Retinoblastoma-binding Protein 4-regulated Classical Nuclear Transport Is Involved in Cellular Senescence*

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Nucleocytoplasmic trafficking is a fundamental cellular process in eukaryotic cells. Here, we demonstrated that retinoblastoma-binding protein 4 (RBBP4) functions as a novel regulatory factor to increase the efficiency of importin α/β-mediated nuclear import. RBBP4 accelerates the release of importin β1 from importin α via competitive binding to the importin β-binding domain of importin α in the presence of RanGTP. Therefore, it facilitates importin α/β-mediated nuclear import.

We showed that the importin α/β pathway is down-regulated in replicative senescent cells, concomitant with a decrease in RBBP4 level. Knockdown of RBBP4 caused both suppression of nuclear transport and induction of cellular senescence. This is the first report to identify a factor that competes with importin β1 to bind to importin α, and it demonstrates that the loss of this factor can trigger cellular senescence.

Nucleocytoplasmic trafficking, which involves transport of cargo through the nuclear pore complexes embedded in the nuclear membrane, is a fundamental cellular process in eukaryotic cells (1). Importin protein family members are known to play essential roles in mediating nuclear import. Importin α functions as an adaptor molecule to connect a classical nuclear localization signal (cNLS)-bearing cargo protein with importin β1, a carrier molecule, in the cytoplasm. The trimeric transport complex of cNLS-cargo/importin α/β1 is then translocated into the nucleus through the nuclear pore complex (2–4). In the nucleus, the binding of the GTP-bound form of Ran (RanGTP) to importin β1 triggers the dissociation of the complex (5). Then, importin α is ferried to the cytoplasm in an export complex containing cellular apoptosis susceptibility gene product (CAS, also known as CSE1L) and RanGTP. The importin β1-RanGTP complex also returns to the cytoplasm, and the importins are reused for the next round of transport.

Importin α is composed of an importin β binding (IBB) domain in the flexible N-terminal region, a core domain composed of 10 tandem armadillo repeats, which contain the major NLS-binding site at ARM2–4 and the minor NLS-binding site at ARM6–8 (3, 6, 7, and a C-terminal domain, which is the binding region for CAS (8, 9). The IBB domain is known to regulate cNLS binding via an autoinhibitory mechanism (6); however, not much is known about the proteins that interact with the IBB domain, except for importin β1.

Cellular senescence was first described as the irreversible growth arrest of normal primary cells after prolonged proliferation in cell culture (10). Recently, it has been shown that retinoblastoma-binding protein 4 (RBBP4, also referred to as RbAp48) is associated with cellular senescence (11, 12). The level of RBBP4 protein is decreased in aged primary human fibroblast cells, and the knockdown of RBBP4 and RBBP7, a molecule closely related to RBBP4, causes aging-associated chromatin defects (11). Furthermore, reduction of RBBP4 has been observed in the aging human hippocampus and is correlated with memory loss. Conversely, the regulation of RBBP4 ameliorated age-related hippocampus-based memory loss (12). These results suggest that RBBP4 is an aging-associated molecule.

RBBP4 is a WD-repeat protein that functions in the nucleus. It binds directly to the histone H3/H4 complex (13) and serves as the histone binding domain of several different complexes, such as the HDAC complex (14), the NuRD complex (15), the CAF-1 complex (16), and the PRC2 (17), all of which regulate chromatin structure and/or function. As mentioned above, although a reduction in RBBP4 level can induce chromatin defects associated with aging (11), it remains unclear how RBBP4 contributes to cellular senescence.

In this study, we show that RBBP4, which was identified as an importin α-binding protein using mass spectrometry in our comprehensive proteomics study (18), accelerates the release of importin β1 from importin α through its interaction with the IBB domain of importin α. Consequently, RBBP4 maintains the efficiency of importin α/β-mediated nuclear transport. Furthermore, knockdown of RBBP4 impairs nuclear import effi-
RBBP4 Regulates Classical Nuclear Transport

Cell Culture and Transfection—Human diploid fibroblasts (TIG-1 cells) were obtained from the Human Science Research Resource Bank (23) or Japanese Collection of Research Biological Resources Cell Bank. TIG-1 cells, HeLa cells, and HEK-293F cells were cultured at 37 °C in 10% CO₂ in Dulbecco's modified Eagles medium (DMEM) (Sigma) supplemented with 10% fetal bovine serum (FBS). TIG-1 cells were classified as young when PDL was <30 and senescent when PDL was >65. The day before transfection, HeLa cells, HEK-293F cells, and TIG-1 cells were plated on 12-well plates (for immunofluorescence, Thermo Scientific, Waltham, MA) with coverslips (MATSUNAMI, Osaka, Japan) or in 6-cm dishes (for collecting cell extracts, Thermo Scientific) to be 70% confluent at the time of transfection. Plasmids were transfected into HeLa cells and HEK-293F cells using Lipofectamine 2000 or Lipofectamine 3000 (Life Technologies, Inc.) according to the manufacturer's instructions. The siRNA sequences for RBBP4 were 5'-CUUGUAGAUCGCAACAAA-3' and 5'-AAUAAGGAAGUACCAUGA-3' (Ambion, Austin, TX). They were transfected into HeLa cells and TIG-1 cells using Lipofectamine RNAiMAX (Life Technologies, Inc.), according to the manufacturer's instructions.

Experimental Procedures

Importin α Gene Nomenclature—The importin α gene nomenclature is complicated due to inconsistencies in the usage of the terms "importin" and "karyopherin." Furthermore, differences in the number of importin α genes between human and mouse have led to homologs with different names. Therefore, we uniformly used the human nomenclature for the three importin α proteins tested in this study as follows: importin α1 (KPN2A), importin α3 (KPN4A), and importin α5 (KPN1A).

Plasmid Construction—The plasmids pGEX6P-2 mouse importin α1 (KPN2A), pGEX4T-3 mouse importin α3 (KPN4A), pGEX4T-3 mouse importin α5 (KPN1A), pGEX4T-3 HA-mouse importin β1, pGEX2T-IBB domain of mouse importin α1 (KPN2A)-GFP, pGEX2T-SV40T-NLS-GFP, and pGEX6P-1 Ran were obtained as described previously (19–21). The gene encoding human RBBP4 CDNA was cloned into the pENTR vector (Life Technologies, Inc.). Deletion mutants and substitution mutants were constructed using a KOD-plus mutagenesis kit (Toyobo Co.). Almost all of the residues are identical in the human and mouse IBB domain of importin α1. The three exceptions are residues 9, 15, and 45. We mutated Glu-45 in mouse IBB domain to lysine as a substitute for Asp-45 in the human IBB domain. Protein expression and purification were performed as described previously (2, 24). GST was cleaved using PreScission protease (GE Healthcare) in cleavage buffer 1 (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA(3Na), 1 mM dithiothreitol (DTT)) containing 1 μg/ml each of aprotinin, leupeptin, and pepstatin) for pGEX6P vector, and thrombin (Sigma) in cleavage buffer 2 (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2.5 mM CaCl₂) for pGEX2T vector. All of the following recombinant proteins were purified in the same manner: all importin α constructs, importin β1, GST-IBB-GFP (GST-GFP fused the IBB domain of mouse-importin α1, gifted from Y. Ogawa), GST-SV40T-NLS-GFP, GST-IBB-GFP, GST-GFP, and GST. The GDP-bound Ran and the GTP-bound Ran Q69L mutant were purified as described previously (25).

GST Pulldown Assay—Plasmids encoding 3×HA-tagged wild type or mutant RBBP4 were transfected into HEK-293F cells, and whole cell lysates were prepared 48 h after transfection. Lysates were pre-cleared with glutathione-Sepharose 4B beads (GSH beads, GE Healthcare) at 4 °C for 1 h, and each pre-cleared lysate was incubated with the GST fusion proteins (50 pmol) and GSH beads at 4 °C for 1 h. The beads were washed extensively with 0.1% Triton X-100 in PBS containing 1 mM DTT and 1 μg/ml each of aprotinin, leupeptin, and pepstatin; and bound proteins were eluted with sample buffer for SDS-PAGE. Bacterially produced recombinant proteins (50 pmol each) were also incubated with each GST fusion protein (50 pmol) immobilized on GSH beads at 4 °C for 1 h. In the experiment shown in the left panel in Fig. 7B, GST-IBB-GFP and importin β1 were mixed first and pre-incubated for 30 min, and RBBP4 was added later. In contrast, in the right panel in Fig. 7B, the three proteins (GST-IBB-GFP, importin β1, and RBBP4) were mixed and incubated simultaneously. Washing and elution of the bound proteins were performed as described above.

Immunoprecipitation Assay—HeLa cell lysates (10 mg/ml) were immunoprecipitated with protein G-Sepharose 4 Fast Flow beads (GE Healthcare) pre-incubated with each primary
antibody (1 μg/ml) or control IgG (normal mouse IgG; sc-2025; mouse; 1.0 μg/ml, Santa Cruz Biotechnology, Santa Cruz, CA) at 4 °C for 2 h. Then the beads were washed following the same protocol described under "GST Pulldown Assay."

**Western Blotting**—Samples were loaded into a 10% SDS-polyacrylamide gel, and the separated proteins in the gel were transferred onto a PVDF membrane using a semi-dry transfer blotting system (Trans-Blot Turbo Transfer System, Bio-Rad). The transferred membrane was blocked in TBS-T (50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.05% Tween 20) containing 5% skim milk for 30 min and incubated in appropriately diluted (in TBS-T containing 5% skim milk) primary antibodies for 2 h at room temperature or overnight at 4 °C. The following antibodies were used in immunoblotting: importin α1 (karyopherin α/Rch1; 610485; mouse mAb; 0.25 μg/ml, BD Biosciences); RBBP4 (RbAp48; ab488; mouse mAb; 0.5 μg/ml, Abcam); HA (sc118674230001; rat mAb; 0.1 μg/ml, Roche Applied Science); importin β1 (karyopherin β; 610559; mouse mAb; 0.5 μg/ml, BD Biosciences or sc-1919; goat mAb; 0.4 μg/ml, Santa Cruz Biotechnology); p21 (556430; mouse mAb; 1 μg/ml, BD Biosciences); p53 (sc-126; mouse mAb; 0.4 μg/ml, Santa Cruz Biotechnology); p16 (sc-468; rabbit polyclonal antibody; 0.4 μg/ml, Santa Cruz Biotechnology); actin (sc-1615; goat polyclonal antibody; 0.4 μg/ml, Santa Cruz Biotechnology); GST (sc38; mouse mAb; 0.2 μg/ml, Santa Cruz Biotechnology); GFP (M048-3; mouse mAb; 1.0 μg/ml, MBL International, San Diego); and histone H3/H4 modifications (H3K9me3, BD Biosciences or sc-1919; goat mAb; 0.4 μg/ml, Santa Cruz Biotechnology); H3K9me1 and H3K9me2; 610559; mouse mAb; 0.5 μg/ml, BD Biosciences or sc-1919; goat mAb; 0.4 μg/ml, Santa Cruz Biotechnology); p16 (sc-468; rabbit polyclonal antibody; 0.4 μg/ml, Santa Cruz Biotechnology); actin (sc-1615; goat polyclonal antibody; 0.4 μg/ml, Santa Cruz Biotechnology); GST (sc38; mouse mAb; 0.2 μg/ml, Santa Cruz Biotechnology); GFP (M048-3; mouse mAb; 1.0 μg/ml, MBL International, San Diego); and histone H3/H4 modifications (H3K9me3, H3K27me3 and H4K20me3; mouse mAb; 0.2 μg/ml respectively, all three antibodies were provided from H. Kimura (26)). Primary antibodies and secondary antibodies were diluted with PBS containing 5% skim milk or Can Get Signal Immunostain Solution (Toyobo Co.). After incubation for 1 h at room temperature with horseradish peroxidase-coupled secondary antibodies (0.8 μg/ml, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), immunoreactive bands were visualized using a chemiluminescence reagent (Pierce Western blotting Substrate, Thermo Scientific, Rockford, IL).

**Quantitative RT-PCR Analysis**—Total RNA was extracted from different PDL of TIG-1 cells using ReliaPrep RNA Cell Miniprep System (Promega, Madison, WI). Reverse transcription and PCR amplification was performed using One-step SYBR PrimeScript PLUS RT-PCR kit (Takara, Shiga, Japan). The primer sequences for RBBP4 are TGAACAAAAC-CTCTTGTTATAGCC (forward) and TGAACCAAATCCAAATTCT (reverse), and primers for hypoxanthine phosphoribosyltransferase (HPRT) are TGACCTTGATTTATTTTG-CTCCAAATTCT (reverse), and primers for hypoxanthine phosphoribosyltransferase (HPRT) are TGACCTTGATTTATTTTG-CTCCAAATTCT (reverse), and primers for hypoxanthine phosphoribosyltransferase (HPRT) are TGACCTTGATTTATTTTG-CTCCAAATTCT (reverse).

**Senescence-associated β-Galactosidase (SA β-Gal) Staining**—The senescence detection kit (BioVision, Mountain View, CA) was used to detect senescent cells according to the manufacturer’s instructions.

**Immunofluorescence and Microscopy**—Cells were fixed with 3.7% formaldehyde in PBS for 15 min. After two washes in PBS, cells were treated with 0.1% Triton X-100 in PBS for 5 min. After washing with PBS twice, the cells were blocked in PBS containing 5% skim milk for 30 min and incubated in appropriately diluted (in PBS containing 5% skim milk) primary anti-bodies for 2 h at room temperature or overnight at 4 °C. The following primary antibodies were used: RBBP4 (RbAp48; ab488; mouse mAb; 2.5 μg/ml, Abcam, Cambridge, UK), and Ki-67 (ab833-500; rabbit polyclonal antibody; 25 μg/ml, Abcam). After subsequent TBS-T washes, cells were incubated with the Alexa-Fluor-488- or Alexa-Fluor-568/594-conjugated secondary antibodies (Molecular Probes, Eugene, OR). Nuclei were counterstained with DAPI (1:10,000 in PBS, Dojindo Laboratories, Kumamoto, Japan) for 10 min. The samples were examined using a Zeiss Axiohot fluorescent microscope (Carl Zeiss, Göttingen, Germany) and a Nikon Eclipse 80i microscope (Nikon, Tokyo, Japan).

**In Vitro Transport Assay and Time-lapse Analysis**—HeLa cells were plated on 8-well multistest slides (MP Biomedicals, Inc., Santa Ana, CA) for 24 h prior to each assay. After washing with ice-cold transport buffer (TB: 20 mM HEPES/NaOH (pH 7.3), 110 mM potassium acetate, 2 mM magnesium acetate, 5 mM sodium acetate, 0.5 mM EGTA/NaOH (pH 7.3), and 2 mM DTT, containing 1 μg/ml each of aprotinin, leupeptin, and pepstatin), HeLa cells were incubated in ice-cold TB containing 20 μg/ml digitonin for 5 min. Cells were then washed and incubated in fresh ice-cold TB for 10 min. The transport mixture (4 μM cargo/substrate with 0.5 μM importin α, 0.5 μM importin β1, 4 μM GDP-bound Ran, 500 μM GTP, and an ATP regeneration system) was mixed in TB containing 2% BSA. The ATP regeneration system (0.33 μM ATP, 1.5 μM phosphocreatine, and 6 units/ml creatine phosphokinase) was adjusted with TB. To generate cytosol extracts for the transport reactions, 4 μM cargo/substrate was mixed with 4 μl of cytosol from cell extracts (10 mg/ml). The transport reactions were conducted in TB containing 2% BSA. The cytosol extracts were prepared from HeLa cells as described previously (27). After applying the transport mixture, semi-intact cells were incubated at 30 °C for 30 min unless noted otherwise. Cells were then washed with TB and fixed with 3.7% formaldehyde in PBS. For the time-lapse analysis, HeLa cells were cultured on a glass bottom dish (PLL-coat, MATSUNAMI) and transfected with siRNAs 48 h before the analysis. Before the cells were permeabilized with digitonin, Hoechst 33342 (1 μg/ml, Molecular Probes) was added to the dishes, followed by incubation for 15 min at room temperature. The cells were permeabilized with digitonin as described above. The dish was set on an inverted confocal microscope (Leica TCS SP8, Leica Microsystems GmbH, Germany, Wetzlar), and the import reaction was performed at 30 °C. Time-lapse images were initiated at the time of the addition of the transport mixtures containing importin α1 and β1 with GST-SV40TNLS-GFP as a cargo. Signal intensities of 10 different nuclei were quantified in three independent experiments and statistically analyzed using Bonferroni’s post hoc test after a two-way analysis of variance. The fluorescent scans were started in time-lapse mode, and the focal plane was adjusted to the nuclei.
Molecular Probes, as an injection marker) were microinjected into the cytoplasm with a final concentration of 1 mg/ml within 1 min. After incubating for the indicated time at room temperature, the cells were fixed with 3.7% formaldehyde. The nuclear (Fn) and cytoplasmic (Fc) fluorescences were quantified for at least 10 different cells to enable the nuclear to cytoplasmic fluorescence ratio (Fn/c) to be determined. Statistical analysis was performed using the Student’s t test. After the fluorescent examination, SA/H9252-gal staining was performed and examined by an OLYMPUS CKX41 inverted microscope (OLYMPUS, Tokyo, Japan).

Multiple Sequence Alignment of Importin α—Sequences of human and mouse importin α homologs were aligned using ClustalW (28). The UniProt database accession numbers are as follows: human importin α1 (hIMPa1, P52292); mouse importin α1 (mIMPaα1, P52293); hIMPa3 (O00629); mIMPa3 (O35343); hIMPa4 (O00505); mIMPa4 (O35344); hIMPa5 (P52294); mIMPa5 (Q60960); hIMPa6 (O15131); hIMPa7 (O60684); mIMPa7 (O35345); hIMPa8 (A9QM74); and mIMPa8 (C0LLJ0).

Results

Importin α1 Binds to RBBP4 but Does Not Mediate Its Nuclear Transport—By proteomic analysis, we previously identified RBBP4 as a potential importin α1-binding protein (18). In addition, another study also shows interaction between karyopherin α2 (importin α1) and RBBP4 (29). To validate the binding between importin α and RBBP4, we performed a GST pulldown assay against three major importin α subtypes, α1, α3, and α5. Whole cell lysates prepared from HEK-293F cells expressing HA-tagged RBBP4 (HA-RBBP4) or GST-SV40T-NLS-GFP was examined in digitonin-permeabilized HeLa cells, in HeLa cell cytosols with or without Q69L-RanGTP (D), or in a reconstitution system that included importin α1, importin β1, RanGDP, and an ATP-regenerating system (E). After 30 min at 30 °C, GFP-tagged proteins were visualized by fluorescence microscopy.
import of GFP-RBBP4, indicating that RBBP4 is transported into the nucleus by some cytosolic factor(s). The addition of a GTPase-deficient mutant of Ran, Q69LRanGTP, inhibited the migration of RBBP4 to the nucleus, indicating that RBBP4 is imported into the nucleus in a Ran-dependent manner (Fig. 1D). Therefore, we used recombinant proteins to test whether RBBP4 is transported into the nucleus in an importin α1-dependent manner. As shown in Fig. 1E, GFP-RBBP4 did not enter the nucleus in the presence of importin α1, whereas SV40 large T antigen NLS fused with GST and GFP (GST-SV40T-NLS-GFP) was efficiently transported into the nucleus. This is consistent with the fact that the cNLS mapper (30) and PSORTII (31) cNLS prediction algorithms did not detect any potential cNLS in the RBBP4 protein, indicating that there are no obvious cNLSs in the coding sequence of RBBP4. These results revealed that importin α1 interacts with RBBP4 but does not mediate its nuclear import.

RBBP4 Has a Novel Binding Domain for Importin α1—Because our results indicated that RBBP4 is unlikely to have a cNLS, we next investigated how RBBP4 binds to importin α1. We constructed 14 deletion mutants of HA-RBBP4 (Fig. 2A) and conducted a pulldown assay using GST-importin α1. All binding assays were analyzed using the same procedure described in (B).

FIGURE 2. Identification of the importin α1-binding site in RBBP4. A, schematic representation of RBBP4 wild type (WT) and its deletion mutants. A, 1–141 aa; B, 142–283 aa; C, 284–425 aa; AB, 1–283 aa; BC, 142–425 aa; AC, 1–141 and 284–425 aa; ΔN5, 6–425 aa; ΔN10, 11–425 aa; ΔN20, 21–425 aa; ΔN30, 31–425 aa; ΔN5, 1–420 aa; ΔC10, 1–415 aa; ΔC20, 1–405 aa; ΔC30, 1–395 aa. B and C, GST pulldown assays for importin α1 and RBBP4 deletion mutants. HEK-293F cells were transfected with the HA-tagged RBBP4 mutants described in A, and proteins bound to GST-importin α1 were detected using an anti-HA antibody (B: WT, A, B, C, AB, and AC; C: WT, ΔN5, ΔN10, ΔN20, ΔN30, ΔC5, ΔC10, ΔC20, and ΔC30). D, amino acid mutations or deletions in the region of 301–315 aa in RBBP4. E and F, GST pulldown assays for importin α1 and RBBP4 mutants (E, WT and Δ305–310; F, WT, K307/309A, K307/309E, L306/308/310A, and L306/308/310E).
shown in Fig. 2, B and C, most of these deletion mutants failed to interact with importin α1. In addition, even a deletion of 20 aa from either the N or C terminus of RBBP4 caused a significant decrease in the ability to bind importin α1. Thus, we could not identify any obvious importin α1-binding domains in RBBP4 by a conventional region deletion procedure.

However, we noticed an unusual cluster of basic amino acids in RBBP4 (301–310, aa WDLRNLKLKL), which is also conserved in mouse, Xenopus, and Drosophila. We constructed a deletion mutant lacking this region (Δ305–310, Fig. 2D), and we found that this region was critical for the interaction of RBBP4 with importin α1 (Fig. 2E). We investigated the role of the basic amino acids in the deleted sequence (NLKLKL) by substituting them with alanine or glutamic acid (K307A/K309A or K307E/K309E, Fig. 2D). However, both of these mutants were able to bind to importin α1. We next focused on the leucine residues. It has been reported that viral protein R of human immunodeficiency virus type 1 binds to importin α and that the substitution of the leucine residues at positions 20, 22, 23, and 26 of viral protein R disrupts this interaction (32). Therefore, we created RBBP4 mutants L306A/L308A/L310A and L306E/L308E/L310E. As shown in Fig. 2F, both of these leucine mutants show a marked decrease in binding to importin α1. These data indicate that these three leucine residues of RBBP4 (Leu-306/-308/-310) are crucial for its interaction with importin α1.

Taken together, these results suggest that RBBP4 uses a novel mode of binding to importin α1, distinct from that of typical cNLS-containing cargoes.

**IBB Domain of Importin α Binds to RBBP4** —Karyophilic proteins containing cNLS(s) are recognized by importin α through its major and/or minor NLS-binding sites in its central region. To test whether RBBP4 also binds to these NLS-binding sites, we first used the ED mutant of importin α (33), which contains mutations in both the major and minor NLS-binding sites (Fig. 3A). Although SV40T-NLS-GFP failed to bind to the ED mutant, RBBP4 bound to the mutant as efficiently as it did to wild type importin α1 (Fig. 3B).

To further clarify this interaction, we used the bimax peptides (bimax1 and bimax2), which inhibit cNLS-containing cargo binding via high affinity interactions with both NLS-binding sites (22). Although the binding of GST-SV40T-NLS-GFP to importin α1 was inhibited by the bimax peptides, there was no effect on the binding between RBBP4 and importin α1 (Fig. 3C). Collectively, these data indicate that importin α1 does not bind to RBBP4 through the major or the minor NLS-binding site.

To identify the RBBP4-binding site in importin α1, we next focused on the IBB domain and the C-terminal domain. We used the following two deletion mutants: the ΔIBB mutant lacking the N-terminal IBB domain (1–60 aa) and the ΔC mutant lacking the C-terminal region (501–529 aa) (Fig. 4A). In a pull-down assay, the ΔIBB mutant showed much weaker binding to HA-RBBP4 expressed in cell lysates and to recombinant RBBP4, compared with the wild type or the ΔC mutant of importin α1 (Fig. 4, B and C). Next, a recombinant protein containing the IBB domain of importin α1 (1–65 aa) fused to GST and GFP (GST-IBB-GFP) was used for the binding assay. As shown in Fig. 4D, the IBB domain of importin α1 binds to RBBP4. These results indicate that the NLKLKL sequence in RBBP4 forms a novel binding site for the IBB domain of importin α1 and that the leucine residues are crucial for binding.

Because we found that RBBP4, like importin β1, binds to the IBB domain of importin α1, we next examined whether RBBP4 can form a ternary complex with cNLS-cargo protein and importin α1. To test this idea, SV40T-NLS-GFP was used as a cNLS substrate and was incubated with GST-importin α1 in the presence of either RBBP4 or importin β1. As shown in Fig. 4E, we found that the ternary complex, SV40T-NLS-GFP:importin α1-RBBP4 was formed. Furthermore, we found that the amount of SV40T-NLS-GFP bound to importin α1 was significantly increased in the presence of RBBP4, just as it is in the presence of importin β1. This implies that the autoinhibition by the IBB domain of importin α1 to its own NLS-binding site is released by RBBP4 binding, just as it is by importin β1 binding (3, 6, 34).

**Biochemical and Structural Analysis of the RBBP4-IBB Domain Interaction**—Next, we examined whether RBBP4 and importin β1 compete with each other to bind to the IBB domain. Initially, we clarified RBBP4 did not directly interact with importin β1 (Fig. 5A). Then GST-IBB-GFP immobilized on a glutathione-Sepharose 4B slurry (GSH beads) was incubated with increasing amounts of importin β1 or GFP (as a negative control) in the presence of a constant amount of RBBP4 (Fig. 5B). As a result, RBBP4 binding was inhibited by
importin β1 but not by GFP. These results showed that importin α1 competes with RBBP4 for binding to the IBB domain, implying that the two proteins bind to the same region of the IBB domain.

We next modeled the binding mode of the RBBP4-IBB domain complex using an in silico method combined with a point mutation analysis. The structure of the importin β1-IBB domain complex (PDB code 1qgk) shows that 8 of the 10 conserved residues in the IBB domain are involved in importin β1 binding (Fig. 5C). We hypothesized that these residues may also be involved in the binding of RBBP4. To identify other potential key interacting residues, we also performed a helical wheel analysis (Fig. 5D) and selected the following set of residues for site-directed mutagenesis: 1) a conserved hydrophobic residue, Leu-38, and neighboring non-conserved hydrophobic residues, Met-27 and Val-34, which we expect to interact with Leu-306/-308/-310; 2) conserved (Arg-31 and Lys-49) and non-conserved (Lys-42) basic residues and a non-conserved acidic residue, Asp-45 (Glu-45 in mouse importin α1), which are all close to Leu-38 on the helix; and 3) conserved residues in a short N-terminal segment, Arg-13 and Lys-18. The results of pulldown assays with the mutant proteins showed that mutation of acidic residues tends to decrease RBBP4 binding to the IBB domain, whereas mutation to basic residues tends to increase the binding (Fig. 6A). The results suggest that all of the mutated residues are involved in RBBP4/IBB domain binding or are located close to the binding site and that both hydrophobic and negatively charged regions in RBBP4 bind to the IBB domain (Fig. 6B).

To examine whether the N or C terminus of RBBP4 is involved in the IBB domain binding (Fig. 2C), we analyzed the structures of the IBB domain in the importin β1-IBB domain complex (PDB code 1qgk) and RBBP4 in the RBBP4-MTA1 complex (PDB code 4pby). We confirmed that the IBB domain can span between Leu-306/-308/-310 and either terminus (Fig. 2, C and F) and that the regions from Leu-306/-308/-310 to both termini are hydrophobic and negatively charged (Fig. 6B), indicating that either terminus may be involved in IBB domain binding. Based on these observations, we propose two structural models of the RBBP4-IBB domain complex (Fig. 6C).

**RBBP4 Accelerates the Release of Importin β1 from the Transport Complex in the Nucleus via a RanGTP-dependent Mechanism**—Because we found that RBBP4 competitively inhibits the binding of importin β1 to importin α1, we next
examed whether RBBP4 can influence the nuclear transport of a cNLS-containing cargo protein (GST-SV40T-NLS-GFP) by inhibiting the formation of the importin α1-β1 complex, using an in vitro transport assay. As shown in Fig. 7A, the nuclear migration of the cargo protein was inhibited by the addition of RBBP4 in a dose-dependent manner. These results suggest that RBBP4 may affect the regulation of importin α1-β1-dependent nuclear transport. However, because RBBP4 is mainly localized in the nucleus in living cells, it is unlikely that RBBP4 competes with importin β1 for binding to importin α in the cytoplasm.

Therefore, we speculated that the interaction of RBBP4 with the IBB domain of importin α1 would occur inside the nucleus, resulting in the dissociation of importin β1 from the cNLS-cargo-importin α1-β1 trimeric complex. To test this, GST-IBB-GFP immobilized on GSH beads was first incubated with importin β1, and then increasing amounts of RBBP4 were added. Interestingly, we found that RBBP4 could not dissociate importin β1 from the IBB domain-importin β1 pre-formed complex (Fig. 7B, left panel), whereas RBBP4 competes with importin β1 when the proteins were mixed simultaneously (Fig. 7B, right panel).

Another important nuclear factor that promotes dissociation of the IBB domain and importin β1 is RanGTP. Therefore, we examined the cooperative effects of RanGTP on these protein interactions. As shown in Fig. 7C, the release of importin β1 from the IBB domain-importin β1 complex was markedly enhanced by the addition of both RBBP4 and Q69LRanGTP as compared with the single addition of Q69LRanGTP. This result indicated that RBBP4 could stimulate the dissociation of importin β1 from the trimeric nuclear complex of cNLS-cargo-importin α1-β1, in cooperation with RanGTP, implying that RBBP4 functions in the nucleus to facilitate the release of importin α from importin β1.

Knockdown of RBBP4 Impairs Nuclear Transport Efficiency—To understand the physiological function of RBBP4 in the classical nuclear transport machinery, the RBBP4 gene was knocked down in HeLa cells using siRNAs, and the effect of this intervention on the nuclear import of GST-SV40T-NLS-GFP was measured using time-lapse confocal microscopy. Two different siRNAs for RBBP4 (si-RBBP4-1 and si-RBBP4-2) were used to transfect HeLa cells, and after 48 h, the cells were permeabilized with digitonin for an in vitro transport assay. The nuclear uptake efficiency of the cargo protein in both the si-RBBP4-1- and si-RBBP4-2-treated cells was significantly lower than in the untreated cells (Fig. 7, D–F). These results strongly suggest that RBBP4 functions in the nucleus to release importin β1 from the trimeric transport complex in the presence of RanGTP, thereby affecting the nuclear transport efficiency of cNLS-containing cargo proteins.

Knockdown of RBBP4 Induces Cellular Senescence by Impairing the Nuclear Transport—RBBP4 was originally identified as an aging-associated molecule (11, 12). This is consistent with our finding that the protein level or mRNA level of RBBP4 decreased in a passage-dependent manner in TIG-1 cells (Fig. 8A). Interestingly, it has been reported that cellular senescence is associated with down-regulation of active nuclear transport (37, 38) and decreased protein levels of several nuclear transport-related factors (23, 39); however, little is known about how the classical nuclear transport machinery is involved in cellular senescence.

To investigate a potential role for RBBP4 in senescence, we first used cytoplasmic microinjection to compare the nuclear import efficiency of GST-SV40T-NLS-GFP in young cells (PDL under 30) with that of senescent cells (PDL over 65). Senescent cells were identified by the expression of SA β-gal, a type of senescence marker. The results showed that the transport efficiency of cNLS-containing cargo was significantly decreased in senescent cells relative to young cells, even at the early time point (Fig. 8, B and C), suggesting that a decrease in the RBBP4 level may induce cellular senescence through the suppression of importin α-β-dependent nuclear transport.

We next investigated whether the knockdown of RBBP4 induces cellular senescence in normal fibroblast TIG-1 cells. As shown in Fig. 8D, both si-RBBP4-1 and si-RBBP4-2 treatments led to a significant increase in the ratio of SA β-gal-positive cells. In addition, the Ki-67 signal, which is known as a cellular proliferation marker, was down-regulated (Fig. 8E), and the senescent marker proteins p21, p53, and p16 were up-regulated in the si-RBBP4-treated cells (Fig. 8F). Additionally, because RBBP4 is known to be a member of some chromatin-modifying complexes, we examined the effect of RBBP4 knockdown on histone modifications known to change with cellular senescence. However, as shown in Fig. 8G, the alterations of histone modifications triggered by the depletion of RBBP4 were not consistent with those observed during cellular senescence of TIG-1 cells or previous reports using different cell lines (40–45). From these findings, we concluded that RBBP4 is a key factor for the maintenance of the classical nuclear transport efficiency, and its decrease triggers cellular senescence in TIG-1 cells. Taken together, we propose that the down-regulation of classical nuclear transport, in which RBBP4 plays an important role, is closely related to cellular senescence.

Discussion

In this study, we demonstrate that RBBP4 is a novel IBB domain-binding protein that regulates the classical nuclear transport machinery and keeps cells young. Inside the nucleus, RBBP4 works in conjunction with nuclear RanGTP to accelerate the dissociation of importin β1 from importin α by binding...
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A. GST pulldown assays for GST-IBB-GFP with RBBP4. Either WT or mutants of GST-IBB-GFP were incubated with RBBP4 and then detected by Western blotting. GST-IBB-GFP proteins are shown by Coomassie staining (mutants of GST-IBB-GFP were incubated with RBBP4 and then detected by Western Brilliant Blue).

B. Structural models of the complex between RBBP4 and the IBB domain. A, GST pull-down assays for GST-IBB-GFP with RBBP4. Either WT or mutants of GST-IBB-GFP were incubated with RBBP4 and then detected by Western blotting. GST-IBB-GFP proteins are shown by Coomassie staining (mutants of GST-IBB-GFP were incubated with RBBP4 and then detected by Western Brilliant Blue).

C. Complex model 1

Complex model 2

FIGURE 6. Structural models of the complex between RBBP4 and the IBB domain. A. GST pull-down assays for GST-IBB-GFP with RBBP4. Either WT or mutants of GST-IBB-GFP were incubated with RBBP4 and then detected by Western blotting. GST-IBB-GFP proteins are shown by Coomassie staining (CBB, Coomassie Brilliant Blue), 8, RBBP4-binding residues in the IBB domain. Left, electrostatic potential and hydrophobicity on the molecular surface of the RBBP4 structure (PDB code 4pyb), which was obtained from the eF-site database (60). The figure is colored to identify negatively charged (red), positively charged (blue), or neutral (white) non-hydrophobic residues and negatively charged (orange), positively charged (green), or neutral (yellow) hydrophobic residues. Upper right, IBB domain structure (PDB code 1gkk), with the putative RBBP4-binding residues shown as ball and stick models. Lower right, electrostatic potential and hydrophobicity on the molecular surface of the IBB domain. We predict that the hydrophobic and positively charged surface in the IBB domain binds to the hydrophobic and negatively charged surface spanning from Leu-306/308/310 to the C terminus of RBBP4. C, two structural models of the RBBP4/IBB complex. We used the interactive molecular viewer, jV (60), and superimposed manually the IBB domain structure in the importin β2-IBB domain complex (PDB code 1gkk) onto the RBBP4 structure in the RBBP4-MTA1 complex (PDB code 4pyb), so that Leu-306/308/310 of RBBP4 would interact with Met-27/Val-34/H9252.

B. Structural models of the complex between RBBP4 and the IBB domain. A, GST pull-down assays for GST-IBB-GFP with RBBP4. Either WT or mutants of GST-IBB-GFP were incubated with RBBP4 and then detected by Western blotting. GST-IBB-GFP proteins are shown by Coomassie staining (mutants of GST-IBB-GFP were incubated with RBBP4 and then detected by Western Brilliant Blue).

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to the IBB domain of importin α1. This acceleration in dissociation facilitates importin αβ-mediated nuclear protein import. This is consistent with our finding that the knockdown of RBBP4 decreased the nuclear transport efficiency of cNLS-containing cargo.

In contrast to the large number of proteins known to bind to the armadillo repeats or the C terminus of importin α, only a few proteins have been shown to bind to the IBB domain. Previously, we reported that Snail, a zinc-finger-containing transcriptional regulator, interacts with a broad region of importin α that includes the IBB domain (46) but is transported into the nucleus by importin β1 alone (47). In addition, we demonstrated that Snail bound to importin α fails to form a complex with importin β1 (46), which means that importin α negatively regulates the importin β-mediated Snail import. However, in contrast to RBBP4, there is no evidence indicating that Snail is involved in the general regulation of importin αβ pathway.

Although RBBP4 is the only known regulator of the importin αβ pathway that binds to the IBB domain of importin α, several other molecules that bind to the other regions of importin α have also been implicated in the regulation of the pathway. Npap60 promotes the release of cNLS-cargo from importin α (48), although its short isoform, Npap60S, stabilizes the complex between importin α and cNLS-containing cargo (49). Recently, it was demonstrated that the interaction of the C-terminal region of importin α with Nup153 promotes translocation of the cNLS-importin αβ1 trimeric complex into the nucleus, suggesting that importin α serves as not only an adapter molecule but also as a transport mediator in the complex (21). Taken together, these results indicate that importin αβ-mediated nuclear transport might be regulated by these importin α-interacting molecules. Further studies will be required to learn how these importin α-binding molecules are related to physiological processes such as cellular senescence.

Cellular senescence is a state of permanent cell cycle arrest. Senescent cells are characterized by a flat and enlarged morphology, increased SA β-gal activity, increased expression of cyclin-dependent kinase inhibitors, and the alteration of chromatin structure (50–53). It has been shown that chromatin modifiers, including RBBP4, are related to cellular senescence (54, 55). In fact, we found that histone H3 modifications (H3K9me3 and H3K27me3) increased in si-RBBP4-treated TIG-1 cells (Fig. 8G), indicating that RBBP4 can affect the chromatin states. However, these changes were not consistent with the previous reports, in which these modifications decrease in senescent cells (40–42). Therefore, we suppose that these changes in histone H3 modifications observed in the si-RBBP4-treated TIG-1 cells might not have a direct effect on cellular senescence. Furthermore, we found that histone H4K20me3, which is known to increase in senescent cells (43), did not change in si-RBBP4-treated TIG-1 cells, although it increased in passage-dependent senescent TIG-1 cells (Fig. 8G). In addition, we found that histone methyltransferase Ezh2 increased in the si-RBBP4-treated senescent TIG-1 cells (Fig. 8G), as observed in proliferative cells (44, 45). Furthermore, it has been shown that the sole knockdown of RBBP7, without simultaneous knockdown of RBBP4, has little effect on the chromatin structure in other types of cells (11). Taken together, we sup-
pose that the knockdown of RBBP4 does not induce cellular senescence primarily through the chromatin modifications. However, cellular senescence is triggered by many causes and regulated through many pathways; therefore, we speculated that both processes, decreasing nuclear transport efficiency and altering chromatin modifications, could potentially contribute to senescence in RBBP4-depleted cells.

Several reports have shown that attenuation of the nuclear transport of macromolecules is closely linked to cellular senescence and aging disorders such as Hutchinson-Gilford progeria syndrome. For example, nuclear localization of mitogen-activated protein kinase (ERK1/2) or galectin-3 is impaired in senescent cells (37, 56). Moreover, Hutchinson-Gilford progeria syndrome cells or lamin A mutant-expressing cells show...
reduced efficiency in importin β-dependent nuclear transport (57, 58). The decrease of nuclear transport-related factors such as importin α, CAS, and Ran is also observed in senescent cells (23, 38). The knockdown of Ran, CAS, or Tpr induces cellular senescence (23, 59). In this study, we showed that RBBP4 acts as a novel key regulator of the classical nuclear transport pathway to keep cells young. We found that the knockdown of RBBP4 not only reduces the efficiency of classical importin α/β-mediated nuclear protein transport in HeLa cells that demonstrated a no senescent state (data not shown) but it also induces cellular senescence in normal fibroblasts, even though there is no change in the protein levels of the nuclear transport factors (data not shown). We also confirmed that nuclear transport efficiency is decreased in senescent cells. Based on these findings, we propose that nuclear transport efficiency must be maintained to keep cells young, and thus a gradual decrease
in nuclear transport efficiency due to a variety of causes, such as the decrease of nuclear transport-related factors and the impairment of positive regulators like RBBP4, induces cellular hypofunction, leading to cellular senescence.

Author Contributions—A. T. carried out all experiments with the exception of the structural models, which Y. T. and K. M. made, and the mass analysis, which C. O. provided. A. T., Y. M., T. M., M. O., and Y. Y. designed the study and wrote the paper with comments from all other authors.

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References
1. Terry, L. J., Shows, E. B., and Wente, S. R. (2007) Crossing the nuclear envelope: hierarchical regulation of nucleocytoplasmic transport. Science 318, 1412–1416
2. Imamoto, N., Shimamoto, T., Takaok, T., Tachibana, T., Kose, S., Matsubae, M., Sekimoto, T., Shimoshini, Y., and Yoneda, Y. (1995) In vivo evidence for involvement of a 58-kDa component of nuclear pore-targeting complex in nuclear protein import. EMBO J. 14, 3617–3626
3. Goldfarb, D. S., Corbett, A. H., Mason, D. A., Harreman, M. T., and Adam, S. A. (2004) Importin α: a multipurpose nuclear-transport receptor. Trends Cell Biol. 14, 505–514
4. Miyamoto, Y., Boag, P. R., Hime, G. R., and Loveland, K. L. (2012) Regulated nucleocytoplasmic transport during gametogenesis. Biochim. Biophys. Acta 1819, 616–630
5. Lee, S. J., Matsuura, Y., Liu, S. M., and Stewart, M. (2005) Structural basis for nuclear import complex dissociation by RanGTP. Nature 435, 695–696
6. Kobe, B. (1999) Autoinhibition by an internal nuclear localization signal revealed by the crystal structure of mammalian importin α. Nat. Struct. Biol. 6, 388–397
7. Fontes, M. R., Teh, T., and Kobe, B. (2000) Structural basis of recognition of monopartite and bipartite nuclear localization sequences by mammalian importin-α. J. Mol. Biol. 297, 1183–1194
8. Herold, A., Truant, R., Wiegand, H., and Cullen, B. R. (1998) Determination of the functional domain organization of the importin α nuclear import factor. J. Cell Biol. 143, 309–318
9. Matsuura, Y., and Stewart, M. (2004) Structural basis for the assembly of a nuclear export complex. Nature 432, 872–877
10. Hayflick, L., and Moorhead, P. S. (1961) The serial cultivation of human diploid cell strains. Exp. Cell Res. 25, 585–621
11. Pegoraro, G., Kuehnen, N., Wickert, U., Goehler, H., Hoffmann, K., and Misteli, T. (2009) Ageing-related chromatin defects through loss of the NURD complex. Nat. Cell Biol. 11, 1261–1267
12. Pavlopoulos, E., Jones, S., Kosmidis, S., Close, M., Kim, C., Kovalerchik, O., Snaill, S. A., and Kandel, E. R. (2013) Molecular mechanism for age-related memory loss: the histone-binding protein RbpA48. Sci. Transl. Med. 5, 209ra115
13. Zhang, W., Tyl, M., Ward, R., Sobott, F., Maman, J., Murthy, A. S., Watson, A. A., Fedorov, O., Bowman, A., Owen-Hughes, T., El Mkami, H., Murzina, N. V., Norman, D. G., and Laue, E. D. (2013) Structural plasticity of histones H3-H4 facilitates their allosteric exchange between RbpA48 and ASFI. Nat. Struct. Mol. Biol. 20, 29–35
14. Delcuve, G. P., Khan, D. H., and Davie, J. R. (2012) Roles of histone deacetylases in epigenetic regulation: emerging paradigms from studies with inhibitors. Clin. Epigenetics 4, 5
15. Denslow, S. A., and Wade, P. A. (2007) The human Mi-2/NuRD complex and gene regulation. Oncogene 26, 5433–5438
16. Kadyrova, L. Y., Rodriges Blanko, E., and Kadyrov, F. A. (2013) Human CAF-1-dependent nucleosome assembly in a defined system. Cell Cycle 12, 3286–3297
17. Margueron, R., and Reinberg, D. (2011) The Polycomb complex PRC2 and its mark in life. Nature 469, 343–349
18. Kimoto, C., Moriyama, T., Tsuji, A., Igashita, Y., Obuse, C., Miyamoto, Y., Oka, M., and Yoneda, Y. (2015) Functional characterization of importin-α8 as a classical nuclear localization signal receptor. Biochim. Biophys. Acta 1853, 2676–2683
19. Kose, S., Imamoto, N., Tachibana, T., Shimamoto, T., and Yoneda, Y. (1997) Ran-unassisted nuclear migration of a 97-kD component of nuclear pore-targeting complex. J. Cell Biol. 139, 841–849
20. Sekimoto, T., Imamoto, N., Nakajima, K., Hirano, T., and Yoneda, Y. (1997) Extracellular signal-dependent nuclear import of Stat1 is mediated by nuclear pore-targeting complex formation with NPI-1, but not Rch1. EMBO J. 16, 7067–7077
21. Ogawa, Y., Miyamoto, Y., Oka, M., and Yoneda, Y. (2012) The interaction between importin-α and Nup153 promotes importin-α/β-mediated nuclear import. Traffic 13, 934–946
22. Kosugi, S., Hasebe, M., Entani, T., Takayama, S., Tomita, M., and Yana-gawa, H. (2008) Design of peptide inhibitors for the importin α/β nuclear import pathway by activity-based profiling. Chem. Biol. 15, 940–949
23. Nagai, M., and Yoneda, Y. (2013) Downregulation of the small GTPase Ras-related nuclear protein accelerates cellular ageing. Biochim. Biophys. Acta 1830, 2813–2819
24. Miyamoto, Y., Hieda, M., Harreman, M. T., Fukumoto, M., Sawaki, T., Hodel, A. E., Corbett, A. H., and Yoneda, Y. (2002) Importin α can migrate into the nucleus in an importin β- and Ran-independent manner. EMBO J. 21, 5833–5842
25. Sekimoto, T., Nakajima, K., Tachibana, T., Hirano, T., and Yoneda, Y. (1996) Interferon-γ-dependent nuclear import of Stat1 is mediated by the GTPase activity of Ran/TC4. J. Biol. Chem. 271, 31017–31020
26. Kimura, H., Hayashi-Takana, Y., Goto, Y., Takizawa, N., and Nozaki, N. (2008) The organization of histone H3 modifications as revealed by a panel of specific monoclonal antibodies. Cell Struct. Funct. 33, 61–73
27. Yasuhara, N., Yamagishi, R., Arai, Y., Mehmood, R., Kimoto, C., Fujita, T., Touma, K., Kaneko, A., Kanikawa, Y., Moriyama, T., Yanagida, T., Kaneko, H., and Yoneda, Y. (2013) Importin α subtypes determine differential transcription factor localization in embryonic stem cells maintenance. Dev. Cell 26, 123–135
28. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680
29. Umegaki-Arao, N., Tamai, K., Nimura, K., Serada, S., Naka, T., Nakano, H., and Katayama, I. (2013) Karyopherin α2 is essential for RNA transcription and protein synthesis in proliferative keratinocytes. PLoS One 8, e76416
30. Kosugi, S., Hasebe, M., Tomita, M., and Yanagawa, H. (2009) Systematic identification of cell cycle-dependent yeast nucleocytoplasmic shuttling proteins by prediction of composite motifs. Proc. Natl. Acad. Sci. U.S.A. 106, 10171–10176
31. Nakai, K., and Horton, P. (1999) PSORT: a program for detecting sorting signals in proteins and predicting their subcellular localization. Trends Biochem. Sci. 24, 34–36
32. Nitahara-Kasahara, Y., Kamata, M., Yamamoto, T., Zhang, X., Miyamoto, Y., Muneta, K., Iijima, S., Yoneda, Y., Tsumetseguyoko, Y., and Aida, Y. (2007) Novel nuclear import of Vpr promoted by importin α is crucial for human immunodeficiency virus type 1 replication in macrophages. J. Virol. 81, 5284–5293
33. Conti, E., Uy, M., Leighton, L., Blobel, G., and Kuriyan, J. (1998) Crystallographic analysis of the recognition of a nuclear localization signal by the nuclear import factor karyopherin α. Cell 94, 193–204
34. Harreman, M. T., Hodel, M. R., Fanara, P., Hodel, A. E., and Corbett, A. H. (2003) The auto-inhibitory function of importin α is essential in vivo. J. Biol. Chem. 278, 5854–5863
35. Cingolani, G., Petosa, C., Weis, K., and Müller, C. W. (1999) Structure of CAF-1-dependent nucleosome assembly in a defined system.
RBBP4 Regulates Classical Nuclear Transport

herd, N. E., Stokes, P. H., Blohel, G. A., Vermeulen, M., et al. (2014) Insight into the architecture of the NuRD complex: structure of the RbAp48-MTAT1 subcomplex. J. Biol. Chem. 289, 21844–21855
37. Kim-Kaneyama, Nose, K., and Shibanuma, M. (2000) Significance of nuclear relocalization of ERK1/2 in reactivation of c-fos transcription and DNA synthesis in senescent fibroblasts. J. Biol. Chem. 275, 20682–20692
38. Pujol, G., Söderqvist, H., and Radu, A. (2002) Age-associated reduction of nuclear protein import in human fibroblasts. Biochem. Biophys. Res. Commun. 294, 354–358
39. Kim, S. Y., Ryu, S. J., Ahn, H. J., Choi, H. R., Kang, H. T., and Park, S. C. (2010) Senescence-related functional nuclear barrier by down-regulation of nucleo-cytoplasmic trafficking gene expression. Biochem. Biophys. Res. Commun. 391, 28–32
40. Bracken, A. P., Kleine-Kohlbrecher, D., Dietrich, N., Pasini, D., Gargiulo, G., Beekman, C., Theiliggaard-Mönch, K., Minucci, S., Porse, B. T., Marine, J. C., Hansen, K. H., and Helin, K. (2007) The Polycomb group proteins bind throughout the INK4A-ARF locus and are disassociated in senescent cells. Genes Dev. 21, 525–530
41. O’Sullivan, R. J., Kubicek, S., Schreiber, S. L., and Karlseder, J. (2010) Reduced histone biosynthesis and chromatin changes arising from a damage signal at telomeres. Nat. Struct. Mol. Biol. 17, 1218–1225
42. Shah, P. P., Donahue, G., Otte, G. L., Capell, B. C., Nelson, D. M., Cao, K., Aggarwala, V., Cruickshanks, H. A., Rai, T. S., McBryan, T., Gregory, B. D., Adams, P. D., and Berger, S. L. (2013) Lamin B1 depletion in senescent cells triggers large-scale changes in gene expression and the chromatin landscape. Genes Dev. 27, 1787–1799
43. Gonzalo, S. (2010) Epigenetic alterations in aging. J. Appl. Physiol. 109, 586–597
44. Tang, X., Milyavsky, M., Shats, I., Erez, N., Goldberg, N., and Rotter, Y. (2004) Activated p53 suppresses the histone methyltransferase EZH2 gene. Oncogene 23, 5759–5769
45. Bai, J., Ma, M., Cai, M., Xu, F., Chen, J., Wang, G., Shuai, X., and Tao, K. (2014) Inhibition enhancer of zeste homologue 2 promotes senescence and apoptosis induced by doxorubicin in p53 mutant gastric cancer cells. Cell Prolif. 47, 211–218
46. Sekimoto, T., Miyamoto, Y., Arai, S., and Yoneda, Y. (2011) Importin α protein acts as a negative regulator for Snail protein nuclear import. J. Biol. Chem. 286, 15126–15131
47. Yamashita, H., Sekimoto, T., Ohkubo, T., Douchi, T., Nagata, Y., Ozawa, M., and Yoneda, Y. (2005) Zinc finger domain of snail functions as a nuclear localization signal for importin β-mediated nuclear import pathway. Gene Cells 10, 455–464
48. Matsuura, Y., and Stewart, M. (2005) Nup50/Npap60 function in nuclear protein import complex disassembly and importin recycling. EMBO J. 24, 3681–3689
49. Ogawa, Y., Miyamoto, Y., Asalley, M., Oká, M., Yasuda, Y., and Yoneda, Y. (2010) Two isoforms of Npap60 (Nup50) differentially regulate nuclear protein import. Mol. Biol. Cell 21, 630–638
50. Funayama, R., and Ishikawa, F. (2007) Cellular senescence and chromatin structure. Chromosoma 116, 431–440
51. Adams, P. D. (2007) Remodeling of chromatin structure in senescent cells and its potential impact on tumor suppression and aging. Gene 397, 84–93
52. Feser, I., and Tyler, J. (2011) Chromatin structure as a mediator of aging. FEBS Lett. 585, 2041–2048
53. Corpet, A., and Stucki, M. (2014) Chromatin maintenance and dynamics in senescence: a spotlight on SAHF formation and the epigenome of senescent cells. Chromosoma 123, 423–436
54. Dimaro, T., and David, G. (2009) Chromatin modifications: the driving force of senescence and aging? Aging 1, 182–190
55. Pegoraro, G., and Misteli, T. (2009) The central role of chromatin maintenance in aging. Aging 1, 1017–1022
56. Openo, K. P., Kadrofske, M. M., Patterson, R. J., and Wang, J. L. (2000) Galectin-3 expression and subcellular localization in senescent human fibroblasts. Exp. Cell Res. 255, 278–290
57. Kelley, J. B., Datta, S., Snow, C. J., Chatterjee, M., Ni, L., Spencer, A., Yang, C. S., Cebra-Potts, C., Matunis, M. J., and Paschal, B. M. (2011) The defective nuclear lamina in Hutchinson-Gilford progera syndrome disrupts the nucleocytoplasmic Ran gradient and inhibits nuclear localization of Ubc9. Mol. Cell. Biol. 31, 3378–3395
58. Busch, A., Kiel, T., Heupel, W. M., Wehnert, M., and Hübner, S. (2009) Nuclear protein import is reduced in cells expressing nuclear envelope-causing lamin A mutants. Exp. Cell Res. 315, 2373–2385
59. David-Watine, B. (2011) Silencing nuclear pore protein Tpr elicits a senescent-like phenotype in cancer cells. PLoS One 6, e22423
60. Kinoshita, K., and Nakamura, H. (2004) eF-site and PDBViewer: database and viewer for protein functional sites. Bioinformatics 20, 1329–1330