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# Importin Alpha Subtypes Determine Differential Transcription Factor Localization in Embryonic Stem Cells Maintenance

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## SUMMARY

**We recently demonstrated that the expression of the importin α subtype is switched from α2 to α1 during neural differentiation in mouse embryonic stem cells (ESCs) and that this switching has a major impact on cell differentiation. In this study, we report a cell-fate determination mechanism in which importin α2 negatively regulates the nuclear import of certain transcription factors to maintain ESC properties. The nuclear import of Oct6 and Brn2 was inhibited via the formation of a transport-incompetent complex of the cargo bound to a nuclear localization signal binding site in importin α2. Unless this dominant-negative effect was downregulated upon ESC differentiation, inappropriate cell death was induced. We propose that although certain transcription factors are necessary for differentiation in ESCs, these factors are retained in the cytoplasm by importin α2, thereby preventing transcription factor activity in the nucleus until the cells undergo differentiation.**

## INTRODUCTION

Cell differentiation is controlled by multiple factors, but the regulation of transcription factor activity through lineage-specific expression is one of the most important factors. The POU transcription factors are a subgroup of the larger homeodomain family. Among the seven classes of POU transcription factors, class III proteins (including Brn1, Brn2, Brn4, and Oct6/Tst-1) are involved in neural development, and class V proteins, such as Oct3/4, function in early embryogenesis.

Oct6 is highly expressed in undifferentiated embryonic stem cells (ESCs) but is predominantly localized in the cytoplasm (Suzuki et al., 1990; Yasuhara et al., 2007). During cell differentiation, Oct6 shuttles between the nucleus and cytoplasm (Yasuhara et al., 2007; Baranek et al., 2005). Thus, although intracellular distribution is an important determinant of Oct6 activity, it is not known how this critical transcription factor is spatially regulated during cell differentiation.

The nuclear envelope separates the nucleus and cytoplasm in eukaryotic cells, and nuclear pores facilitate nucleocytoplasmic transport in these cells. Most karyophilic proteins contain specific nuclear localization signals (NLSs); the consensus sequences of these NLSs determine the cargo-specificity of nuclear transport factors. A monopartite classical NLS consists of a single stretch of basic amino acids (Kalderon et al., 1984), whereas bipartite classical NLSs are composed of two stretches of basic amino acids separated by a gap of several amino acids (Robbins et al., 1991). Importin (karyopherin) α proteins, a family of well-characterized transport factors, bind both types of classical NLS to form a trimeric complex of cargo, importin α, and importin β1 (Görlich and Mattaj, 1996; Imamoto et al., 1995). Complex translocation through the nuclear pore requires the interaction of importin β1 with nucleoporins (Radu et al., 1995; Rexach and Blobel, 1995; Hu et al., 1996). Binding of the GTP-bound form of Ran (RanGTP) to importin β1 dissociates the complex and induces the recycling of importin β1/RanGTP to the cytoplasm (Lee et al., 2005). Nup50/Npap60 binding to importin α induces cargo release (Matsuura and Stewart, 2005), facilitating the return of importin α to the cytoplasm via binding to CAS, a specific export factor, in conjunction with RanGTP (Kutay et al., 1997).

Importin α has two characteristic domains: an importin β1 binding (IBB) domain in the flexible N-terminal region and a core domain containing ten armadillo (ARM) repeats and two NLS binding sites (Fontes et al., 2000, 2003; Conti et al., 1998; Conti and Kuriyan, 2000). The major NLS binding site interacts with monopartite NLSs and a longer stretch of basic residues in bipartite NLSs, whereas the minor NLS binding site binds a shorter stretch of basic residues in bipartite NLSs. CAS interacts

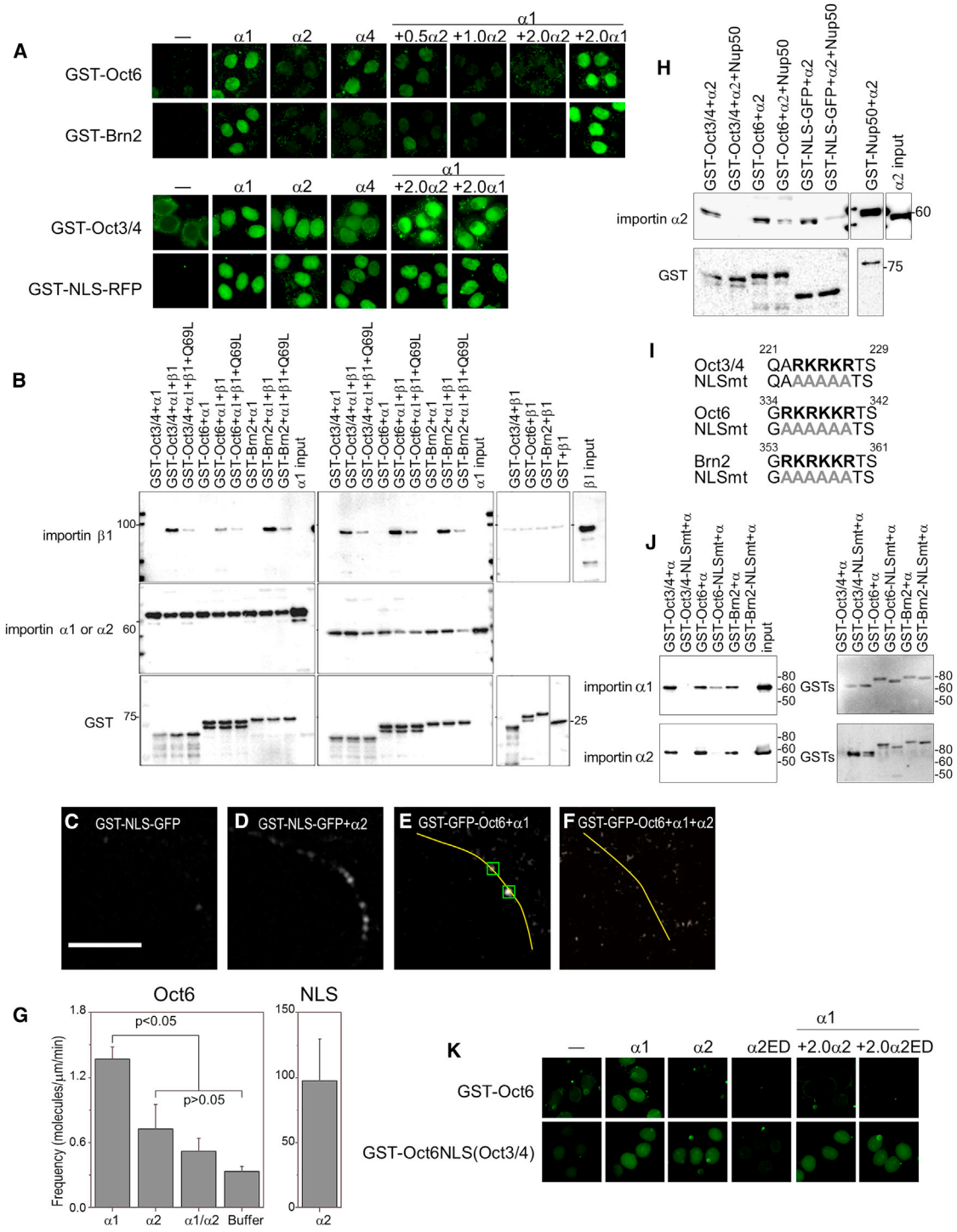



**Figure 1. Importin α2 Inhibits the Nuclear Import of Specific POU Transcription Factors**

(A) We performed an in vitro transport assay using HeLa cells to examine the effects of importin α2 on the import of GST-Oct6, GST-Brn2, GST-Oct3/4, and the control GST-NLS(SV40 TAg)-RFP. The indicated importin α was added to POU proteins alone (upper panel) or in the presence of importin α1 (lower panel). The numbers +0.5, +1.0, and +2.0 indicate the molar ratios of each importin α compared with importin α1. Importin β1, Ran, NTF2, and an ATP regeneration system were added to all samples. (—), no addition of importin α. After 1 hr incubation, the cells were stained with an anti-GST antibody.

(B) The binding and release of importin β1 cannot overcome the inhibitory effect of importin α. We examined the binding of importin β1 to importin α subtypes in the presence of POU proteins. We also tested the release of importin β following the addition of a constitutively active mutant of Ran-GTP. After incubation, the resulting protein complexes were precipitated using GST-affinity beads. The bound proteins were separated through SDS-PAGE, followed by immunoblotting with antibodies against GST and importin α1 or α2 independently.

*(legend continued on next page)*

with the tenth ARM repeat, whereas Nup50 binds two sites in importin α that partially overlap with the minor NLS and CAS binding sites (Matsuura and Stewart, 2005).

There are several importin α family members in mammals. Here, the name "importin α2" is used to refer to the product of the *KPNA2* gene, called importin-α1 in our previous report (Yasuhara et al., 2007; Yasuda et al., 2012). Similarly, we use the names importin α1, α3, α4, and α6 to refer to the products of the *KPNA1*, *KPNA3*, *KPNA4*, and *KPNA6* genes, respectively, which we previously called importin-α5, α4, α3, and α7, respectively (Yasuhara et al., 2007; Yasuda et al., 2012). Individual importin α proteins exhibit cargo specificity (Miyamoto et al., 1997; Kamei et al., 1999; Köhler et al., 1999), and the expression of these proteins is regulated during cell differentiation (Yasuhara et al., 2007; Hogarth et al., 2006; Köhler et al., 2002; Hall et al., 2011). Undifferentiated ESCs express a high level of importin α2 and low levels of importins α1 and α4, an expression pattern that is reversed upon the induction of neural differentiation. Changes in importin α expression are essential for ESC neural differentiation, and the recapitulation of this expression pattern in vitro triggers neural differentiation (Yasuhara et al., 2007).

The knockdown of importin α2 expression in ESCs decreases the expression of transcription factors that maintain pluripotency (e.g., Oct3/4, Nanog, and Sox2) and induces cell differentiation into several lineages. However, it is unknown how the downregulation of importin α2 in ESCs triggers differentiation.

This study indicates that importin α2 exerts dominant-negative activity via an unknown, C-terminal NLS-binding site. In addition, this activity must be downregulated upon ESC differentiation or inappropriate cell death is induced. We propose that importin α2 plays a crucial role in the maintenance of undifferentiated ESCs through a regulatory mechanism that inhibits the nuclear import of specific transcription factors, promoting the cytoplasmic retention of transcription factors that induce cell differentiation.

## RESULTS

### Importin α2 Inhibits the Nuclear Import of Oct6 and Brn2

We previously demonstrated that three POU transcription factors, Oct3/4, Oct6, and Brn2, are differentially imported into the nucleus by several importin α subtypes in an in vitro transport assay using digitonin-treated HeLa cells and ESCs (Yasuhara et al., 2007). We also observed that the complex of importin α1 and importin β1 (importin α1/β1) mediates the import of all three POU proteins, whereas only Oct3/4 is imported through importin α2/β1. Thus, although Oct6 and Brn2 have classical NLSs, these proteins are not transported by importin α2.

Therefore, we speculated that the characterization of the molecular basis underlying the importin α2-mediated nuclear import of Oct3/4, but not that of Oct6 and Brn2, might provide insight into the increased expression of importin α2 in undifferentiated ESCs. Therefore, we first performed a pull-down assay to examine the binding modes of importin α2. Unexpectedly, we observed that importin α2 strongly bound to Oct6 and Brn2 despite an inability to promote the nuclear import of these proteins (Figure 1B). To clarify the biological significance of this finding, we examined the effects of importin α2 on the importin α1/β1-mediated nuclear import of POU factors in vitro. The results showed that the addition of equimolar amounts of importin α2 strongly inhibited the importin α1/β1-mediated nuclear import of Oct6 and Brn2 (Figure 1A), although the nuclear import of Oct3/4 or a control SV40 TAg NLS (PKKKRKV)-containing protein was unaffected by the addition of importin α2 (Figure 1A).

Single-molecule imaging confirmed that the control GFP-SV40 TAg NLS-containing protein accumulated at the nuclear envelope in the presence of importin α2/β1 (Figures 1C and 1D), and GFP-Oct6 similarly accumulated in the presence of importin α1/β1, which was reduced by the addition of importin α2 (Figures 1E and 1F; Movie S1 available online). Thus, we propose that importin α2 specifically inhibits the nuclear import of certain transcription factors via a direct molecular interaction.

### Importin α2 Differentially Regulates the Subcellular Localization of Oct3/4 and Oct6 in Undifferentiated ESCs

Oct6 is expressed in undifferentiated ESCs, although it is involved in neural differentiation and not stem cell pluripotency. Oct6 is primarily localized to the cytoplasm of undifferentiated ESCs, but during cell differentiation, Oct6 shuttles between the nucleus and cytoplasm (Suzuki et al., 1990; Yasuhara et al., 2007). Thus, intracellular distribution is an important determinant of transcription factor activity.

Therefore, we characterized the dynamic behaviors of transcription factors, such as Oct6 and Oct3/4, in undifferentiated

(C–F) Importin α2 interrupts the nuclear pore targeting of Oct6. Single-molecule observation of GST-NLS(SV40TAg)-GFP alone (C) or in the presence of importin α2 (D) (see also Movie S1). In this assay, the proteins undergoing nuclear import appear as GFP-positive spots on the nuclear envelope. Bright spots along the rim of the nuclear membrane indicate the binding of GST-NLS-GFP to the nuclear pore complex. Scale bar represents 5 μm. Single-molecule observation of GST-Oct6 was similarly performed in the presence of importin α1 (E; Movie S1) and in the presence of both importin α1 and importin α2 (F; Movie S1).

(G) The number of GST-GFP-Oct6 or GST-NLS-GFP molecules bound to the nuclear pore complex under different conditions. From left to right, the samples were incubated in the presence of importin α1 (n = 6), α2 (n = 7), a mixture of α1 and α2 (number of cells [n] = 10), buffer alone as a control (n = 5), and the NLS (n = 4). The data are expressed as the mean ± SEM (Student's t test). Student' t test was performed for statistical analysis.

(H) NLS release by Nup50 cannot overcome the inhibitory effect of importin α. Recombinant Nup50 protein was added to a mixture of cargo proteins containing importin α1. The antibodies for proteins indicated on the left were used.

(I) The NLS sequences of POUs and mutants used in this study.

(J) We performed interaction assays using recombinant proteins by mixing importin α subtypes with wild-type or NLS-mutated POUs (NLSmt). For the input, one-fifth of the volume of proteins was added to the reaction mixture.

(K) We performed an in vitro transport assay using HeLa cells to examine the effects of importin α2 and importin α2ED on the import of GST-Oct6NLS(Oct3/4) and the control GST-Oct6. The indicated importin α was added to POU proteins. The number 2.0 indicates the molar ratio of each importin α compared to importin α1. Importin β1, Ran, NTF2, and an ATP regeneration system were added to all samples. (–), no addition of importin α. After 1 hr incubation, the cells were stained with an anti-GST antibody.

See also Figure S1.

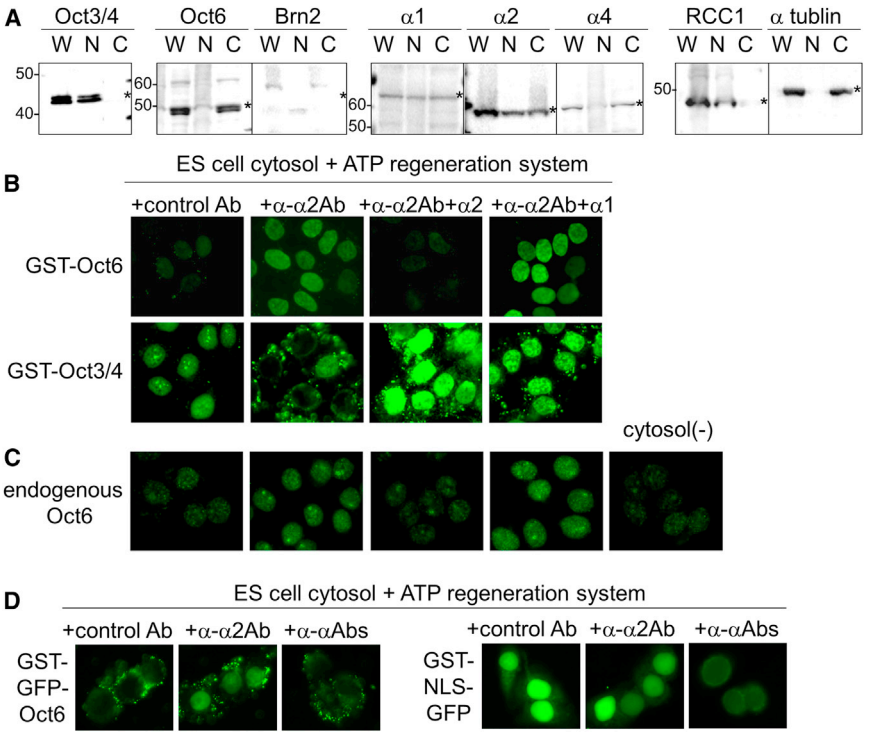



**Figure 2. Endogenous Importin α2 Inhibits Oct6 Nuclear Import**

(A) The nuclear and cytoplasmic fractions of undifferentiated ESCs were examined by immunoblotting for POU and importin α subtypes. The antibodies for proteins indicated were used. The accuracy of the fractionation was confirmed by the localization of RCC1 to the nuclear fraction and α tubulin to the cytoplasmic fraction. The expected size of each protein is marked with an asterisk in the right of the figure.

(B and C) An in vitro transport assay was performed to determine the effects of endogenous importin α2 on the import of GST-Oct6, GST-Oct3/4 (B), and endogenous Oct6 (C). The cytosolic fraction of undifferentiated ESCs was preincubated in the presence or absence of anti-importin α2 or control (anti-transportin1) antibodies at a concentration of 0.05 mg/ml for 1 hr, followed by the addition of recombinant importin α1 or α2. The fractions were mixed with an ATP regeneration system with (B) or without (C) POU proteins; these mixtures were then added to digitonin-treated HeLa cells. After incubation, the cells were stained with anti-GST (B) or anti-Oct6 (C) antibodies.

(D) Transport assays were performed as described in (B) using GST-GFP-Oct6 and GST-NLS(SV40 TAg)-GFP, with the addition of antibodies in the lysate as indicated. The following antibodies were used: control Ab, anti-transportin1 with normal goat IgG and mouse IgG; α2 Ab, anti-importin α2 with normal goat IgG and mouse IgG; and anti-importin α1, α2, α3, α4, α1/6. The total amount of IgG was constant in each sample.

ESCs. We performed an in vitro transport assay using undifferentiated ESC lysate. We observed that Oct6 was predominantly detected in the cytosolic fraction of ESCs, Brn2 was not detected, and Oct3/4 was present in the nuclear fraction (Figure 2A). Upon the addition of cytosolic extracts from undifferentiated ESCs to the reaction mixtures, recombinant Oct3/4 was imported into the nucleus of digitonin-treated HeLa cells (Figure 2B). The immunodepletion of importin α2 abrogated the nuclear import of Oct3/4, and the addition of exogenous importin α2 restored this import, suggesting that importin α2 is the predominant transport factor mediating Oct3/4 nuclear import in undifferentiated ESCs. This conclusion is consistent with a report demonstrating that the overexpression of a mutant importin α2 lacking the ability to interact with importin β1 reduces the nuclear localization of Oct3/4 in ESCs (Young et al., 2011).

In contrast, recombinant Oct6 was excluded from the nucleus in the presence of cytosolic extracts from undifferentiated ESCs. However, the immunodepletion of importin α2 mediated the nuclear import of Oct6, which was inhibited by the addition of importin α2 (Figure 2B). Oct6 import in importin α2-immunodepleted extracts was blocked by the addition of antibodies against importins α1, α3, α4, and α6 (Figure 2D), suggesting that some of these importins mediate the import of Oct6 in the absence of importin α2 in ESC cytosolic extracts. Digitonin-treated cells were also incubated with cytosolic extracts in the absence of recombinant proteins to examine the localization of endogenous Oct6 (Figure 2C), which was similar to the localization of the recombinant Oct6 protein. These results indicate that importin α2 retains Oct6 in the cytoplasm by negatively regulating Oct6 nuclear import in undifferentiated ESCs.

## Together with Importin β1, Importin α2 Forms a Transport-Incompetent Ternary Complex with Oct6 and Brn2 In Vitro

Nuclear import is a multistep process. First, the NLS is recognized by importin α and subsequently forms a trimeric complex with importin β1. Second, importin β1 interacts with nucleoporins to initiate nuclear pore translocation. Ran-GTP binding dissociates importin α from importin β1, and the NLSs bound to the major or/and minor NLS binding site of an importin α protein are released by Nup50, which binds along the NLS binding groove of the importin α protein (Matsuura and Stewart, 2005). We examined whether importin β1 forms a trimeric complex with importin α and the POU protein cargo (Figure 1B). All of the cargo proteins examined formed a trimeric complex, although the binding efficiency varied. The addition of a constitutively active Ran mutant, Ran(Q69L)-GTP (Bischoff et al., 1994; Klebe et al., 1995), induced importin β1 release.

We next performed a competition assay using Nup50 to determine whether the POU proteins were released from importin α2. As shown in Figure 1H, Oct3/4 and Oct6 were both dissociated from importin α2 by the addition of Nup50. These results indicate that the importin α2-mediated inhibition of Oct6 and Brn2 nuclear import is not associated with impaired trimer formation or dissociation.

### POU Proteins Bind to Importin α Subtypes via Their NLSs

We next examined the binding of POU proteins to importin α using an in vitro pull-down assay. A monopartite-type NLS is conserved among POU transcription factors, including Oct3/4, Oct6, and Brn2 (Yasuhara et al., 2007; Sock et al., 1996). We generated a series of mutant NLSs by substituting alanine for lysine or arginine, which abrogated protein binding by importins α1 and α2 (Figure 1I). Whereas the NLS-mutated Oct6 retained a weak interaction with importin α1, all three NLS mutant POU proteins completely lost the ability to interact with importin α2 (Figure 1J). Thus, these POU proteins bind to importin α2 via their NLSs, although Oct6 might bind importin α1 at multiple binding sites.

Next, we generated a mutant Oct6 protein, the NLS of which was substituted with that of Oct3/4, Oct6-NLS(Oct3/4, RKRKR), to assess whether the NLS sequence affects nuclear import. As a result, Oct6-NLS(Oct3/4) was imported into the nucleus by importin α2 (Figure 1K), indicating that importin α2 can distinguish the difference between RKRKKR and RKRKR. Thus, we concluded that the NLS sequence is the key determinant of selective inhibition by importin α2.

### POU Proteins Bind to Different Sites of Importin α Subtypes

Typical NLSs bind the major and minor NLS binding sites within the ARM repeats of importin α proteins, and it is possible that cargo molecules could compete for NLS binding to importin α. An NLS peptide derived from SV40 TAg binds the major NLS binding site of importin α. Therefore, to determine how importin α subtypes bind to POU proteins, we examined whether the POU proteins competed for the major NLS binding site of importin α using the SV40 TAg NLS peptide in vitro. The binding of a control SV40 TAg NLS-GST-fusion protein and Oct3/4 to all of the importin α proteins was strongly inhibited by the SV40 TAg peptide (Figure S2A), indicating that these proteins bind to the major NLS binding site of importin α. In contrast, the binding of importin α2 to Oct6 and Brn2 was not affected by the addition of the SV40 TAg NLS peptide, whereas the binding of importin α1 was clearly abrogated (Figure S2A). These results indicate that importin α2 binds Oct6 and Brn2 at a site independent of the major NLS binding site.

The replacement of two amino acids in importin α2 (importin α2 ED mutant) blocks its recognition of classical NLSs (Gruss et al., 2001) but does not disrupt the interaction of importin α2 with importin β1 (Figure S1C). Therefore, we tested the ability of this mutant to bind the POU proteins. As shown in Figure S1A, Oct3/4 and the control SV40 TAg NLS did not efficiently bind the importin α2 ED mutant, but the interaction of Oct6 and Brn2 was maintained. Under this condition, a trimeric complex of importin β1/importin α2 ED mutant/Oct6 was formed (Figure S1B). Additionally, Oct6-NLS(Oct3/4) did not bind the importin α2 ED mutant (Figure S1B), suggesting that the NLS sequence of Oct6 is critical for the binding to importin α2. Thus, Oct6 and Brn2 bind importin α2 via site(s) other than the major and minor NLS binding sites, and these data suggest that importin α2 contains an additional NLS binding site specific for the NLSs of Oct6 and Brn2. Moreover, the importin α2 ED mutant inhibited the nuclear import of Oct6 similar to the inhibition observed with wild-type importin α2 (Figure 1K), further indicating the existence of another Oct6 binding site other than the canonical NLS binding site in importin α2, which mediates its dominant negative activity.

### The C-Terminal Region of Importin α2 Inhibits the Nuclear Import of Oct6 and Brn2

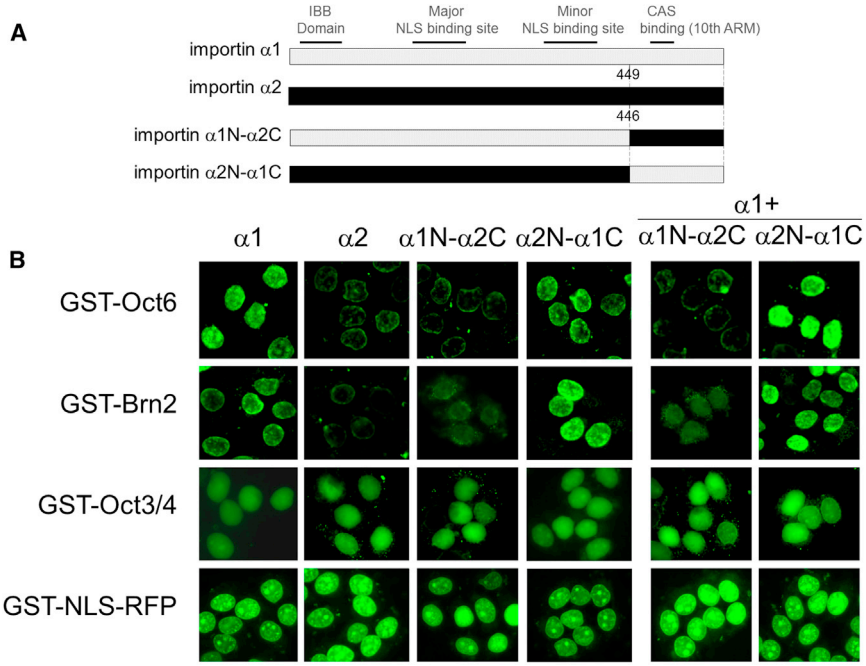
The interaction of importin α with importin β1 is mediated through the N terminus of importin α protein, and the conventional NLS binding sites in the ARM repeats are highly conserved. Accordingly, Nup50, which binds to this region of importin α, competed with Oct6 (Figure 1H); therefore, we speculated that Oct6 and Brn2 binding might be mediated by the C terminus of importin α2. To test this hypothesis, we produced two chimeric importin α proteins by swapping the C-terminal regions, including the CAS-binding site (Matsuura and Stewart, 2005), between importins α1 and α2 to generate the constructs importin α1N-α2C and importin α2N-α1C (Figure 3A).

As shown in Figure S2E, the importin α2N-α1C protein bound Oct3/4, Oct6, and Brn2, and this binding was strongly inhibited by the addition of the SV40 TAg NLS peptide, indicating that the major NLS binding site is responsible for these interactions. Additionally, these data suggest that the major NLS binding site of importin α2 also binds Oct6 and Brn2 when the C-terminal region is lacking. In contrast, although importin α1N-α2C bound the three POU proteins, the addition of the NLS peptide only abrogated the interaction with Oct3/4. The binding of importin α1N-α2C to Oct6 was only slightly inhibited by the SV40 TAg NLS, and the interaction with Brn2 was not affected (Figure S2B). Thus, the major NLS binding site of importin α1N-α2C binds Oct3/4, but Oct6 and Brn2 interact with the C-terminal region of importin α1N-α2C, suggesting that the predominant site of interaction of importin α2 with Oct6 and Brn2 lies within the C-terminal region.

As shown in Figure 3B, both Oct3/4 and the control substrate were imported into the nucleus in the presence of the importin β1/α chimeras. Although importin α2N-α1C mediated the nuclear import of Brn2 and Oct6, nuclear import was minimal in the presence of importin α1N-α2C. The addition of equimolar amounts of importin α1N-α2C to importin α1/β strongly inhibited the nuclear import of Oct6 and Brn2. Thus, the C terminus of importin α2 preferentially interacts with Brn2 and Oct6 independently of the major NLS binding site, which inhibits the nuclear import of these transcription factors.

### Glu458 and Surrounding Amino Acids of Importin α2 Function as an NLS Binding Site

To identify the Oct6 binding site in the C terminus of importin α2, we used the known crystal structures of importin α (Protein Data Bank [PDB] ID: 1Q1T) (Conti and Kuriyan, 2000) and yeast importin (PDB ID:1BK6) (Kutay et al., 1997) to generate a structural model of importin α1. Using these structures, we modeled NLS binding from the known binding characteristics (Figure 3C). The NLS of Oct3/4 is bound by the major NLS binding site of importin α2, and the NLSs of Oct3/4 and Oct6 are bound by the major site of importin α1. Based on the structure of the Nup50/mouse importin α2 complex (PDB ID: 2C1M) (Matsuura and Stewart, 2005) and our chimeric protein binding assays, we predicted a binding site for the Oct6 NLS



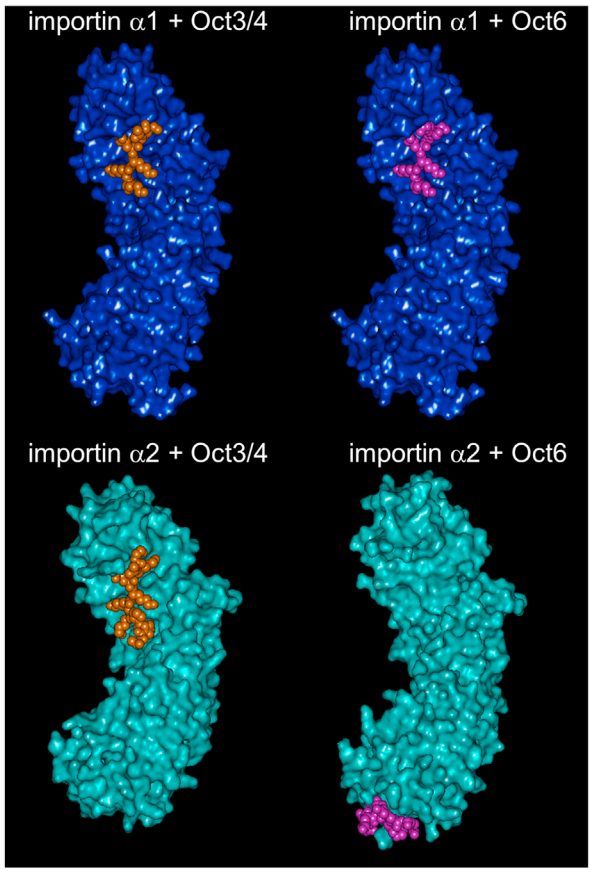




**Figure 3. The C-Terminal Region of Importin α Proteins Determines the Binding and Import of Partner Proteins**

(A) Schematic representations of the chimeric importin α proteins.

(B) The indicated importin α proteins were mixed with POU proteins and added to digitonin-treated HeLa cells. The molar ratio of the importin α proteins to importin α1 was 2:1 for all samples. Importin β1, Ran, NTF2, and an ATP regeneration system was added to all samples. After incubation, the cells were stained with an anti-GST antibody.

(C) Model structures of the importin α and cNLS complexes. The binding of the following protein pairs is shown: the cNLS of Oct3/4 (orange) and the major site of importin α2 (cyan) (lower left), the cNLS of Oct6 (magenta) and the C terminus of importin α2 (lower right), the cNLS of Oct3/4 and the major site of importin α1 (blue) (upper left), and the cNLS of Oct6 and the major site of importin α1 (upper right).

See also Figure S2.

distinct from the major NLS binding site in importin α2 (Figure 3C).

A more detailed analysis of the structural model of the importin α2/Oct6 NLS complex revealed that importin α2-Glu458 forms a salt bridge with the sixth basic amino acid in the Oct6 NLS (Figures 4A and 4C). The corresponding residue in importin α1, an asparagine (Figures S3B, S3D, and S3A), cannot interact with the Oct6 NLS in the same manner due to weakened electrostatic interactions.

To determine whether importin α2-Glu458 is biologically critical, we mutated Glu458 in importin α2 to asparagine (E458N) (Figures 4B and 4D). We confirmed that this mutation did not impair the binding of Nup50 or the NLS release by Nup50 (Figure S2D). Unlike wild-type importin α2, importin α2-E458N mediated the nuclear import of Oct3/4, Oct6, and Brn2 to levels comparable to those by importin α1 (Figure 4E). Conversely, mutating importin α1-Asn466 to glutamic acid (N466E), which mimics the interactions of importin α2-Glu458, restored the inhibition of nuclear import of Oct6 but not Oct3/4 (Figure 4E). Competitive binding assays using the SV40 TAg NLS peptide revealed that although the major NLS binding site of importin α1-N466E bound Oct3/4, interactions with Oct6 and Brn2 occurred at the C terminus (Figure S3B). Importin α2-E458N bound all of the cargo proteins via the major NLS binding site (Figure S3B). Thus, we defined critical residues in the C-terminal region of importin α subtypes that confer specific transcription factor binding and transport activity.

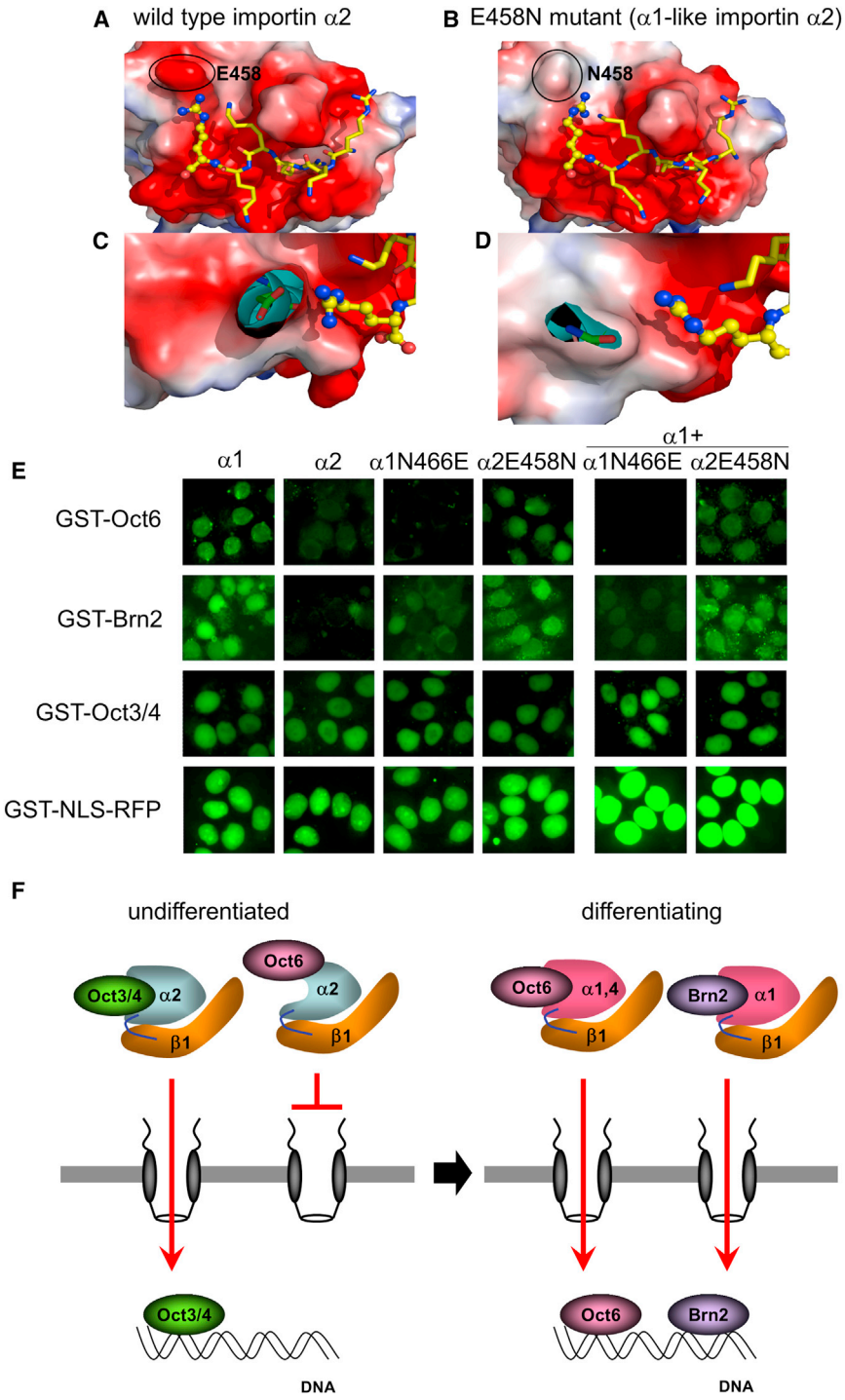



**Figure 4. A Single Amino Acid in Importin α Proteins Controls the Binding and Import of Partner Proteins**

(A and B) C-terminal electrostatic interactions. The charge-smoothed surface potential of the molecular surfaces of importin α proteins is shown. The dark-red color indicates a negative charge. The cNLS of Oct6 is represented, with the atom types shown in different colors (carbon, yellow; oxygen, red; and nitrogen, blue); the sixth basic amino acid (arginine) is shown as a ball-and-stick representation.

(C and D) A close-up view of the interactions between the mutated site (Glu458) of importin α2 and the sixth basic amino acid of the Oct6 cNLS. Residue 458 is shown in a cutaway view and is presented as sticks.

(E) Combinations of importin α proteins were mixed with POU proteins and NLS-bearing cargo proteins as indicated and subsequently added to digitonin-treated HeLa cells. The molar ratio of the added importin α proteins to importin α5 was 2:1 for all samples. Importin β1, Ran, NTF2, and an ATP regeneration system were added to all samples. After incubation, the cells were stained with an anti-GST antibody.

(F) A model for cell fate determination driven by the control of importin α expression. See the body of the text for details.

See also Figure S3.

protein reduced the binding of GST-SV40 TAg NLS to importin α2 and hardly coprecipitated with GST-SV40 TAg NLS (Figure S3C). Although the small SV40 TAg NLS peptide bound to the importin α2/Oct6 complex (Figure S2A), these results indicate that Oct6 does not inhibit the transport of other NLS proteins mediated by importin α2 through tetramer cargo complex formation but rather competes importin α2 with other cargo proteins, which bind to the major NLS site of importin α2 (see Discussion).

## Importin α2 Bound to Oct6 via the C Terminus Does Not Simultaneously Bind Other NLS Proteins to Its Major NLS Binding Site

As Oct6 binds the NLS binding site in the C-terminal region of importin α2, importin α2 could simultaneously bind Oct6 and another NLS protein, which binds to the canonical NLS binding site. Therefore, we examined whether Oct6 is coprecipitated with GST-SV40 TAg NLS in the presence of importin α2. Oct6

## Constitutive Expression of Importin α2 Induces Cell Death upon Neural Differentiation

To elucidate the in vivo impact of importin α2 on ESC fate, we developed ESC lines in which the expression of exogenous wild-type or mutant importin α was controlled through a tet-off system (Figure S4A) (Masui et al., 2005). Under normal growth conditions, the induction of importin α transgene expression had little effect on ESCs, with the exception of a slight increase in proliferation, as demonstrated by larger colony sizes (Figures S4B and S4C). In contrast, when neural differentiation was induced, the morphological transition to neural lineages was repressed in cells expressing the importin α2 transgene (Figure 5A), which expressed higher levels of Oct3/4 than the control cells (Figure 5C). Whereas

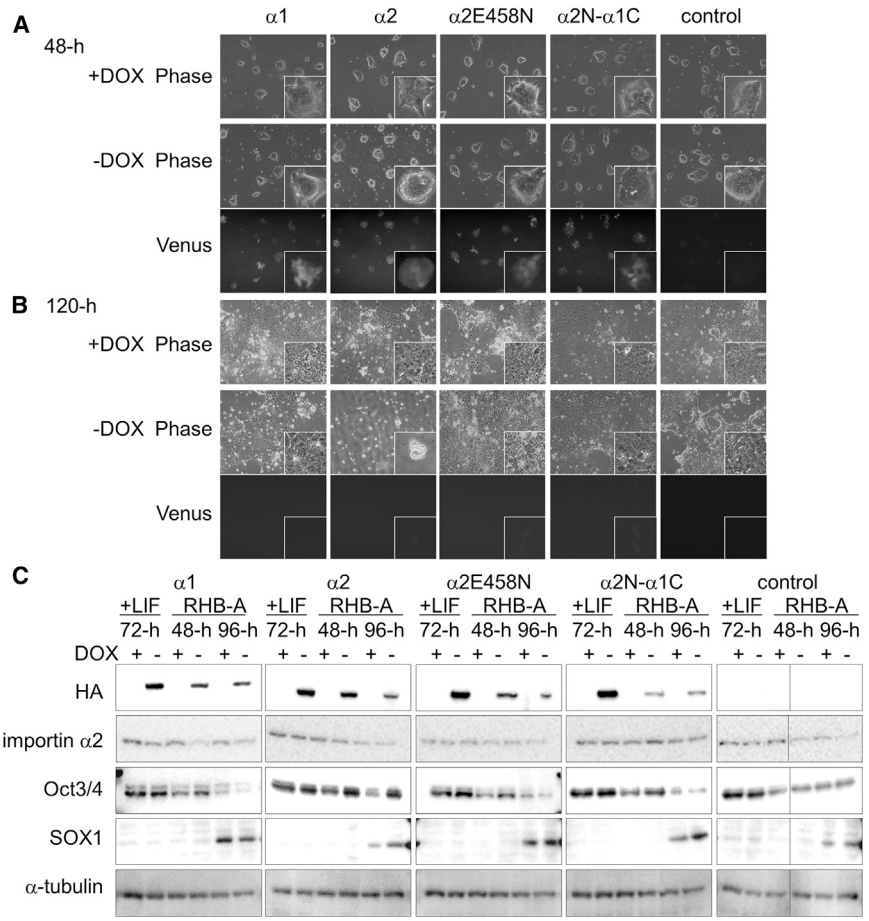



**Figure 5. Importin α2 Inhibits the Progression of Neural Differentiation**

Mouse ESC lines bearing the indicated inducible importin α genes were cultured with or without doxycycline (+ or −DOX). In the absence of DOX, HA-importin α transgenes were expressed, represented by Venus expression from the IRES construct.

(A and B) Neural differentiation was induced using RHB-A media after the induction of importin α transgenes; the morphology was subsequently observed after 48 (A) or 120 hr (B) (see also Figure S4). A typical colony is shown in the inset window in each picture. Venus expression was observed in the cells with the induced transgenes.

(C) HA-importin α, Oct3/4, SOX1, and α-tubulin were also detected. The cells under normal growth conditions (+LIF) or under conditions for neural differentiation (RHB-A), with or without doxycycline (+ or − DOX), were collected at the indicated times after the start of culture in media and analyzed by western blotting. The antibodies for proteins indicated on the left were used.

See also Figure S4.

the expression of the neural marker SOX1 in cells constitutively expressing the importin α2 transgene was upregulated upon differentiation (Figure 5C), most cells died and displayed fragmentation and detachment (Figures 4B, 6B, and 6C). These effects on cell fate were not observed in cells exogenously expressing importin α2-E458N, α2N-α1C, or importin α1 (Figures 5A–5C). However, when importin α2ED was exogenously expressed, a large number of ESCs died during neural differentiation (Figures 6A, 6B, and S5A), and SOX1 upregulation and Oct3/4 downregulation were observed (Figures 6D and S5B). A few cells escaped from cell death and exhibited a flat morphology different from the control cells, which were differentiating into neural cells (Figures 6B, 6C, and S5A). These results indicate that the C-terminal dominant-negative activity of importin α2ED prevented neural differentiation by inhibiting the activity of transcription factors, such as Oct6, thus inducing cell death or differentiation into a cell type other than neural cells. Moreover, the transport activity of importin α2 is necessary to maintain Oct3/4 expression. These results indicate that the function of importin α2 must be inhibited for ESCs to differentiate. Otherwise, ESCs die due to the disorder in the transcriptional regulation of cell fate determination.

## DISCUSSION

The expression of importin α subtypes is regulated during cell differentiation, including the differentiation of ESCs into neural cells (Yasuhara et al., 2007), the differentiation of human promyelocytic leukemia HL60 cells into either macrophages or granulocytes (Köhler et al., 2002; Suzuki et al., 2008), spermatogenesis (Hogarth et al., 2006), and the growth of muscle cells (Hall et al., 2011). Unlike other importin α subtypes, importin α2 is downregulated at the onset of differentiation, which is necessary for cell differentiation to proceed. We previously demonstrated that the forced downregulation of importin α2 leads to unregulated cell differentiation (Yasuhara et al., 2007). In this study, we attempted to identify the mechanism by which importin α2 downregulation affects cell differentiation. We determined that importin α2 negatively regulates the activity of certain transcription factors through cytoplasmic retention and contains a NLS binding site in the C terminus of certain transcription factors.

Three POU transcription factors, Oct3/4, Oct6, and Brn2, are differentially imported into the nucleus by importin α subtypes. In this study, we demonstrated that importin α2 directly interacts with Oct6 and Brn2 in an NLS-dependent manner to regulate the nuclear import of these proteins in a dominant-negative manner. Within the importin α family, the conventional NLS binding sites are located in the central groove, but the inhibitory interaction of Oct6 and Brm2 with importin α2 is mediated through C-terminal residues. In the absence of the importin α2 C terminus, the Oct6 and Brn2 NLSs are bound by the conventional importin α2 NLS binding site. A structural comparison of several importin α/transcription factor NLS complexes by homology modeling revealed a NLS binding site in the C terminus of importin α2 that distinguishes the Oct6-type NLS from the Oct3/4-type NLS. Surprisingly, we observed that the mutation of a single, critical amino acid (importin α2-E458) could switch the features of the importin α family proteins, suggesting that the small molecule

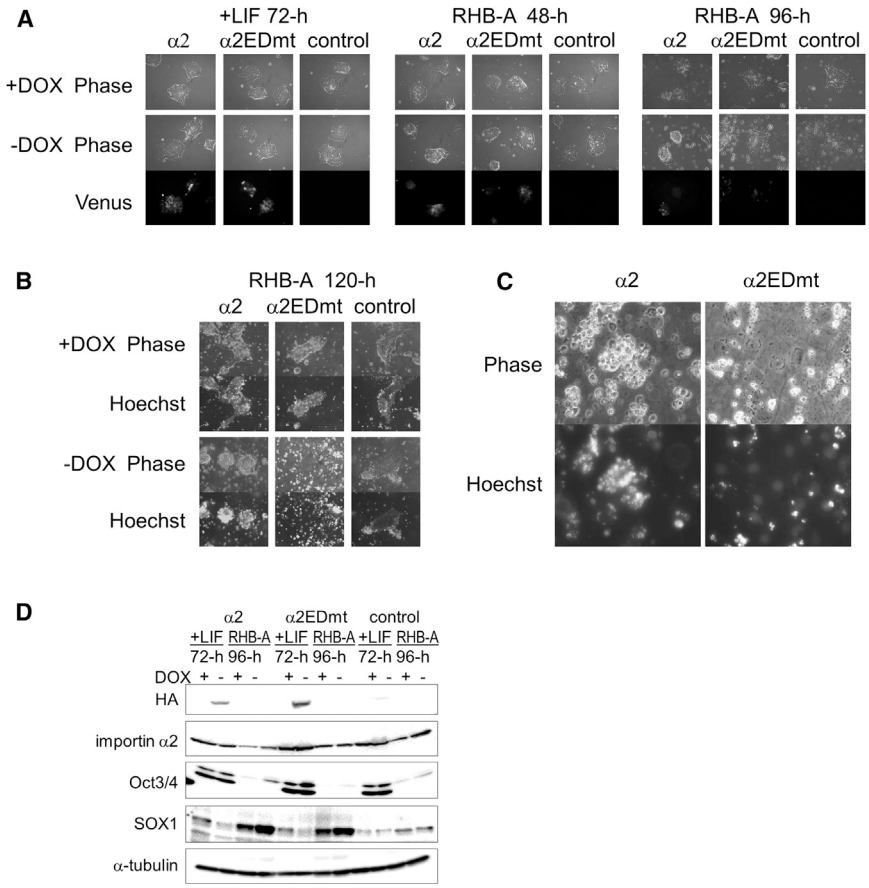



**Figure 6. The C-Terminal NLS Binding Site of Importin α Protein Inhibits Neural Differentiation**

Mouse ESC lines bearing the indicated inducible importin α genes were cultured with or without doxycycline (+DOX or −DOX). In the absence of DOX (−DOX), *HA-importin α* transgenes were expressed, represented by Venus expression from the IRES construct.

(A) Neural differentiation was induced using RHB-A media after the induction of *importin α* transgenes; the morphology was observed after 48 or 96 hr.

(B) Cells under the same conditions (A) were stained with Hoechst to observe the nuclear shape after 120 hr.

(C) Enlarged picture of the cells in (B). HA-importin α, importin α2, Oct3/4, SOX1, and α-tubulin were also detected. The cells under normal growth conditions (+LIF) or under conditions for neural differentiation (RHB-A), with or without doxycycline (+ or − DOX), were collected at the indicated times after the start of culture in media and analyzed by western blotting. The antibodies for proteins indicated on the left were used.

(D) HA-importin α, Oct3/4, SOX1, and α-tubulin were also detected. The cells under normal growth conditions (+LIF) or under conditions for neural differentiation (RHB-A), with or without doxycycline (+ or − DOX), were collected at the indicated times after the start of culture in media and analyzed by western blotting. The antibodies for proteins indicated on the left were used.

See also Figure S5.

disruption of NLS binding could be a possible target for drug discovery in regenerative medicine.

Nuclear import is a complex multistep process: the NLS is recognized by importin α, followed by the formation of a trimeric complex with importin β1 to initiate nuclear pore translocation. Subsequently, Ran-GTP binding induces the dissociation of importin α from importin β1, and the cargo is released from importin α. Using in vitro protein binding assays, we showed that the trimeric complex formation and Ran-GTP-mediated importin β1 dissociation of the POU proteins are comparable with cargo binding to the conventional NLS binding site of importin α1. However, single molecule imaging indicated that the Oct6/importin α2/β1 ternary complex was not targeted to the nuclear pore, leading to its cytoplasmic retention. Furthermore, when not imported, Oct6 was aggregated, suggesting the possibility of noncanonical import complex formation. These results suggest that the binding of the cargo to the C-terminal Oct6-NLS binding site of importin α2 might induce structural changes in importin β1 that suppress its interaction with nucleoporins.

Cargo release is inhibited under conditions of strong cargo-transport factor binding, leading to the nuclear accumulation of cargo-transport factor complexes (Engelsma et al., 2004; Kosugi et al., 2008). The C termini of importin α proteins interact with Nup50 and CAS to mediate cargo release and recycling; thus, it is possible that Oct6 or Brn2 binding at these sites impairs cargo release. However, the in vitro protein binding assays revealed that Nup50 dissociates Oct6 from importin α2. However, it was previously reported that when NLS-containing proteins were injected into the cytoplasm of tsBN2 cells expressing a temperature-sensitive mutant of RCC1 (a guanine-nucleotide exchange factor for Ran), the injected substrates were diffusely distributed throughout the cytoplasm but not in the nucleus (Tachibana et al., 1994). The loss of RCC1 should lead to reduced nuclear RanGTP levels and impaired importin α dissociation. Therefore, the diffuse cytoplasmic distribution of endogenous Oct6 in ESCs (Yasuhara et al., 2007) does not exclude the possibility of impaired substrate release from importin α2. Further studies might reveal the detailed mechanisms by which importin α2 C-terminal binding retains cargo proteins within the cytoplasm.

Oct6 induces neural differentiation but is expressed in undifferentiated ESCs (Suzuki et al., 1990). In addition, Oct6 is retained in the cytoplasm of undifferentiated cells and shuttles between the nucleus and the cytoplasm as differentiation proceeds (Yasuhara et al., 2007), suggesting that the intracellular distribution of Oct6 regulates its activity (Baranek et al., 2005). Our data provide a model by which the critical amino acid residue importin α2-E458 sequesters certain transcription factors, such as Oct6, in the cytoplasm of undifferentiated ESCs (Figure 3F). We hypothesize that under conditions of high importin α2 expression in undifferentiated ESCs, Oct6 binds the C-terminal NLS binding site of importin α2, leading to its retention in the cytoplasm via the formation of a transport-incompetent trimer with importin β1. Under these conditions, the importin

α2 molecule bound to Oct6 via the C terminus does not simultaneously bind other NLS proteins at the NLS binding site. Thus, the direct binding of Oct6 to the trimeric complex does not inhibit the nuclear import of other cargo protein with importin α2. Instead, Oct6 competes for the importin α2 protein with other cargo, which bind to the major NLS site of importin α2. However, the increased expression of importin α2 abolishes the competition in undifferentiated ESCs; thus, the Oct6 expression in undifferentiated ESCs has little effect on cell survival or the maintenance of undifferentiated ESCs. As cell differentiation begins, importin α2 is downregulated, leading to the release of Oct6. Oct6 subsequently binds other importin α family members and enters the nucleus to activate transcription, thereby inducing neural differentiation. In contrast, the nuclear localization of overexpressed Oct6 in undifferentiated ESCs did not induce differentiation but did lead to cell death (data not shown). Although it is unclear why ESCs died after Oct6 overexpression, one hypothesis is that when Oct6 escapes importin α2 inhibition, it enters the nucleus to activate genes at an inappropriate time, leading to cell death. Similar to Oct6, the nuclear import of Brn2 is negatively regulated by importin α2. Thus, importin α2 maintains stem cells in an undifferentiated state by blocking the nuclear import of transcription factors that do not contribute to pluripotency.

Importin α2 also regulates the expression levels of Oct3/4. The constitutive expression of importin α2 inhibited ESC differentiation and led to cell death. Importin α2 inhibited morphological changes and the downregulation of Oct3/4 upon neural differentiation but had no effect on the upregulation of SOX1. Although it remains unclear how importin α2 maintains Oct3/4 expression, one possibility is that the sustained import of Oct3/4 might maintain expression levels through positive feedback on Oct3/4 regulation. As Oct3/4 is transported into the nucleus by importins α1 and α2, the positive feedback on Oct3/4 might also work in cells expressing importin α1 and α2 mutants lacking the C-terminal Oct6-NLS binding site, in which Oct3/4 expression was temporally maintained after the induction of neural differentiation. However, as these importin α proteins do not negatively influence the transport of transcription factors, such as Oct6 and Brn2, other machineries that downregulate Oct3/4 expression, such as epigenomic regulation or posttranscriptional regulation by microRNA, are potentially activated, and the expression of Oct3/4 is not maintained as is in importin α2-expressing cells. Alternatively, the import of other functional proteins that regulate Oct3/4 levels might be regulated by importin α2. However, the constitutive expression of the importin α2 ED mutant lacking the transport activity did not affect the morphological changes nor the downregulation of Oct3/4, but cell death and abnormal differentiation were induced. Taken together, these results show that the dual activity of importin α2 on the nuclear transport of functional proteins maintains the undifferentiated state of ESCs.

Nuclear transport is affected by both the affinity of the cargo for importin α and the concentration of importin α (Riddick and Macara, 2005; Hodel et al., 2006; reviewed in Lange et al., 2007). These findings suggest that the relative expression of each importin α protein regulates the distribution and function of certain transcription factors. Taken together, it is likely that the activities of a specific subset of transcription factors are simultaneously regulated through the expression of importin α subtypes. It is also tempting to speculate that multiple functional proteins, such as transcription factors preexisting in the cytoplasm, can quickly and efficiently enter the nucleus to respond to extracellular and/or intracellular signals without protein synthesis. Thus, we propose a regulatory mechanism in which importin α subtypes control cell differentiation by regulating the intracellular distribution of functional proteins. Further studies are required to identify examples other than Oct6, whose subcellular localization is regulated by importin α subtypes during cell differentiation.

The regulatory mechanisms that we identified have important evolutionary implications. Homologs of importin α1 are present in multiple eukaryotes, suggesting that importin α1 might be the ancestral importin α (Goldfarb et al., 2004; Mason et al., 2009). In contrast, importins α2 and α4 have been identified only in metazoans. As the ancestral form, the function and mechanism of importin α1 are likely essential and straightforward. However, as multicellular organisms become more complex, factors that provide greater spatiotemporal control of nuclear transport are likely beneficial. Using the evolutionary trace method (Innis et al., 2000), we demonstrated that importin α2-Glu458 and the corresponding importin α1-Asn466 are conserved among species, but there is little conservation of importin α4 (Figure S4A). The corresponding residue in importin α3 is serine, which, unlike glutamic acid, has no negative charge, suggesting that importin α3 might behave similarly to importin α1. This redundancy might explain the lack of a phenotype for importin α2-deficient mice (Shmidt et al., 2007).

Taken together, these results suggest that a specific subset of transcription factors is spatiotemporally regulated via the differential expression of importin α subtypes to determine cell fate depending on a critical amino acid located in the C-terminal region of importin α. These results provide insights into the interplay between transcription and protein trafficking.

## EXPERIMENTAL PROCEDURES

### In Vitro Transport Assay

In vitro nuclear transport assays were performed as previously described (Yokoya et al., 1999; Adam et al., 1990), except that the incubation time was extended to 1 hr. Cargo proteins (0.05 μg/μl) and importin α proteins (0.035, 0.0175, or 0.07 μg/μl) were used. The cytoplasmic fraction from ESCs was prepared by incubating the cells on ice for 15 min in an equal volume of hypotonic buffer (5 mM HEPES, pH 7.3, 10 mM potassium acetate, 2 mM magnesium acetate, 2 mM DTT, 1 mM PMSF, and 1 μg/ml each aprotinin, pepstatin, and leupeptin) prior to homogenization. The extract was centrifuged at 1,500 × *g* for 15 min. The supernatants were used as the cytoplasmic fraction after the addition of 20-fold concentrated transport buffer, and the pellet was used as the nuclear fraction.

### Cell Culture

The ESC line E14Tg2a was maintained in Knockout DMEM (Invitrogen) supplemented with 14% KSR (Invitrogen), 1% FCS, ESGRO (Millipore), nonessential amino acids (Invitrogen), 2 mM L-glutamine (Nakarai), and 1 mM 2-mercaptoethanol (Invitrogen). The cells were cultured in 2i media, 3 μM Chiron (Wako), and 1 μM PD03F (Wako) in N2B27 (Stem Cells) for 4 days after thawing from cryopreservation.

Stable ESC lines harboring the HA-importin α transgene were generated from EBRTcH3 cells using the ROSA-TET system according to a previously described method (Masui et al., 2005). For the maintenance of stable ESC lines, the cells were cultured in DMEM (Sigma) supplemented with

10% FBS, ESGRO, nonessential amino acids, pyruvate, and 1 mM 2-mercaptoethanol supplemented with 1 μg/ml doxycycline (BD Bioscience) and 1 μg/ml puromycin on gelatin-coated culture dishes. For neural differentiation, the cells were first cultured under normal conditions with or without doxycycline at a density of $4 \times 10^3/cm^2$. After 24 hr in culture, the cells were washed five times with RHB-A (Stem Cells) and cultured in RHB-A with media changes every other day.

### Fluorescence Imaging

Single-molecule imaging was performed using inclined illumination (30) based on objective-type total internal reflection fluorescence microscopy. An inverted fluorescence microscope (Eclipse Ti-E, Nikon) and other optics were set on a vibration-free table. The cells were placed on the sample holder and maintained at 37°C using a heating stage (MIU-IBC-IF-2, Tokai Hit). A 488-nm blue-laser (Sapphire 488 LP, 20 mW, Coherent) for EGFP excitation was expanded and focused on the back focal plane of the objective lens (60×, N.A. 1.49) through the back port of the microscope using a dichroic mirror (FF495-Di02-25x36, Semrock). The incident light was shifted through the edge of the objective lens using a mirror to form the inclined illumination. EGFP fluorescence was passed through the emission filter (FF01-520/35-25, Semrock) and recorded using an EMCCD Camera (iXon+ DU-897BV, Andor Technology) with an exposure time of 0.1 s. The number of fluorescent spots on the nuclear membrane was counted using ImageJ software.

### Homology Modeling

Homology modeling of the four importin α/transcription factor complexes was performed according to the following protocol: (1) model-building using template structures, (2) side-chain optimization, and (3) energy minimization. Model building for complexes of importin α1 and Oct6 NLS, importin α2 and Oct3/4 NLS, and importin α5 and Oct3/4 NLS was performed using the crystal structures of the complex of importin α2 from *Mus musculus* and SV40 T Antigen NLS (PDB ID: 1Q1T) (Fontes et al., 2003) and importin α from *Saccharomyces cerevisiae* and SV40 T Antigen NLS (PDB ID: 1BK6) (Conti et al., 1998) as templates for homology modeling. The model of the complex between importin α2 and Oct6 NLS was built using the crystal structure of the complex between importin α2 from *Mus musculus* and Nup50 (PDB ID: 2C1M) (Matsuura and Stewart, 2005) as a template. Modeler 9v1 (Šali, 1995) was used for all modeling. The conformations of side chains were optimized using the side-chain prediction program SCWRL3.0 (Canutescu et al., 2003). Energy minimization was performed on the four models using CHARMM (Brooks et al., 1983). The figures were prepared using PyMOL (http://www.pymol.org) and Discovery Studio (Accelrys).

### Immunoprecipitation

The cells ($2 \times 10^8$) were lysed in 1.6 ml hypotonic buffer, and the lysates were incubated on ice for 15 min. NP40 (1%) was added, and the suspension was vortexed for 10 s followed by centrifugation at 15,000 rpm for 30 s. The supernatant was used with the addition of 20× concentrated transport buffer and 1% glycerol. Four micrograms of each antibody was added to 400 μl of the fractionated lysate, and the reactions were incubated at room temperature. Protein G Sepharose (Amersham Bioscience) was used to pull down the antibodies. The antibodies used are described in detail in the Supplemental Experimental Procedures.

### Immunostaining

For immunofluorescence microscopy, the cells were fixed in 3.7% formaldehyde for 10 min at room temperature, permeabilized with 0.5% Triton X-100 for 5 min at room temperature and incubated with primary antibodies, followed by incubation with secondary antibodies according to the manufacturers' protocol. The antibodies used are described in detail in the Supplemental Experimental Procedures. The images were captured using a Cool SNAP CCD camera (Roper Scientific) and Openlab software (Improvision).

### Plasmid Construction

Wild-type importins α2 and α1 were cloned as previously described (Imamoto et al., 1995). Importins α2N-α1C and α1N-α2C were cloned as previously described (Nardozzi et al., 2010). The importin α1 ED mutant was obtained as previously described (Gruss et al., 2001). Both the N466E and E458N mutants were generated through site-directed mutagenesis using conventional methods. N466E was subcloned into the pGEX4T-3 vector, and E458N was subcloned into the pGEX6p-2 vector for recombinant protein purification. NLS mutant POU genes were created as previously described (Yasuhara et al., 2007), and NLS-swapped Oct6 was generated through site-directed mutagenesis using conventional methods.

### Protein Purification

Recombinant importins α1, α2, α2N-α1C, α1N-α2C, GST-NLS-GFP, GST-NLS-RFP, Ran, Ran(Q69L), and NTF2 were expressed and purified as previously described (Imamoto et al., 1995). GST-POU proteins were expressed in BL21 cells following the induction of plasmid expression with 0.1 μM IPTG. The cells were lysed in 100 μg/ml lysozyme and 0.5% NP-40 and frozen at −180°C. After thawing, the lysates were sonicated, and GST-containing proteins were precipitated with glutathione Sepharose 4B (GE Healthcare) according to the manufacturer's protocol. The proteins were stored in transport buffer (20 mM HEPES, pH 7.3, 110 mM potassium acetate, 2 mM magnesium acetate, 5 mM sodium acetate, 0.5 mM EGTA, 2 mM DTT, and 1 μg/ml each aprotinin, pepstatin, and leupeptin).

### Protein Binding Assay

Recombinant proteins (2 μg importin α subtype, 2 μg GST-POU, 1 μg GST and 1 μg NLS peptide, 2 μg importin b1, 30 μg Nup50, 10 μg Oct6, and 2 μg GST-NLS-GFP) were mixed with 20 μl glutathione Sepharose 4B in 100 μl transport buffer and rotated at 4°C for 30 min. The beads were subsequently washed four times with transport buffer, and after the addition of sample buffer, the samples were analyzed by immunoblotting.

### Antibodies

See the Supplemental Experimental Procedures.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2013.06.022.

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