A stem cell-derived gene (Sddr) negatively regulates differentiation of embryonic stem cells

A stem cell-derived gene, Sddr, negatively regulates differentiation of embryonic stem cells

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ABSTRACT

Embryonic stem (ES) cells, derived from the inner cell mass of blastocysts, are pluripotent and continue to self-renew.   To understand the molecular mechanism of self-renewal, we have been searching for a gene(s) that is specifically expressed in self-renewing ES cells.   Here we report the isolation and characterization of a novel gene, Sddr (stem cell-derived differentiation regulator).   Sddr was highly expressed in undifferentiated ES cells, and its expression was downregulated upon differentiation. In addition to ES cells, Sddr expression was observed strongly in ovary, and weakly in lung.   Immunostaining and cellular fractionation analyses suggested that Sddr is a cytoplasmic protein associated with the cytoskeleton.   Sddr-null ES cells showed no remarkable abnormalities in their undifferentiated state.   In differentiating Sddr-null cells, by contrast, induction of several differentiation-associated markers was enhanced, and downregulation of self-renewal marker genes was accelerated, as compared with wild-type cells.   These results suggest that although it is dispensable for ES cell self-renewal, Sddr is a negative regulator of ES cell differentiation.

Introduction

Embryonic stem (ES) cells were established from inner cell mass (ICM) of mammalian blastocysts (Evans and Kaufman, 1981; Martin, 1981). Mouse ES cells require leukemia inhibitory factor (LIF) for self-renewal (Smith *et al.*, 1988; Williams *et al.*, 1988). LIF, which belongs to the interleukin-6 cytokine family, acts through a receptor complex composed of a low-affinity LIF receptor and gp130 (Hibi *et al.*, 1990; Gearing *et al.*, 1991). We previously reported that the tyrosine residue of gp130 responsible for STAT3 activation is necessary for self-renewal in mouse ES cells (Matsuda *et al.*, 1999). Using a fusion protein between STAT3 and the ligand-binding domain of estrogen receptor (STAT3ER), we also demonstrated that STAT3 activation is sufficient to maintain the undifferentiated state of ES cells. Furthermore, it has been shown that suppression of STAT3 activity results in differentiation of ES cells (Niwa *et al.*, 1998). These observations indicate that STAT3 plays a critical role in the self-renewal of mouse ES cells.

Oct3/4 and Nanog are other important transcription factors for self-renewal of ES cells. Oct3/4 is a POU transcription factor expressed in early embryo cells and germ cells. Oct3/4-deficient embryos fail to form ICM (Nichols *et al.*, 1998). In ES cells, a reduced level of Oct3/4 leads to trophoectodermal differentiation, while its overexpression induces differentiation into primitive endoderm and mesoderm (Niwa *et al.*, 2000), indicating that Oct3/4 is indispensable for ES cell self-renewal. Nanog is a homeoprotein that has been identified as a self-renewal-promoting gene (Chambers *et al.*, 2003) and as a gene specifically

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expressed in ES cells (Mitsui *et al*, 2003). Constitutive expression of Nanog enables self-renewal of ES cells even in the absence of LIF, and Nanog-deficient ICM failed to generate epiblast and produced only parietal endoderm-like cells, suggesting the importance of Nanog in ES cell self-renewal. A recent report, however, has demonstrated that Nanog-deficient ES cells can self-renew indefinitely, although showing the tendency to differentiate (Chambers *et al*., 2007). These observations suggest that Nanog is a promoting factor, rather than an indispensable factor, in ES cell self-renewal.

In this study, to understand the molecular mechanism of self-renewal in ES cells, we searched for a gene(s) specifically expressed in self-renewing ES cells, and we isolated Sddr (stem cell-derived differentiation regulator). Although disrupting this gene had no effect on the maintenance of ES cells, it promoted ES cell differentiation, suggesting that Sddr regulates a switching between self-renewal and differentiation in ES cells.

Results

Identification of Sddr as a self-renewing ES-specific gene

To identify self-renewal-specific genes, we performed microarray analysis to search for a gene(s) whose expression is upregulated by both LIF stimulation and STAT3ER activation, and found 2410146L05Rik, which encodes a novel protein of 164 amino acids with no known motif (Fig. 1A). We named this gene Sddr (stem cell-derived differentiation regulator) to reflect its properties: the gene is highly expressed in self-renewing ES cells and is involved in regulating ES cell differentiation (see below).

The expression of Sddr in self-renewing ES cells was verified by Northern blot analysis and quantitative RT-PCR (Fig. 1B). Robust expression of Sddr was detected in ES cells cultured with LIF, while LIF removal led to quick downregulation of this expression. STAT3ER-expressing ES cells possessed a higher level of Sddr in the presence of 4HT compared with those cultured without 4HT (Fig. 2A). ES cells transfected with a dominant-negative mutant of STAT3 showed a lower expression level of Sddr compared with cells transfected with wild-type STAT3 (Fig. 2B). When we examined the expression level of Sddr in ZHBTc4 ES cells, in which Oct3/4 expression can be artificially regulated by addition of tetracycline (Tet) (Niwa *et al.*, 2000), Oct3/4 and Sddr were strongly expressed (Fig. 2C). As Oct3/4 expression was downregulated by addition of Tet, Sddr expression gradually decreased. When Oct3/4 expression was recovered by removal of Tet, downregulation of Sddr mRNA ceased, but was not restored. When we suppressed

expression of Nanog by RNA interference (RNAi), the expression level of Sddr was not changed despite Nanog downregulation (Fig. 2D).   Taken together, the results indicate that Sddr is a self-renewal-specific gene, and suggest the possibility that expression of Sddr may be regulated by STAT3 and Oct3/4.

Expression of Sddr in adult tissues

To examine whether the expression of Sddr is restricted to ES cells, we prepared total RNAs from several adult mouse tissues and performed Northern blot analysis.   As shown in Fig. 3A, Sddr transcript was strongly expressed in ovary, which corresponds well with the recent report by Joshi *et al.* (2007).   In addition, RT-PCR analysis suggested that Sddr is expressed also in lung (Fig. 3B).

Sddr is localized in the cytoplasm

To determine the localization of Sddr in ES cells, we constructed Sddr fusion protein with EGFP (Sddr-EGFP).   When the expression of Sddr-EGFP was driven by the CAG promoter, this protein localized in the cytoplasm of ES cells (Fig. 4A).   Cytoplasmic localization of Sddr is also observed in HeLa cells expressing myc-tagged Sddr (Fig. 4B).   Furthermore, cellular fractionation analysis revealed that, in ES cells, Sddr exists in the cytoskeleton fraction together with a cytoskeleton marker, vimentin (Fig. 4C).   These data suggest that Sddr is localized in the cytoplasm of ES cells and is associated with the cytoskeleton.

Targeted disruption of the sddr gene in ES cells

Since Sddr is expressed in undifferentiated ES cells, it is possible that Sddr is involved in the maintenance of ES cell self-renewal. To explore this possibility, we established Sddr-null ES cells with two targeting constructs to replace the entire open-reading frame region with the β-galactosidase (LacZ) and neomycin-resistance (neo) genes or hygromycin-resistance (hygro) gene (Fig. 5A). The LacZ-neo targeting vector was introduced into ES cells by electroporation, and neomycin-resistant cells were selected in medium containing G418. Of 360 G418-resistant clones screened, four clones were isolated as Sddr$^{+/-}$ clones. The homologous recombinations in all clones were confirmed by PCR analysis (Fig. 5B), Southern blotting (Fig. 5C), and X-gal staining (data not shown). To obtain homozygous mutant ES cells, we next introduced the hygro targeting vector into Sddr$^{+/-}$ ES cells. Of 360 G418- and hygromycin-resistant clones, two clones were isolated as Sddr$^{-/-}$ clones by PCR (Fig. 5B). In both clones (#54-2, #78-1), the homologous recombinations were confirmed by Southern blot analyses (Fig. 5C). Furthermore, Northern blot analysis showed that Sddr mRNA is absent in both clones (Fig. 5D).

Sddr is dispensable for self-renewal, but it plays a negative role in regulation of

differentiation in ES cells

When we compared Sddr$^{-/-}$ cells with the wild-type ES cells, we found that Sddr$^{-/-}$

cells could be maintained with LIF and were normal in morphology (Fig. 6A), Oct3/4 and Nanog expression (Fig. 6B), cell-cycle structure (Fig. 6C), and proliferation (Fig. 6D). These results indicate that Sddr is dispensable for the self-renewal of ES cells. However, we noticed that the expression level of Gata4 was slightly higher in Sddr-null cells than in the wild-type cells (Fig. 6B), suggesting the possible involvement of Sddr in repressing ES cell differentiation. To assess this possibility, Sddr$^{-/-}$ ES cells were subjected to *in vitro* differentiation through embryoid body formation. No apparent differences in the efficiency of forming embryoid bodies were observed between the wild-type and Sddr$^{-/-}$ cells (data not shown). However, self-renewal marker genes, Oct3/4 and Nanog, were more quickly downregulated in differentiated Sddr$^{-/-}$ cells than in the wild-type cells (Fig. 6E). We next compared the expression levels of differentiation-associated markers between the wild-type and Sddr-null cells. Since we detected a slight upregulation of Gata4 in self-renewing Sddr-null cells, we first examined the effect of Sddr deficiency on induction of Gata4, and found that Gata4 is induced more strongly in Sddr-null cells. Similarly, induction of other endoderm markers, Gata6 and Sox17, was apparently accelerated in differentiated Sddr-null cells. Furthermore, Sddr deficiency also promoted induction of other germ layer markers, Fgf5 (ectoderm), Pax6 (neuroectoderm), T, Tbx5 (mesoderm), Cdx2 and Hand1 (trophectoderm). The observed phenotype was due to Sddr deficiency, because ectopic expression of Sddr suppressed downregulation of self-renewal markers, as well as induction of differentiation markers (Fig. 6F). These data suggest that disruption of the Sddr gene promotes

differentiation of ES cells.

Discussion

Pluripotency is maintained during ES cell self-renewal through the prevention of differentiation and the promotion of proliferation. It is well-established that LIF is a key factor preventing differentiation for mouse ES cells. But, how is the self-renewal of mouse ES cells maintained at the molecular level? In this study, we isolated Sddr as a self-renewal–specific molecule in ES cells. Although there is no known motif in Sddr protein, we inferred that Sddr is a cytoplasmic protein associated with the cytoskeleton. Knockout analysis indicated that Sddr is dispensable for self-renewal but does play a role in repression of differentiation in ES cells.

Since expression of Sddr is restricted to self-renewing ES cells, it is reasonable to assume that its expression is controlled by important transcription factors for self-renewal, such as STAT3, Oct3/4, and Nanog. Indeed, we demonstrated that suppression of STAT3 activity resulted in downregulation of Sddr, using the dominant-negative mutant of STAT3 and STAT3ER (Fig. 2A and B). However, since downregulation of STAT3 causes ES cell differentiation, we cannot determine whether downregulation of Sddr is due to inactivation of STAT3 or due to differentiation of ES cells. Therefore, although the present data suggest that Sddr is a putative target of STAT3, more detailed analysis, such as promoter analysis and chromatin immunoprecipitation assay, will be required to determine whether Sddr is a direct target of STAT3. As for the relationship between Sddr and Oct3/4, it seems that Oct3/4 indirectly regulates Sddr expression, since the influence of Oct3/4 repression on Sddr expression was quite small (Fig. 2C). In agreement with this conclusion, by combining the microarray data of ZHBTc4 cells with ChIP-chip and ChIP-PET data, Matoba *et al*. (2006)

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have reported that 2410146L05Rik (Sddr) is not the primary but rather the secondary or even

tertiary target of Oct3/4.   As for Nanog, knockdown experiments suggested that this

transcription factor is not involved in the regulation of Sddr expression, although we cannot

exclude the possibility that suppression of Nanog might be insufficient to influence Sddr

expression (Fig. 2D).

As compared with wild-type cells, the expression of Oct3/4 was reduced more

rapidly in differentiating Sddr-null ES cells (Fig. 6E).   Sddr deficiency also promoted

downregulation of Nanog expression, as well as the inductions of multiple differentiation

markers, during differentiation (Fig. 6E).   Furthermore, we observed that Sddr-null ES cells

produced more beating cells than parental ES cells when they underwent differentiation (data

not shown).   These results suggest that Sddr somehow controls a switching between

self-renewal and differentiation in ES cells.   In addition, the present observation that Sddr is

expressed also in ovary and lung (Fig. 3) suggests that this molecule may act as a

differentiation regulator in types of cells other than ES cells.   In particular, the expression of

Sddr in female germ cells may imply a role of Sddr in other stem cells.

Our data suggest that Sddr localizes in the cytoplasm and associates with the

cytoskeleton.   How then does this protein regulate expression of differentiation-associated

genes?   Several studies have shown that cytoskeleton-associated proteins regulate

transcription factors by trapping them in the cytoplasm.   For example, Keap1, an

actin-binding protein, controls the transactivity of Nrf2 by retaining Nrf2 in the cytoplasm

(Itoh *et al*., 1999). LMP-4, which is associated with the actin cytoskeleton, indirectly

regulates the transcription of Tbx5 target genes, Fgf10 and ANF, through trapping Tbx5 in the

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cytoplasm (Camarata *et al*., 2006).   Therefore, it is possible that Sddr sequesters an essential molecule for ES cell differentiation in the cytoskeleton to prevent expression of differentiation-associated genes.   Identification of an Sddr-interacting protein would provide a clue to understanding the role of Sddr in ES cells.

In conclusion, the present data suggest that Sddr regulates a switching step from self-renewal to differentiation in ES cells.   Further analysis of Sddr may open up new insights for understanding how self-renewing ES cells are prevented from differentiation.   In addition, Sddr may be an attractive target for regulation of ES cell differentiation in the future.

Materials and Methods

Cell Culture

ES cell lines A3-1 (Azuma and Toyoda, 1991) and ZHBTc4 (Niwa *et al*., 2000) were maintained on gelatin-coated dishes in the absence of feeder cells, as described previously (Matsuda *et al*., 1999). STAT3ER-expressing ES cells were cultured with 4-hydroxytamoxifen (4HT, Sigma). HeLa cells were cultured in Dulbecco's modified Eagle medium containing 10% fetal bovine serum.

For *in vitro* differentiation, 1×10^6 ES cells were transferred to a 6-cm petri dish and cultured without LIF to undergo embryoid body formation. Cells were harvested after three and six days.

Plasmid construction and transfection

Construction of expression vectors for the wild-type STAT3 (pCAG-wtSTAT3-IP) and dominant-negative mutant of STAT3 (pCAG-dnSTAT3-IP) was described previously (Akagi *et al*., 2005). The coding sequence of Sddr (GenBank accession number **AB283026**) was amplified from cDNAs synthesized with total RNA of A3-1 cells using sense (5'-TAA GAA TTC ACC ATG GCA TCC CAC ACG GCT GAT GC-3') and antisense (5'-TTA CGC CGG CGT TAA GAC TCC ATC TGT GTT TCT CTT C-3') oligonucleotide primers. pCAG-myc-Sddr-IP and pEGFP-N2-Sddr were constructed by inserting myc-tagged Sddr coding fragment into the mammalian expression vectors, pCAG-IP (Yoshida-Koide *et al*.,

2004) and pEGFP-N2 (Clontech), respectively. pEFlacIP-hrGFP and pEFlacIP-sddr were

constructed by inserting cDNAs of GFP and Sddr into pEFlacIP, respectively. pEFlacIP was

produced by transferring a DNA fragment carrying internal ribosome entry site (IRES)

sequence and puromycin resistance gene from pCAG-IP into pEF-LACAB (Yamazaki *et al.*,

2001). The target sequence (5'-GGT GCT TGC TTG TCC TTG G-3') of RNA interference

for Nanog was cloned into the *Apa*I and *Eco*RI sites of pSi-puro (Akagi *et al.*, 2005). ES

cells were transfected by lipofection using LipofectAMINE 2000 (Invitrogen) , and then

selected with 1 µg/ml puromycin at 48 hr post-transfection. HeLa cells were transfected by

electroporation (240V, 500 µF) using Gene Pulser II (Bio Rad).

Northern blot, RT-PCR, and real-time PCR analyses

The total RNAs of individual cultured cells were extracted using Trizol Reagent.

Northern blot analysis was carried out as previously described (Akagi *et al.*, 2005). Probes

corresponding to the entire coding regions of Sddr and glyceraldehyde-3-phosphate

dehydrogenase (GAPDH) were labeled with [α-^{32}P]dCTP using Megaprime DNA Labeling

System (Amersham Biosciences).

For RT-PCR analysis, cDNA synthesis was performed with SuperScriptIII Reverse

Transcriptase (Invitrogen) as described previously (Kajihara *et al.*, 2003). Real-time PCR

analysis was done with FullVelocity SYBR Green QPCR Master Mix (Stratagene) using the

Mx3000p System (Stratagene). The amount of Sddr mRNA was determined from the

appropriate standard curve and divided by the amount of GAPDH mRNA for normalization.

Primers for Sddr were 5'-CTGGTGGTTCCCAATTCAGGAAGT-3' and 5'-CCTTCACAGCTCTTTGGAGTTCGT-3'.   Primers for GAPDH, Oct3/4, Nanog, Gata4, Gata6, Sox17, Fgf5, Pax6, T (Brachyury), Tbx5, Cdx2, Hand1 were described before (Niwa *et al*., 2000; Yoshida-Koide *et al*., 2004; Akagi *et al*., 2005; Kinoshita *et al*., 2007; Ura *et al*., 2008).

Immunostaining

Immunostaining of cultured cells was carried out as previously described (Ogino and Yasuda, 1998).   Briefly, the cells were fixed and incubated with mouse monoclonal anti-myc antibody (Santa Cruz Biotechnology), and then with Alexa Fluor 488 rabbit anti-mouse IgG (Molecular Probes).   Hoechst (bisBENZIMIDE, Sigma) was added to final wash solution at the final concentration of 10 μg/ml.

Cellular fractionation and Western blot analysis

A3-1 ES cells were transfected with pCAG-myc-Sddr-IP and harvested at 48 h post-transfection.   Cellular fractions of the transfected cells were extracted using a Subcellular ProteoExtract Kit S-PEK (Calbiochem).   Western blotting was carried out using mouse monoclonal anti-α-tubulin (ICN), rabbit polyclonal anti-calnexin (H-70, Santa Cruz Biotechnology), goat polyclonal anti-laminB (M-20, Santa Cruz Biotechnology), goat

polyclonal anti-vimentin (C-20, Santa Cruz Biotechnology), and mouse monoclonal anti-myc (Upstate) antibodies.

Targeted disruption of Sddr

The 5'-arm (4712 bp) and 3'-arm (2584 bp) were isolated by PCR. To construct the two gene-targeting vectors for Sddr locus, the neomycin phosphotransferase (neo) and LacZ genes, or phosphoglycerate kinase (PGK) promoter and the hygromycin resistance gene (hygro) were inserted into the Sddr open-reading frame (ORF) region. A diphtheria toxin A gene was inserted at the end of the 3' short arm of the targeting vectors for negative selection. Targeted ES cells were identified by PCR screening using Sddr antisense primer (3'AS: 5'-GCTAGGCCTGCATCATGGAGTTGTTGCTTC-3') in combination with neo sense (NS: 5'-AGCAGCCGATTGTCTGTTGTGCCCAGTCAT-3') or hygro sense (HS: 5'-AGAAGTACTCGCCGATAGTGGAAACCGACG-3') primers. Independent clones, which had undergone homologous recombination at the Sddr locus, were isolated. Their genotypes were verified by Southern blot hybridization analyses using a partial 5' long arm region of Sddr (881 bp) as a probe, and by PCR for neo or hygro genes (Akagi *et al*., 2005). The 881-bp probe was isolated by PCR with 5'-AACTCACAGAGATCTACTGC-3' and 5'-TACTCACTGAGTCTGGTCC-3'.

Acknowledgments

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Figure Legends

Fig. 1. Sddr is specifically expressed in self-renewing ES cells. (A) *Amino acid sequence of Sddr.* **(B)** *Downregulation of Sddr mRNA during ES differentiation.   Left panel, ES cells were cultured with LIF for three days or without LIF for five days and subjected to Northern blot analysis.   GAPDH was used as an internal control (Willems* et al.*, 2006). Right panel, ES cells were cultured in the absence of LIF for the indicated days and subjected to real-time PCR.   The value at day 0 is set to 1.0.   Bars represent the means and standard errors of triplicates.*

Fig. 2. Relationship of Sddr with STAT3, Oct3/4 and Nanog. (A) *STAT3ER-expressing ES cells were incubated with (+) or without (-) 4HT for four days.* **(B)** *ES cells were transfected with either wild-type STAT3 (wtSTAT3) or dominant-negative mutant of STAT3 (dnSTAT3) and selected for two days in the presence of puromycin (1 μg/ml).   Cells were cultured for two more days in the absence of puromycin and harvested.   In each experiment, total RNA (10 μg) was loaded to 1.5% agarose gel and subjected to Northern blot analysis.* **(C)** *ZHBTc4 cells were incubated with (+) or without (-) tetracycline (Tet) for 24 or 48 h and subjected to RT-PCR analysis.   Control, ZHBTc4 cells maintained in the absence of Tet.* **(D)** *RNAi construct for Nanog (Nanog RNAi) or an empty vector, pSi-puro (control), was introduced into ES cells.   After 24 h culture, cells were transferred to new dishes and incubated for another 24 h, and the medium was exchanged for a new medium containing 1 μg/ml puromycin.   Cells were cultured for three more days and subjected to RT-PCR analysis.*

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Fig. 3.   Tissue distribution of Sddr.   *Expression of Sddr in the indicated tissue was examined by Northern blot analysis* **(A)** *and RT-PCR analysis* **(B)**.

Fig. 4. Localization of Sddr in ES cells.   **(A)** *ES cells were transfected with pEGFP-N2 (EGFP) or pEGFP-N2-Sddr (Sddr-EGFP), and cultured for 24 h.   Cells were pelleted onto glass slides using a Cyto-spin and analyzed with a fluorescence microscope.*   **(B)** *HeLa cells were transfected with pCAG-myc-IP (Myc) or pCAG-myc-Sddr-IP (Myc-Sddr) and cultured for two days.   Myc-Sddr was detected by anti-myc antibody.*   **(C)** *Western blot analysis of cellular fractions from myc-Sddr-transfected ES cells.   Lane 1, cytoplasm; lane 2, membrane/organella; lane 3, nucleus; lane 4, cytoskeleton.   Tubulin, calnexin, laminB, and vimentin were used as markers for cytoplasm, membrane/organella, nucleus, and cytoskeleton fractions, respectively.   Myc-Sddr was detected with an anti-myc antibody.*

Fig. 5. Targeted disruption of Sddr gene.   **(A)** *Schematic representations of the wild-type allele, targeted neo-mutant allele, and targeted hygro-mutant allele.   The solid box in the wild-type allele represents the coding sequence.   Arrows show oligonucleotide primers used in PCR screening.*   **(B)** *Selection of heterozygous and homozygous ES cells by PCR with primers for neo and hygro genes, and mutant alleles (NS-3'AS, HS-3'AS).*   **(C)** *Southern blot analysis of mutant clones using probe for the 5' region of the Sddr gene.   Upper panel,*

EcoRI digesta; wild-type allele 10.3 kb, neo mutant allele 11.4 kb, hygro mutant allele 8.4 kb.

Lower panel, HindIII digesta; wild-type allele 10.2 kb, neo mutant allele 7.7 kb, hygro mutant

allele 9.7 kb. **(D)** *Northern blot analysis of Sddr transcripts. Total RNAs (10 μg) were*

loaded to 1.5% agarose gel. Sddr mRNA was detected as a 0.8 kb band. +/+, wild-type

cells; +/-, neo heterozygous mutant cells; -/-, homozygous mutant cells.

Fig. 6. Phenotypes of Sddr-null cells. **(A)** *Morphology of Sddr-null cells. Wild-type*

(+/+) and Sddr-null cells (#78-1) (-/-) were cultured with LIF for three days. **(B)**

Expression levels of Sddr, Oct3/4, Nanog, Gata4, T, and Fgf5 in Sddr-null ES cells.

Wild-type (+/+) and Sddr-null ES cells (#78-1) (-/-) were cultured in the presence of LIF for

three days, and the expression level of each gene was compared by RT-PCR. **(C)** *Analysis of*

cell-cycle distribution. Wild-type (+/+) and Sddr-null ES cells (#78-1) (-/-) were cultured

for 24 h. The numbers represent the percentage of cells in the G1, S, and G2 to M phases of

the cell cycle. **(D)** *Proliferation of Sddr-null ES cells. Cells were plated to a 6-well plate*

at 3×10^4 cells per well. Cell numbers were counted daily for six days. **(E)** *Expression*

levels of self-renewal and differentiation marker genes in differentiated Sddr-null ES cells.

Wild-type (+/+) and Sddr-null ES cells (#54-2 and #78-1) were allowed to form embryoid

bodies for the indicated days and subjected to RT-PCR analysis. **(F)** *Suppression of*

accelerated differentiation of Sddr-null ES cells by ectopic expression of Sddr. Sddr-null ES

cells (#78-1) were transfected with pEFlacIP-hrGFP (control) or pEFlacIP-sddr (Sddr), and

stable clones were established by puromycin selection. Each clone was allowed to form

embyoid bodies for six days and subjected to RT-PCR analysis.

A

MASHTADADA KPDSDSQKLL NVLPVSLRLR

TRPWWFPIQE VSNPLVLYME AWVAERVIGT

DQAEISEIEW MCQALLTVDS VNSGNLAEIT

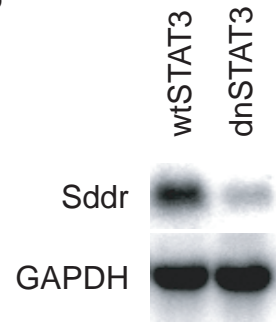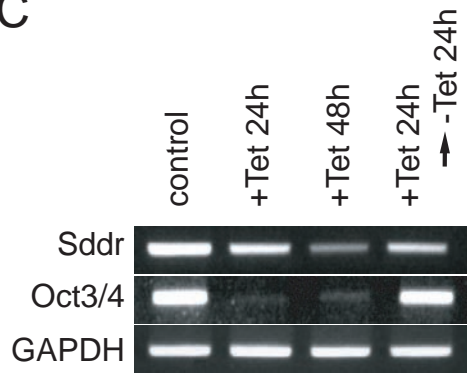**IFGQPSAQTR MKNILLNMAA WHKENELQRA**

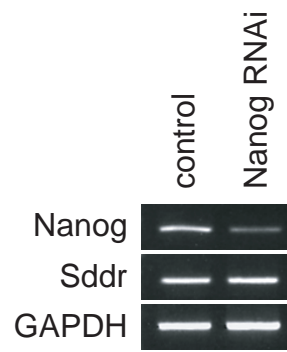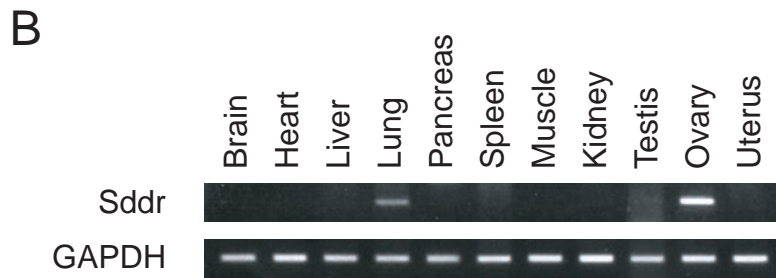**VKVKEVEEFL KIRASSILSK LSKKGLKLAG**

FPLPLEGRET QMES

B

A

+4HT
-4HT

Sddr

GAPDH

B

wtSTAT3
dnSTAT3

Sddr

GAPDH

C

control
+Tet 24h
+Tet 48h
+Tet 24h ↑ -Tet 24h

Sddr

Oct3/4

GAPDH

D

control
Nanog RNAi

Nanog

Sddr

GAPDH

A

	EGFP	Sddr-EGFP
Phase		
Hoechst		
EGFP		
Merge		

B

	Myc	Myc-Sddr	
Phase			
Hoechst			
anti-Myc			
Merge			

C

	1	2	3	4
tubulin				
calnexin				
laminB				
vimentin				
myc-Sddr				

A

wild-type allele

neo mutant allele

NLS-LacZ   neo

NS   3'AS

hygro mutant allele

hygro

HS   3'AS

E: EcoRI
H: HindIII

B

neo
hygro
NS-3'AS   3.5 kb
HS-3'AS   3.7 kb

C

EcoRI

11.4kb
10.3kb
8.4kb

HindIII

10.2kb
9.7kb
7.7kb

D

Sddr
GAPDH