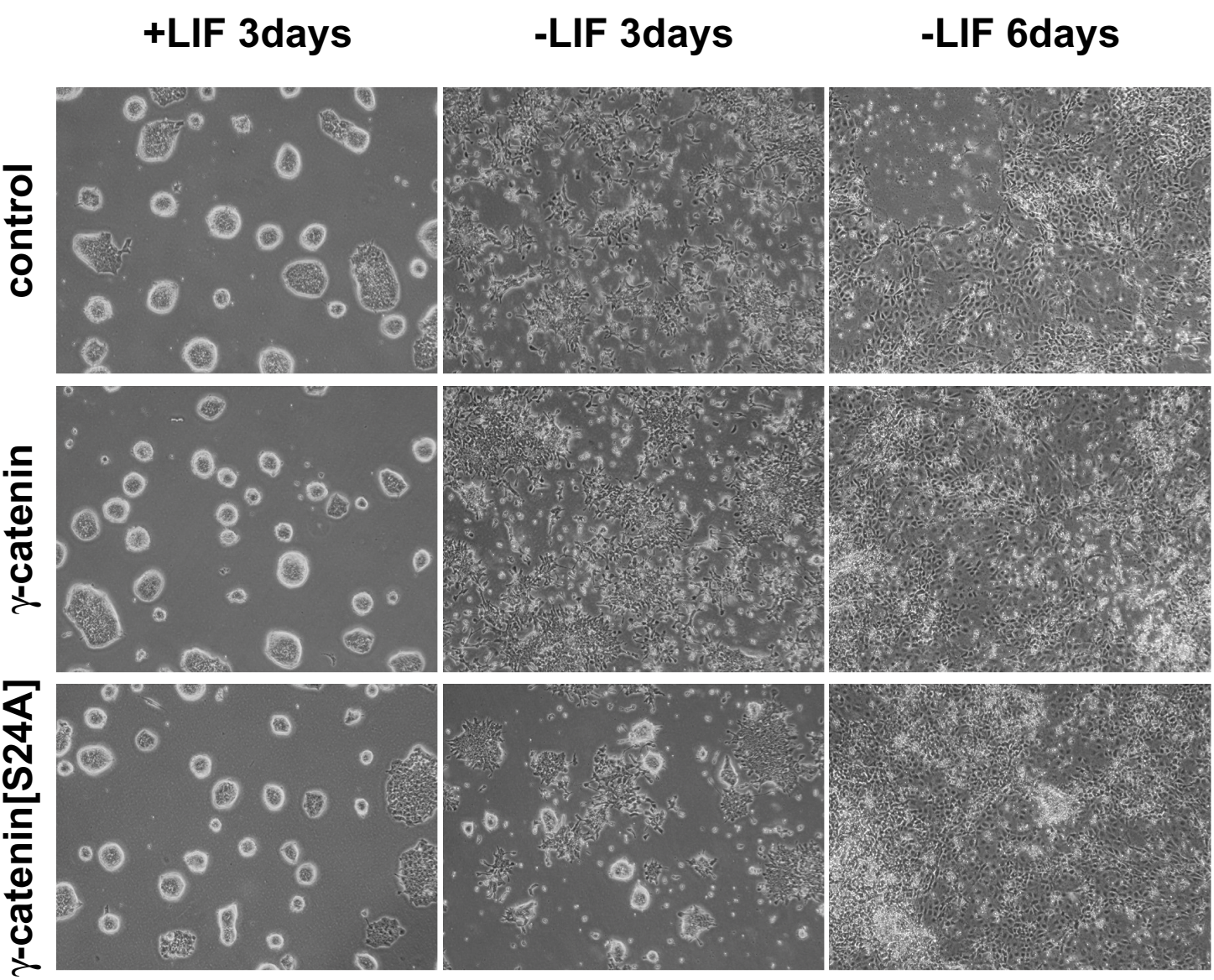
Fugure S2

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