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Identification of Importin α 7 Specific Transport Cargoes Using a Proteomic Screening Approach*⦾

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The importin α:β complex is responsible for the nuclear import of proteins bearing classical nuclear localization signals. In mammals, several importin α subtypes are known to exist that are suggested to have individual functions. Importin α 7 was shown to play a crucial role in early embryonic development in mice. Embryos from importin α 7–depleted females stop at the two-cell stage and show disturbed zygotic genome activation. As there is evidence that individual importin α subtypes possess cargo specificities, we hypothesized that importin α 7 binds a unique set of intracellular proteins. With the use of a collection of *in vitro* and *in vivo* binding assays, importin α 7 interaction partners were identified that differed from proteins found to bind to importin α 2 and 3. One of the proteins preferentially binding importin α 7 was the maternal effect protein Brg1. However, Brg1 was localized in oocyte nuclei in importin α 7–deficient embryos, albeit in reduced amounts, suggesting additional modes of nuclear translocation of this factor. An additional SILAC-based screening approach identified Ash2l, Chd3, Mcm3, and Smarcc1, whose nuclear import seems to be disturbed in importin α 7–deficient fibroblasts. *Molecular & Cellular Proteomics* **13: 10.1074/mcp.M112.026856, 1286–1298, 2014.**

The nuclear compartment is spatially separated from the cytoplasm by the nuclear envelope. The nuclear pores, which are embedded in the nuclear membrane, are the gateway for intracellular molecules that must traverse the nuclear envelope to enter or exit the nucleus. Small molecules can pass through the nuclear pores via passive diffusion; molecules weighing more than 40 kDa must be transported actively through the nuclear pore (1). According to the transport direction, carrier proteins that mediate these nuclear trafficking events are called importins or exportins, known collectively as karyopherins. Nuclear trafficking mediated by the importin α:importin β heterodimer is perhaps the best characterized nuclear import pathway. Here, importin α (or karyopherin α) serves as an adaptor molecule that binds cargoes containing classical nuclear localization signals (NLSs)[1] in their primary amino acid sequence. Upon cargo binding, importin α binds to importin β (karyopherin β 1), forming a trimeric transport complex that moves through the nuclear pore into the nucleus. In the nucleoplasm, RanGTP binds to importin β, leading to a conformational change in importin β and to the dissociation of the transport complex. The cargo is released to the nucleoplasm and can fulfill its function, whereas importins α and β are recycled back to the cytoplasm, where they can perform the next round of import (for reviews, see Refs. 2–4).

There is only one importin α and one importin β protein present in yeast. However, multiple importin α isoforms, each transcribed from a different gene, are found in higher eukaryotes. Three importin α subtypes have been identified in *Caenorhabditis elegans* and *Drosophila melanogaster*, and up to seven importin α isoforms have been identified in mammals (5–7). These importin α isoforms can be grouped into three subfamilies based on sequence similarity (8). Little is known as to why multiple importin α isoforms exist in higher eukaryotes, but there is evidence that each importin α subtype has a tissue-specific expression pattern and distinct cargoes containing classical NLSs (9–12).

We have recently shown that importin α 7 is required for embryonic development in mice (13). Oocytes from importin α 7 null females ovulate but produce embryos that fail to develop beyond the two-cell stage. To elucidate the molecular mechanisms behind this phenotype, we were especially interested in the identification of importin α 7 binding partners.

[1] The abbreviations used are: NLS, nuclear localization signal; Brg1, Brahma-related gene 1; SILAC, stabile isotope labeling by amino acids in cell culture; MEF, murine embryonic fibroblast; LFQ, label-free quantification; GV, germinal vesicle.

Therefore, the aim of this study was to combine *in vivo* and *in vitro* screens to identify an importin α 7 subtype-specific cargo set. Through GST pull-down and co-immunoprecipitation experiments, we were able to identify a unique set of importin α 7 interaction partners that are involved in RNA processing, chromosome organization, and chromatin modification. Among them we found Brahma-related gene 1 (Brg1), also known as smarca4 or Baf190a, a known maternal effect protein required for early development in the mouse (14). An additional approach utilizing stable isotope labeling by amino acids in cell culture (SILAC) was used to further narrow down the list of potential importin α 7 specific cargoes. Hereby, we identified Ash2l, Chd3, Mcm3, Mcm5, and Smarcc1, whose nuclear levels were clearly decreased in importin α 7–deficient fibroblasts.

EXPERIMENTAL PROCEDURES

Cell Culture—Wild-type and importin α 7 knockout (−/−) murine embryonic fibroblasts (MEFs) were prepared from embryos harvested from pregnant females on embryonic day 13.5; cells isolated from wild-type and importin α 7 −/− mouse (13) embryos were immortalized by repeated passaging. MEFs and NIH3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Darmstadt, Germany) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin.

Plasmids and Recombinant Proteins—For mammalian expression, the open reading frames of murine importin α isoforms were inserted into pcDNA3.1(-) with a 3′-mycHis tag. For bacterial expression, the open reading frame of murine importin α 7 was inserted into a modified pQE60 vector with a 3′-GST tag (10). The human importin β-pQE60 clone was generated as described elsewhere (11). The *Escherichia coli* BL21 (DE3) strain containing the importin α 7 expression vector was cultured at 17 °C overnight in the presence of 0.05 mM isopropyl 1-thio-β-D-galactopyranoside. Importin α 7-GST and GST-control proteins were applied to a GST-Trap 4B column and eluted with 15 mM reduced glutathione. Importin β-His was applied to a Ni^{2+}-charged His-trap Crude FF column and eluted by 250 mM imidazole. Further purification of recombinant proteins was done using a gel filtration 26/60 Superdex 200 prep-grade column. Recombinant human importin α proteins were generated as described previously (15).

GST Pull-down—15 mouse ovaries were homogenized in 0.5 ml of 1× lysis buffer (Cell Signaling, Frankfurt, Germany) supplemented with protease inhibitor mixture without EDTA (Roche, Mannheim, Germany) using a FastPrep-24 device (MP Biomedicals, Eschwege, Germany). 30 μg of importin α 7-GST or GST-control protein was coupled to 100 μl of glutathione Sepharose (Qiagen, Hilden, Germany) overnight at 4 °C. The next day, beads were washed and blocked with 2 mg/ml BSA. Afterward, the ovary protein lysate was mixed with GST beads together with 45 μg of importin β-His and incubated overnight at 4 °C. Finally, GST beads were washed and bound proteins were eluted by 1× SDS sample buffer and 5 min of heating at 95 °C.

Transfection—One day before transfection, 1.5×10^6 NIH3T3 cells were seeded onto a 10-cm dish and cultivated in DMEM supplemented with 10% FBS, without antibiotics. On the next day, cells were transfected with 20 μg of importin α-pcDNA vector using 60 μl of lipofectamine LTX and PLUS reagent (Invitrogen, Darmstadt, Germany).

Co-immunoprecipitation—After 2 days of transfection, cells were harvested and co-immunoprecipitation of binding partners was per-

formed using the μMACS Epitope Tag Protein Isolation kit from Miltenyi (Bergisch Gladbach, Germany) according to the manufacturer's instructions. Before the cell extract was loaded onto the columns, anti-His beads were blocked with 2 mg/ml BSA.

Mass Spectrometry—The eluted proteins from the GST pull-down experiment were separated on a 10% SDS gel. After staining with Coomassie Blue, the gel lane was cut into 12 slices. Proteins in each of the slices were converted to peptides by in-gel digestion with trypsin (16). The recovered peptides were separated on an in-house-packed 15-cm reverse-phase column (3-μm beads, Reprosil, Dr. Maisch HPLC GmbH, Ammerbuch-Entringen, Germany) using a 10%–50% acetonitrile linear gradient on an easy-nLC system (Proxeon, Dreieich, Germany). The separated peptides were directly applied to an LTQ-OrbiTrap mass spectrometer (Thermo Scientific, Dreieich, Germany). The recorded spectra were analyzed using the MaxQuant software package (version 1.2.2.5) (17) by matching the data to the IPI mouse database (version 3.84, 59,995 entries) with a false discovery rate of 1% (peptides and proteins), allowing two missed cleavages. Mass tolerances were set to default values. The fixed modifications were carbamidomethylation of cysteines, and the variable modifications were methionine oxidation and N-terminal protein acetylation.

Brg1 Binding Assay—Full-length murine Brg1 cDNA was transcribed and translated *in vitro* in the presence of [^{35}S]-methionine (TNT Quick Coupled Transcription/Translation Systems, Promega, Mannheim, Germany) according to the manufacturer's instructions. The Brg1 binding assay was done as previously described (18). Signals were quantified via densitometry.

Immunocytochemical Staining—Wild-type and importin α 7 −/− MEFs were washed two times with PBS, fixed with 4% paraformaldehyde for 10 min, washed again, and permeabilized with 0.1% Triton–PBS for 10 min. Afterward, cells were blocked in 5% normal donkey serum–PBS for 30 min and incubated with anti-Brg1 antibody (sc-10768, Santa Cruz, Heidelberg, Germany) 1:50 diluted in 0.1% Triton–PBS at 4 °C overnight. Cells were then washed two times with PBS and incubated with donkey anti-rabbit Cy3 antibody (711–165-158, Jackson ImmunoResearch, Suffolk, UK) 1:500 diluted in 0.1% Triton–PBS at room temperature for 1 h. After final washing, cells were mounted with DAPI mounting medium (Vector, Lörrach, Germany) and analyzed using a Keyence microscope (Biorevo, Berlin, Germany). Signal quantification was done using the Keyence Analyzer II Hybrid Cell Count software. An unpaired *t* test was performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, CA).

Immunocytochemical staining of oocytes was performed as previously described (13).

SILAC-based Screening—In order to label the proteomes of wild-type and importin α 7 −/− MEFs with heavy and light amino acids, respectively, cells were cultured in SILAC medium composed of DMEM high glucose (4.5 g/l) supplemented with 10% dialyzed FCS (Sigma-Aldrich, Munich, Germany), 1% penicillin/streptomycin (GIBCO, Darmstadt, Germany), L-glutamine (4 mM), and light (arginine-0, 28 μg/ml; lysine-0, 48 μg/ml) or heavy (arginine-10, 28 μg/ml; lysine-8, 48 μg/ml) amino acids (Sigma-Aldrich, Munich, Germany). Cells were cultured for 2 to 3 weeks and were split 1:4 at least two times per week. Nuclear fractions were analyzed via mass spectrometry. Identified proteins were ranked according to heavy/light signal intensities. Proteins that were quantified with fewer than three SILAC counts were excluded.

RESULTS

Importin α 7 Binding Partners from Ovary Are Involved in RNA Processing, Chromosome Organization, and Chromatin Modification—A previous study had shown that importin α 7 is

TABLE I

Top five enriched Gene Ontology terms among importin α 7 binding partners identified via GST pull-down from ovary lysate. 807 proteins were analyzed using the ToppFun analysis software from the ToppGene Suite. List entries are ranked according to p value; p < 0.01

	I.D.	Name	*p* value	Term in query	Term in genome
		(A) Biological process			
1	GO:0006396	RNA processing	1.54E-31	109	680
2	GO:0051276	Chromosome organization	5.37E-22	100	751
3	GO:0016071	mRNA metabolic process	2.31E-20	88	631
4	GO:0006397	mRNA processing	3.23E-20	70	418
5	GO:0016568	Chromatin modification	2.72E-19	73	468
		(B) Cellular component			
1	GO:0016585	Chromatin remodeling complex	2.12E-23	42	133
2	GO:0044451	Nucleoplasm part	5.36E-21	107	906
3	GO:0030529	Ribonucleoprotein complex	1.12E-15	75	594
4	GO:0016591	DNA-directed RNA polymerase II, holoenzyme	1.32E-14	28	92
5	GO:0005694	Chromosome	2.81E-14	78	671

a maternal effect gene and importin α 7 knockout females are infertile because of a very early embryonic developmental arrest (13), so we set out to identify importin α 7 interaction partners. For this purpose, we performed an importin α 7-GST pull-down experiment using mouse ovary lysate as a source of prey proteins. High-resolution mass spectrometry allows a quantitative analysis of identified proteins from GST pull-down via label-free quantification (LFQ) (19).

The LFQ intensities of proteins bound to importin α 7-GST and to the GST-control were determined, and importin α 7 binding partners were ranked according to their relative LFQ intensity in comparison with the GST-control (log2 LFQ intensity importin α 7/LFQ intensity control). We identified 807 proteins that bound to importin α 7 and not or very weakly to the GST-control protein (supplemental Table S1).

Candidate importin α 7 binding partners identified in GST pull-down experiments were further examined according to their cellular localization and biological processes based on Gene Ontology terms. A cellular component enrichment analysis using the ToppGene Suite service (20) revealed significant enrichment (*p* < 0.01) of nuclear and nucleolar proteins present in the list of importin α 7-GST binding partners (Table I, "biological process"). Among these, factors involved in RNA processing, chromosome organization, and chromatin modification were found to be significantly enriched (*p* < 0.01; Table I, "cellular component").

Identification of Importin α 7 Binding Partners from Fibroblast Cells via Co-immunoprecipitation—GST pull-down assays are a powerful tool for identifying binding partners in an *in vitro* context. To identify proteins that require physiological conditions in order to form a complex with importin α 7, we performed another proteomic screen. A mycHis-tagged version of importin α 7 was overexpressed in murine fibroblast cells, and its expression could be detected in the cytoplasm as well as in the nucleus, as expected (supplemental Fig. S1). Importin α 7-mycHis-bound proteins were co-immunoprecipitated via the His tag.

The LFQ intensities of identified proteins were determined, and importin α 7-mycHis binding partners were ranked according to LFQ intensity relative to the mycHis control. 299 proteins bound to importin α 7-mycHis and not or very weakly to the mycHis control (supplemental Table S2). Again, cellular component enrichment analysis revealed a significant (*p* < 0.01) accumulation of nuclear proteins (supplemental Table S3B), of which most were involved in the same biological processes identified in the first importin α 7 binding partner screen from mouse ovary (supplemental Table S3A).

36% of Importin α 7 Binding Partners Identified via Co-immunoprecipitation from Fibroblast Cells Overlapped with GST Pull-down Results from the Ovary—Out of 299, 107 proteins identified via importin α 7-mycHis co-immunoprecipitation from fibroblast cells also showed up in the list of potential importin α 7 interaction partners identified via GST pull-down from ovary (supplemental Table S4). These proteins seemed to be robust importin α 7 binding partners and thus interesting candidates for further studies. The top 20 importin α 7 binding partners overlapping in both screening result lists are shown in Table II.

Importin α 7 Binding Partners Differ from Importin α 2 and α 3 Substrates—Another important question we wanted to address was whether the identified importin α 7 binding partners differ from those of other importin α subtypes. For this purpose, we performed a co-immunoprecipitation experiment with importin α 2 and α 3, which belong to different importin α subfamilies than importin α 7 (5).

Identified proteins were ranked according to their LFQ intensity relative to the mycHis control.

We identified 266 proteins binding to importin α 2 and 276 proteins binding to importin α 3 that did not bind or bound very weakly to the mycHis control (supplemental Tables S5 and S6). The heat map in Fig. 1 shows an overview of the different intensities of proteins found to bind to importin α 2, 3, or 7. The Gene Ontology analysis of potential importin α 2 or α 3 cargoes showed again a clear enrichment of nuclear

TABLE II

Top 20 overlapping importin α 7 binding partners from ovary and fibroblast cells. Comparison of 807 importin α 7 binding partners identified via GST pull-down from ovary lysate and 299 proteins identified via co-immunoprecipitation from fibroblast cells. Candidate genes were ranked according to their LFQ intensity from the importin α 7-GST pull-down experiment using ovary lysate

	Gene name (Protein name)	Function		Gene name (Protein name)	Function
1	Lmnb1 (Lamin B1) *	scaffolding, interaction with chromatin	11	Npap60 (Nuclear pore complex protein Nup50)	facilitates disassembly of importin-alpha:beta-cargo complex
2	Chd4 (Chromodomain-helicase-DNA-binding protein 4) *	remodeling of chromatin	12	Hdac2 (Histone deacetylase 2)	modification of chromatin
3	Lmn1 (lamin 1) *	scaffolding, interaction with chromatin	13	Nup153 (Nuclear pore complex protein Nup153) *	Possible DNA-binding subunit of the NPC
4	Kiaa0398 (mRNA cap guanine-N7 methyltransferase)	mRNA-capping	14	Gatad2b (Transcriptional repressor p66-beta)	transcriptional repressor activity, targets MBD3 to discrete loci in nucleus
5	Mta1l1 (Metastasis-associated protein MTA2)*	modification of chromatin	15	Ddx21 (Nucleolar RNA helicase 2) *	rRNA processing
6	Mcm3 (DNA replication licensing factor MCM3) *	replicative helicase essential for 'once per cell cycle' DNA replication	16	Dhm1 (5'-3' exoribonuclease 2) *	termination of transcription by RNA polymerase II
7	Taf2s (Transcription elongation regulator 1) *	Transcription factor that binds RNA polymerase II and inhibits elongation of transcripts	17	Baf190a (Transcription activator Brg1) *	remodeling of chromatin
8	Cdc46 (DNA replication licensing factor MCM5) *	replicative helicase essential for 'once per cell cycle' DNA replication	18	Rbap48 (Histone-binding protein RBBP4)	remodeling of chromatin
9	Ars2 (Serrate RNA effector molecule homolog) *	mediator between cap-binding complex and primary microRNAs processing machinery during cell proliferation	19	Rbap46 (Histone-binding protein RBBP7)	remodeling of chromatin
10	Baf170 (SWI/SNF complex subunit SMARCC2) *	remodeling of chromatin	20	Hnrnpc (Heterogeneous nuclear ribonucleoproteins C1/C2) *	Binds pre-mRNA and nucleates assembly of 40S hnRNP particles

Information taken from the Nuclear Protein Database. SWI/SNF, switch/sucrose non-fermentable.
[a] Proteins with known NLS.
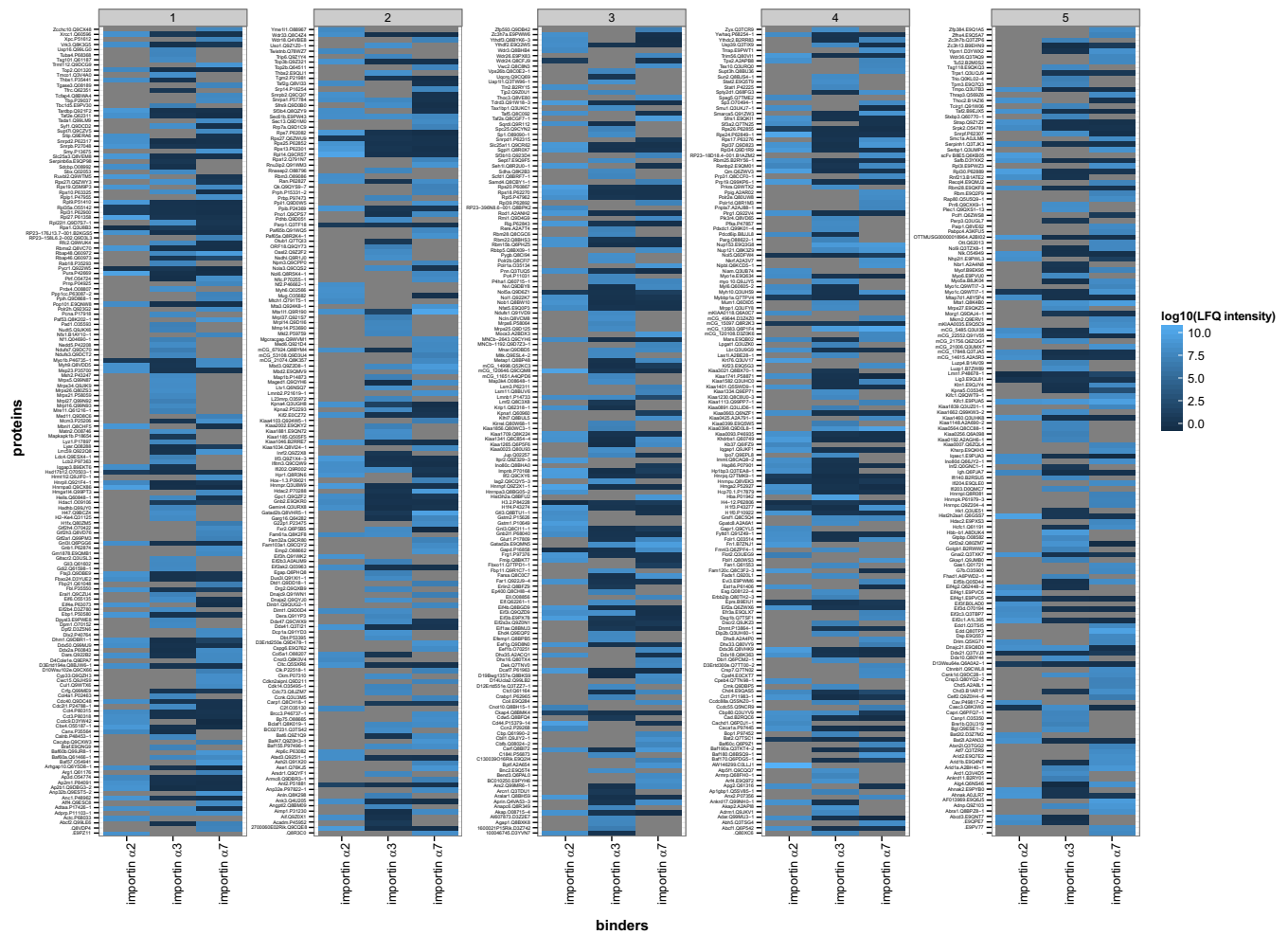
FIG. 1. **Comparative overview of importin α 2, 3, and 7 interactomes in fibroblast cells.** Binding partners of importin α 2–, 3–, and 7–mycHis found via co-immunopreciptation in NIH3T3 cells were identified via mass spectrometry. The heat map shows the abundance of interaction partners based on their relative LFQ intensities (log10 LFQ intensity importin α/LFQ intensity control) found in the individual samples. The blue shading represents the LFQ intensity of a detected protein: the lighter the blue, the greater the LFQ intensity of the binding partner. Gray means no protein binding was detected. Binding partners are displayed by gene name and UniProt I.D.

factors, but ribosomal proteins also were abundant (Tables III and IV). Like those of importin α 7, importin α 2 and α 3 binding partners seem to be involved in RNA processing. However, the Gene Ontology analysis revealed a significant strong enrichment of factors that differed according to linked biological processes from those of importin α 7 binding partners (Tables III and IV).

Approximately 10% overlap was found between importin α 2 or α 3 and α 7 binding partners.

Brg1 Is a Potential Importin α 7 Specific Cargo—Our aim was to identify cargo proteins that bind importin α 7 more effectively than other importin α subtypes. Therefore, we searched for proteins that were more abundant in the importin α 7 binding partner screen than in the importin α 2 or α 3 screen. For this purpose, importin α 2 and α 3 binders having a greater LFQ intensity than importin α 7 were excluded from supplemental Table S2. Potential specific importin α 7 binding partners were compared with importin α 7 bound proteins

from the ovary. The top 10 preferential importin α 7 binding partners also found in ovary tissue are displayed in Table V (for the complete list, see supplemental Table S7).

Among candidate proteins, Brg1 (also known as smarca4 or Baf190a) was identified as an importin α 7 interacting protein in both the GST pull-down from ovary and the co-immuno-precipitation experiments (Table II). Furthermore, Brg1 did not seem to bind importin α 2, and not only was it much more abundant in the importin α 7 binding partner screen than in that of importin α 3 (Fig. 1, panel 4; here annotated as Baf190a), but it also belonged to the 10 most abundant binding partners of importin α 7 in ovary lysate (Table V). Like importin α 7, Brg1 is reported to be a maternal effect gene whose depletion leads to a two-cell arrest in murine embryos with disturbed zygotic genome activation (14). Therefore, we selected Brg1 for further analysis.

Brg1 Is Still Imported into Nuclei of Importin α 7 −/− MEFs—In order to determine the requirements of importin α 7

TABLE III

Top five enriched Gene Ontology terms among importin α 2–mycHis binding partners identified via co-immunoprecipitation from NIH3T3 cells. 266 proteins were analyzed using the ToppFun analysis software from the ToppGene Suite. List entries are ranked according to p value; p < 0.01

	I.D.	Name	p value	Term in query	Term in genome
		(A) Biological process			
1	GO:0016071	mRNA metabolic process	9.94E-41	68	631
2	GO:0006396	RNA processing	2.19E-34	64	680
3	GO:0006397	mRNA processing	1.69E-26	46	418
4	GO:0006413	Translational initiation	2.39E-25	32	166
5	GO:0022613	Ribonucleoprotein complex biogenesis	1.32E-21	34	254
		(B) Cellular component			
1	GO:0030529	Ribonucleoprotein complex	6.51E-43	67	594
2	GO:0005681	Spliceosomal complex	1.61E-17	24	149
3	GO:0022626	Cytosolic ribosome	1.35E-15	19	93
4	GO:0071013	Catalytic step 2 spliceosome	1.66E-15	18	80
5	GO:0044391	Ribosomal subunit	5.65E-14	21	148

TABLE IV

Top five enriched Gene Ontology terms among importin α 3–mycHis binding partners identified via co-immunoprecipitation from NIH3T3 cells. 276 proteins were analyzed using the ToppFun analysis software from the ToppGene Suite. List entries are ranked according to p value; p < 0.01

	I.D.	Name	p value	Term in query	Term in genome
		(A) Biological process			
1	GO:0006412	Translation	1.37E-06	28	532
2	GO:0043933	Macromolecular complex subunit organization	5.58E-06	45	1299
3	GO:0065003	Macromolecular complex assembly	2.67E-05	40	1128
4	GO:0034622	Cellular macromolecular complex assembly	2.51E-04	26	594
5	GO:0006396	RNA processing	9.81E-04	27	680
		(B) Cellular component			
1	GO:0030529	Ribonucleoprotein complex	4.14E-11	35	594
2	GO:0005840	Ribosome	3.61E-09	20	215
3	GO:0016581	NuRD complex	7.68E-09	8	17
4	GO:0000118	Histone deacetylase complex	8.37E-06	9	51
5	GO:0016585	Chromatin remodeling complex	9.85E-06	13	133

for Brg1 nuclear import, we examined the intracellular localization of Brg1 in importin α 7 −/− MEFs. For this purpose, MEFs were prepared from importin α 7 −/− embryos, and the absence of importin α 7 expression was verified via PCR (supplemental Fig. S2). In wild-type MEFs, immunocytochemical staining of Brg1 was exclusively nuclear. However, the nuclei of importin α 7 −/− MEFs also showed strong Brg1 staining (Fig. 2*A*). Surprisingly, quantification of the nuclear–cytoplasmic ratio revealed a 13% higher nuclear Brg1 signal in importin α 7 −/− MEFs relative to control cells (Fig. 2*B*).

Normal Brg1 Nuclear Localization in Importin α 7 −/− Oocytes—In murine oocytes and zygotes, only certain importin α subtypes are expressed (importin α 1, 2, 4, and 7) (6, 13). Based on the developmental phenotype of embryos from importin α 7 −/− mice, we considered an importin α 7–dependent nuclear import of Brg1 in pre-implantation embryos. Therefore, the subcellular localization of Brg1 in unfertilized germinal vesicle (GV) oocytes was examined via immunocytochemistry. However, in GV oocytes from importin α 7 null females, Brg1 staining was clearly detected in the nucleus (Fig. 3*A*). Quantification of the nuclear–cytoplasmic ratio revealed that importin α 7 −/− oocytes showed ~14% less Brg1 in the nucleus than wild-type oocytes (Fig. 3*B*).

Brg1 Does Bind Importin α 7 in Vitro, but Not the Other Maternally Expressed Importin α Subtypes—Besides importin α 7, importin α 1, 2, and 4 have been shown to be expressed in oocytes and zygotes (6, 13). To figure out whether one of the other maternally expressed importin α subtypes can bind to Brg1 as well, we performed an *in vitro* binding assay. The analysis showed high amounts of Brg1 binding to importin α 7-GST (set to 100%), and only low levels binding to importin α 1-GST (10.9%) or α 4-GST (11.5%) (Fig. 4).

Decreased Nuclear Levels of Ash2l, Chd3, Mcm3, Mcm5, and Smarcc1 in Importin α 7 −/− MEFs—In order to narrow down the number of potential importin α 7 specific cargoes,

TABLE V

Top 10 proteins that bound importin α 7 preferentially and overlapped with importin α 7 binding partners identified from ovary. Importin α 7, 2, and 3 binding partners identified from fibroblast cells via co-immunoprecipitation were ranked according to their LFQ intensities relative to the mycHis-control. Importin α 2 or α 3 binding partners with a greater LFQ intensity than importin α 7 were excluded from the list of importin α 7 binding partners. Displayed are the top 10 preferential importin α 7 binding proteins identified in ovary and fibroblast cells ranked according to LFQ intensity based on the ovary GST pull-down dataset (the complete list of proteins is found in supplementary Table S7)

|	Gene name	Protein name	Localization	Description	Biological process	LFQ intensity
1	Hnrnpu[a]	Heterogeneous nuclear ribonucleoprotein U	Cytoplasm, nucleus, spliceosome	Component of coding region determinant–mediated complex	mRNA processing	3.76E+10
2	Lmnb1[a]	Lamin-B1	Intermediate filament, membrane, nucleus	Components of the nuclear lamina	Positive regulation of JNK cascade	3.29E+10
3	Hnrnpl[a]	Heterogeneous nuclear ribonucleoprotein L	Cytoplasm, nucleus, spliceosome	Component of heterogeneous nuclear ribonucleoprotein (hnRNP) complexes	mRNA processing	9.71E+09
4	Mcm3[a]	DNA replication licensing factor MCM3	Nucleus	Component of MCM complex, putative replicative helicase essential for "once per cell cycle" DNA replication initiation and elongation in eukaryotic cells	Cell cycle, DNA replication	6.73E+09
5	Anp32b[a]	Acidic leucine-rich nuclear phosphoprotein 32 family member B	Cytoplasm, nucleus	Required for progression from the G1 to the S phase	Chaperone	6.58E+09
6	Taf2s[a]	Transcription elongation regulator 1	Nucleus	Transcription factor that binds RNA polymerase II and inhibits the elongation of transcripts from target promoters	Transcription regulation	5.1E+09
7	Cdc46[a]	DNA replication licensing factor MCM5	Nucleus	Component of MCM complex, putative replicative helicase essential for "once per cell cycle" DNA replication initiation and elongation in eukaryotic cells	Cell cycle, DNA replication	3.8E+09
8	Dhm1[a]	5'-3' exoribonuclease 2	Nucleus	May promote termination of transcription by RNA polymerase II	Transcription regulation, mRNA processing	3.73E+09
9	Smarcc2[a]	SWI/SNF complex subunit SMARCC2	Nucleus	Chromatin remodeling	Transcription regulation	3.45E+09
10	Smarca4[a]	Transcription activator Brg1	Nucleus	Chromatin remodeling	Transcription regulation	2.21E+09

SWI/SNF, switch/sucrose non-fermentable.
[a] Proteins with known NLS.
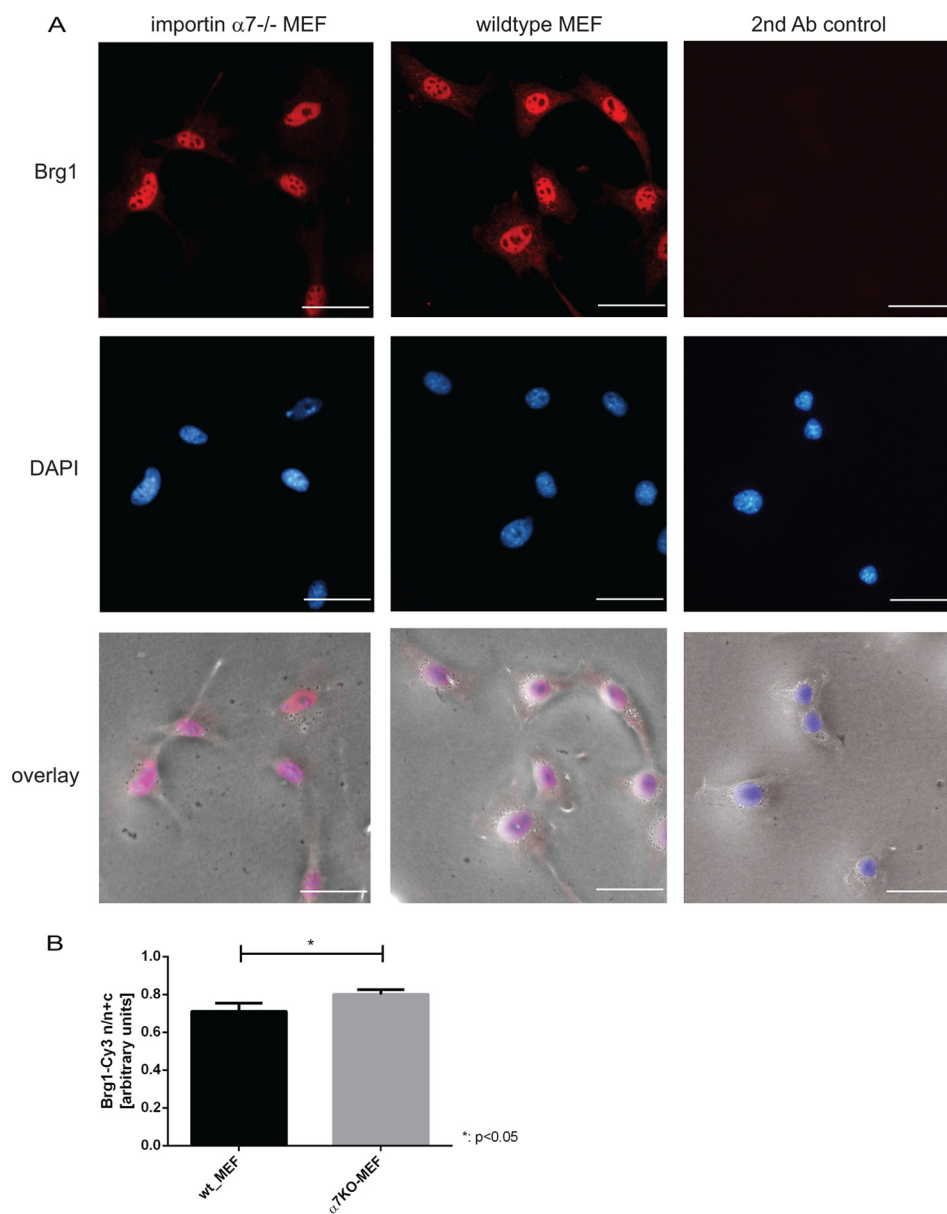
FIG. 2. **Subcellular localization of Brg1 in importin α 7 −/− MEFs.** *A*, wild-type and importin α 7 −/− MEFs were probed with anti-Brg1 antibody (upper panel). DAPI served as a nuclear marker (middle panel). The lower panel shows an overlay of the Brg1-Cy3, DAPI, and bright field. For the secondary antibody control (2nd Ab control), no primary antibody was used (20× magnification; scale bar, 50 μm). *B*, the nuclear Brg1 level was measured by dividing the nuclear (n) by the nuclear plus cytoplasmic (n+c) ratio of the Cy3 fluorescence signal. The bar chart shows the mean of 17 cells.

an additional SILAC-based screen was performed. The objective of this screen was to find importin α 7 binding partners from ovary and cells that are less abundant in the nuclei of importin α 7–depleted MEFs, suggesting disturbed importin α 7–dependent nuclear import. Thus, we identified five candidate proteins, Ash2l, Chd3, Mcm3, Mcm5, and Smarcc1, whose nuclear levels are decreased in importin α 7 −/− MEFs and which had been characterized as binding partners of importin α 7 in GST pull-down and co-immunoprecipitation experiments (Table V, rows 4 and 7; supplemental Table S7, rows 38, 44, and 46). Western blot analysis confirmed these findings and showed significantly lower nuclear levels for all five proteins (Fig. 5). Cytoplasmic levels of Ash2l, Chd3, and Mcm3 were not significantly changed (Figs. 5A–5C). Smarcc1 and Mcm5 were not detectable via Western blotting in cytoplasmic fractions (data not shown). Real-time PCR analysis of wild-type and importin α 7 −/− MEFs did not show any significant changes in mRNA levels of Ash2l, Chd3, Mcm3, and Smarcc1 (supplemental Fig. S3). The mRNA expression of Mcm5, however, was down-regulated, suggesting lower overall Mcm5 protein expression in importin α 7 −/− MEFs (supplemental Fig. S3*D*).
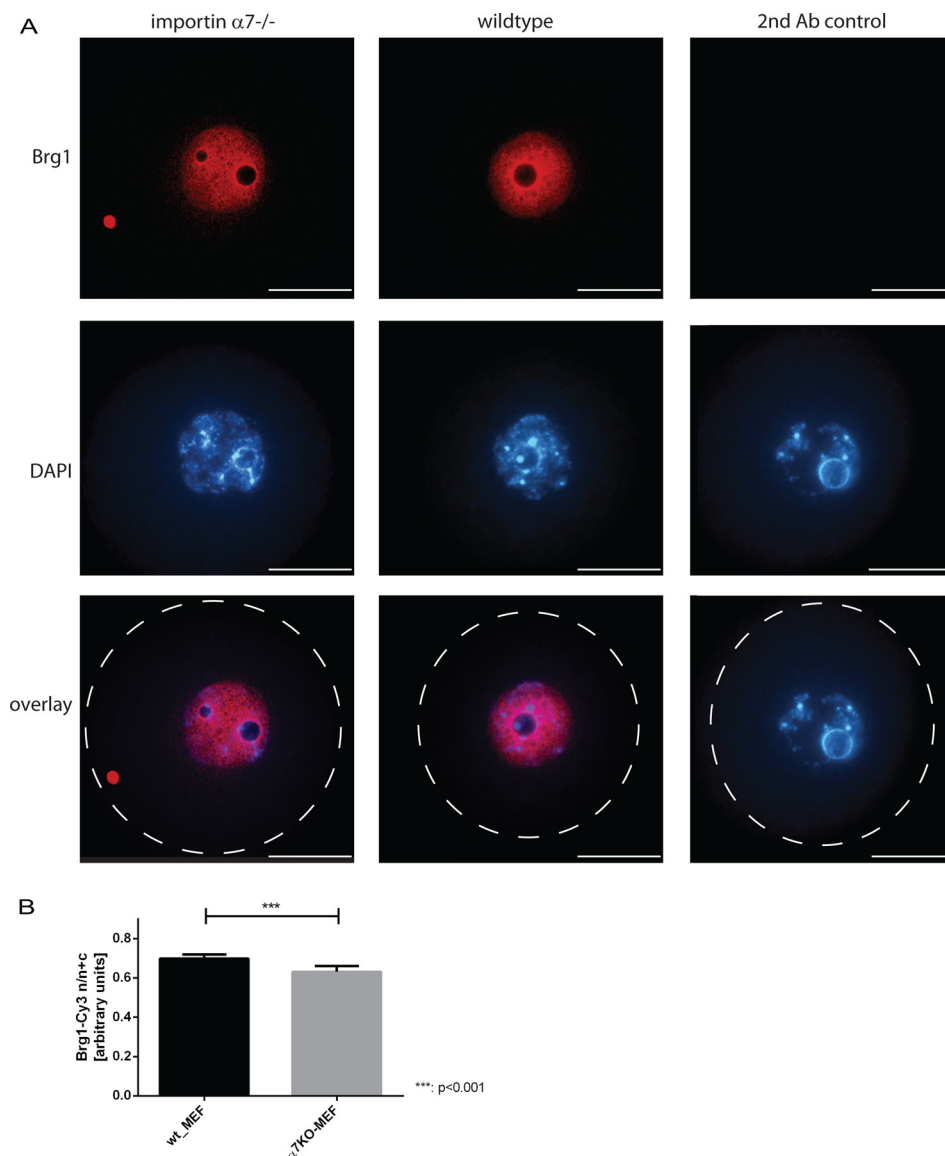
FIG. 3. **Nuclear Brg1 staining in importin α 7 $-/-$ germinal vesicle (GV) oocytes.** GV oocytes from wild-type and importin α 7 $-/-$ embryos were probed with anti-Brg1 antibody (upper panel). DAPI staining served as a nuclear marker (middle panel). The lower panel shows an overlay of the Brg1-Cy3 and DAPI. The contours of GV oocytes are displayed by a dashed line that was generated by overexposure of the Cy3 signal. For the secondary antibody control (2nd Ab control), no primary antibody was used (scale bar: 50 μm). *B*, the nuclear Brg1 level was measured by dividing the nuclear (n) by the nuclear plus cytoplasmic (n+c) ratio of the Cy3 fluorescence signal. The bar chart shows the mean of four cells.

DISCUSSION

Substrate specificities among importin α subtypes have been postulated to allow the cell more flexible control over nuclear cargo import in response to different environmental stimuli (21).

Although most importin α isoforms are ubiquitously expressed in different organs, their expression levels differ, and the expression of some importin α subtypes is restricted to specific tissues (5, 6) and particular time points of development (22). It is therefore likely that importin α subtypes import individual cargoes. However, the identities of these cargoes remain largely unknown. The first evidence of cargo preferences for individual importin α isoforms *in vivo* came from a cell culture assay showing that importin α 3 mediates the nuclear import of the Ran guanine nucleotide exchange factor RCC1 (12). Furthermore, cargo specificities may become obvious only under special conditions. For example, in the mTOR signaling pathway, latent STAT1 depends on importin α 5 for nuclear import under conditions of reduced mitogen or nutrition levels (23).

Interestingly, the specific use of single importin α subtypes has also been described for viruses, such as the influenza
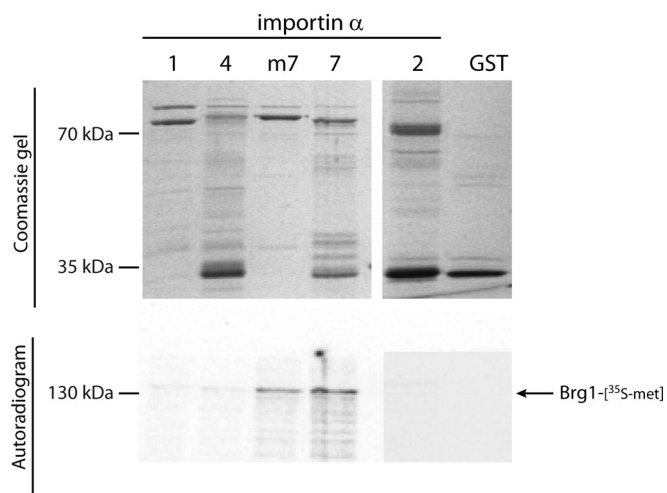
FIG. 4. **Importin α-GST *in vitro* binding assay.** *In vitro* transcribed and translated Brg1 was incubated with human recombinant importin α-GST proteins immobilized on glutathione Sepharose beads. Murine importin α 7 (m7) was examined as well. Detection of bound radioactively labeled Brg1 (Brg1-[^{35}S-met]) was performed via autoradiography. Only maternally expressed importin α subtypes were analyzed. Recombinant GST served as a negative control.

virus, whose polymerase subunit PB2 and nucleoprotein are specifically imported to the nucleus by importin α 7, offering novel opportunities for antiviral therapies (24).

The phenotype of importin α 7–deficient mice strongly supports the hypothesis that importin α isoforms transport unique subsets of intracellular cargoes in higher eukaryotic organisms. Embryos derived from female mice lacking importin α 7 show developmental arrest at the two-cell stage (13) despite the presence of other α importins, so there may be intracellular cargoes that rely on this particular adapter protein for nuclear import. Therefore, we set out to use GST pull-down and co-immunoprecipitation approaches to screen for possible importin α 7 specific binding partners.

In GST pull-down experiments, we identified importin α 7 binding partners from murine ovary lysate that might shed light on the role of importin α 7–mediated trafficking during oocyte and embryo development. As expected for a nuclear transport receptor, Gene Ontology analysis regarding cellular localization of identified importin α 7 binders revealed a high proportion of nuclear proteins. Interestingly, the majority of these nuclear proteins appeared to be involved in RNA processing, chromosome organization, and chromatin modification; such processes are known to be dynamically regulated during early embryo development and during zygotic genome activation.

To get a more comprehensive picture of importin α 7 cargoes, we searched for importin α 7 binding partners from a cultured murine cell line using a co-immunoprecipitation screening approach. 36% of identified proteins matched the list of importin α 7-GST interaction partners from ovary lysate. Given that both screening approaches can cover only a sub-

set of proteins present in a cell or tissue, 36% overlapping proteins is a relatively high degree of congruency. Almost all overlapping proteins are known to localize to the nucleus. Moreover, approximately half of these factors carry an NLS and are therefore likely to be imported by importin α 7. Among NLS cargoes, nuclear membrane components such as Lmnb1 or Lmn1 also appeared. These proteins are supposed to be targeted to the inner nuclear membrane via the classical nuclear protein import pathway (25, 26). Furthermore, the importin α 7 binding partner list contained Npap60, which is known to support cargo release from importin α (27–29).

Like importin α 7 binding partners, importin α 2 and α 3 binding partners seem to be involved in RNA processing. However, we found strong enrichment of factors that differed according to linked biological processes among importin α 2 and α 3 binders relative to importin α 7 binding partners, highlighting the uniqueness of importin α 7 transported cargoes.

In a recent study, importin α 5 binding partners were identified from mouse brain (30). Out of 48 proteins binding to importin α 5, only 3 proteins also showed up in our importin α 7 screens (FUBP1, HnrpK, and Nup50). The low degree of congruence between the importin α 5 and α 7 binding partner lists may be explained by the different tissues used as a source of prey proteins. However, this finding also supports the idea of importin α subtype-specific cargo sets.

Brg1 was found in both the GST pull-down and the co-immunoprecipitation screen as a protein that interacted with importin α 7. Interestingly, Brg1 is one of the first identified maternal effect genes in mice and part of the chromatin-remodeling switch/sucrose non-fermentable complex. Like embryos from importin α 7 knockout females, zygotes from Brg1-depleted females display a two-cell arrest (14). Furthermore, it has been shown that Brg1 regulates zygotic genome activation and controls the expression of genes involved in transcription, RNA processing, and cell cycle regulation. Thus, we decided to analyze Brg1 in more detail to determine whether the depletion of importin α 7 perturbs the intracellular localization of endogenous Brg1. However, nuclear detection of Brg1 in importin α 7–depleted MEFs showed that Brg1 was not solely dependent on nuclear import by this particular transport receptor. Against our expectations, the nuclear Brg1 level seemed to be slightly higher in importin α 7 −/− MEFs than in control cells. The biological reasons for this finding are unclear. Furthermore, although murine oocytes and zygotes lack at least some of the importin α–dependent pathways, Brg1 can enter the nucleus in GV oocytes. Nevertheless, nuclear Brg1 levels were slightly but significantly decreased in importin α 7 knockout oocytes. This finding fits with the observed binding preference of Brg1 for importin α 7. However, we assume that this marginal decline in brg1 protein level has no biological relevance in importin β 7 knockout oocytes. Despite the almost negligible *in vitro* binding of Brg1 to the remaining maternally expressed importin α subtypes,
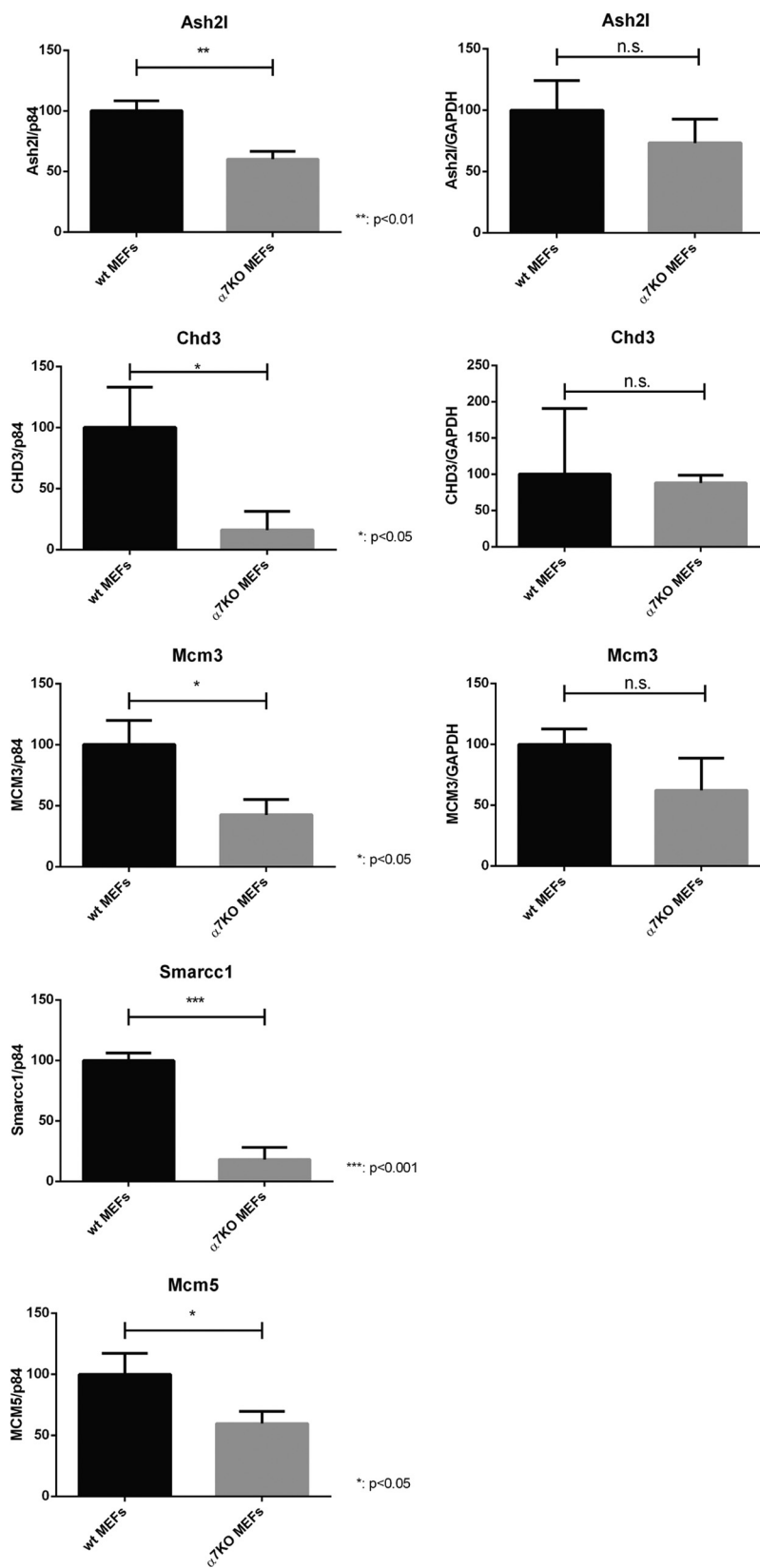
FIG. 5. **Nuclear and cytoplasmic levels of Ash2l, Chd3, Mcm3, Mcm5, and Smarcc1 in importin α 7 −/− MEFs.** Three independent nuclear and cytoplasmic fractions were prepared from wild-type (wt) and importin α 7 −/− (a7KO) MEFs and analyzed via Western blotting. Quantification of signals was performed with Odyssey System software (LiCor, Bad Homburg, Germany), and statistics were analyzed using GraphPad Prism 6 software. Nuclear protein signals were normalized to the nuclear marker protein p84, and cytoplasmic signals to the cytoplasmic marker protein GAPDH. The bar diagrams show the mean of three different sample preparations, setting the mean wild-type value as 100%.

we cannot rule out the possibility that Brg1 nuclear import can also partially be taken over by other importin α proteins in pre-implantation embryos. Moreover, Brg1 may enter the nucleus in a complex with other NLS-bearing importin α substrates, or Brg1 could be transported by an alternative nuclear import receptor. Nevertheless, we cannot rule out a role for importin α 7 in regulating the amount of Brg1 that enters the nucleus at critical times in development, or the possibility that individual splice variants of Brg1 are trafficked specifically by importin α 7 *in vivo*.

By using an additional SILAC-based screening approach, we identified five importin α 7 binding partners from ovary and fibroblasts whose nuclear levels were significantly reduced in importin α 7–deficient MEFs: Ash2l, Chd3, Mcm3, Mcm5, and Smarcc1. Cytoplasmic levels of Ash2l, Chd3, and Mcm3 were not increased in importin α 7 knockout MEFs, suggesting a compensatory degradation mechanism that protects the cell from abnormally high cytoplasmic amounts of these proteins. As expected for mainly nuclear proteins, Smarcc1 and Mcm5 were not detected in cytoplasmic fractions in Western blots. Because mRNA levels were not significantly changed, we conclude that the reduced nuclear amount of Ash2l, Chd3, Mcm3, and Smarcc1 is the consequence of disturbed nuclear import in importin α 7–deficient MEFs. In contrast, Mcm5 mRNA was down-regulated, suggesting transcriptional impairment of Mcm5 expression in importin α 7 $-/-$ MEFs.

In summary, this study demonstrates that binding specificities of importin α subtypes can be reproduced using proteomic approaches. The finding that different α-importins may serve proteins associated with distinct biological processes suggests that they may be key components regulating different parts of the cellular signaling network. To confirm this idea, further studies will be required to compare binding partners from all importin α subtypes. Moreover, the newly identified importin α 7 cargoes Ash2l, Chd3, Mcm3, and Smarcc1 will be analyzed in more detail according to their roles in the phenotype of importin α 7–deficient mice (13) in the near future.

REFERENCES

1. Wente, S. R., and Rout, M. P. (2010) The nuclear pore complex and nuclear transport. *Cold Spring Harb. Perspect. Biol.* **2,** 14

2. Lange, A., Mills, R. E., Lange, C. J., Stewart, M., Devine, S. E., and Corbett, A. H. (2007) Classical nuclear localization signals: definition, function, and interaction with importin alpha. *J. Biol. Chem.* **282,** 5101–5105

3. Marfori, M., Mynott, A., Ellis, J. J., Mehdi, A. M., Saunders, N. F., Curmi, P. M., Forwood, J. K., Boden, M., and Kobe, B. (2011) Molecular basis for specificity of nuclear import and prediction of nuclear localization. *Biochim. Biophys. Acta* **9,** 25

4. Jamali, T., Jamali, Y., Mehrbod, M., and Mofrad, M. R. (2011) Nuclear pore complex: biochemistry and biophysics of nucleocytoplasmic transport in health and disease. *Int. Rev. Cell Mol. Biol.* **287,** 233–286

5. Kohler, M., Ansieau, S., Prehn, S., Leutz, A., Haller, H., and Hartmann, E. (1997) Cloning of two novel human importin-alpha subunits and analysis of the expression pattern of the importin-alpha protein family. *FEBS Lett.* **417,** 104–108

6. Tejomurtula, J., Lee, K. B., Tripurani, S. K., Smith, G. W., and Yao, J. (2009) Role of importin alpha8, a new member of the importin alpha family of nuclear transport proteins, in early embryonic development in cattle. *Biol. Reprod.* **81,** 333–342

7. Tsuji, L., Takumi, T., Imamoto, N., and Yoneda, Y. (1997) Identification of novel homologues of mouse importin alpha, the alpha subunit of the nuclear pore-targeting complex, and their tissue-specific expression. *FEBS Lett.* **416,** 30–34

8. Goldfarb, D. S., Corbett, A. H., Mason, D. A., Harreman, M. T., and Adam, S. A. (2004) Importin alpha: a multipurpose nuclear-transport receptor. *Trends Cell Biol.* **14,** 505–514

9. Fagerlund, R., Melen, K., Kinnunen, L., and Julkunen, I. (2002) Arginine/lysine-rich nuclear localization signals mediate interactions between dimeric STATs and importin alpha 5. *J. Biol. Chem.* **277,** 30072–30078

10. Friedrich, B., Quensel, C., Sommer, T., Hartmann, E., and Kohler, M. (2006) Nuclear localization signal and protein context both mediate importin alpha specificity of nuclear import substrates. *Mol. Cell. Biol.* **26,** 8697–8709

11. Kohler, M., Speck, C., Christiansen, M., Bischoff, F. R., Prehn, S., Haller, H., Gorlich, D., and Hartmann, E. (1999) Evidence for distinct substrate specificities of importin alpha family members in nuclear protein import. *Mol. Cell. Biol.* **19,** 7782–7791

12. Quensel, C., Friedrich, B., Sommer, T., Hartmann, E., and Kohler, M. (2004) In vivo analysis of importin alpha proteins reveals cellular proliferation inhibition and substrate specificity. *Mol. Cell. Biol.* **24,** 10246–10255

13. Rother, F., Shmidt, T., Popova, E., Krivokharchenko, A., Hugel, S., Vilianovich, L., Ridders, M., Tenner, K., Alenina, N., Kohler, M., Hartmann, E., and Bader, M. (2011) Importin alpha7 is essential for zygotic genome activation and early mouse development. *PLoS One* **6,** e18310

14. Bultman, S. J., Gebuhr, T. C., Pan, H., Svoboda, P., Schultz, R. M., and Magnuson, T. (2006) Maternal BRG1 regulates zygotic genome activation in the mouse. *Genes Dev.* **20,** 1744–1754

15. Depping, R., Steinhoff, A., Schindler, S. G., Friedrich, B., Fagerlund, R., Metzen, E., Hartmann, E., and Kohler, M. (2008) Nuclear translocation of hypoxia-inducible factors (HIFs): involvement of the classical importin alpha/beta pathway. *Biochim. Biophys. Acta* **1783,** 394–404

16. Shevchenko, A., Tomas, H., Havlis, J., Olsen, J. V., and Mann, M. (2006) In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat. Protoc.* **1,** 2856–2860

17. Cox, J., and Mann, M. (2008) MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat. Biotechnol.* **26,** 1367–1372

18. Depping, R., Schindler, S. G., Jacobi, C., Kirschner, K. M., and Scholz, H. (2012) Nuclear transport of Wilms' tumour protein Wt1 involves importins alpha and beta. *Cell Physiol. Biochem.* **29,** 223–232

19. Zhu, W., Smith, J. W., and Huang, C. M. (2010) Mass spectrometry-based label-free quantitative proteomics. *J. Biomed. Biotechnol.* **840518,** 10

20. Chen, J., Bardes, E. E., Aronow, B. J., and Jegga, A. G. (2009) ToppGene Suite for gene list enrichment analysis and candidate gene prioritization. *Nucleic Acids Res.* **37,** W305-W311

21. Riddick, G., and Macara, I. G. (2007) The adapter importin-alpha provides flexible control of nuclear import at the expense of efficiency. *Mol. Syst. Biol.* **3,** 118

22. Hogarth, C. A., Calanni, S., Jans, D. A., and Loveland, K. L. (2006) Importin alpha mRNAs have distinct expression profiles during spermatogenesis. *Dev. Dyn.* **235,** 253–262

23. Fielhaber, J. A., Tan, J., Joung, K. B., Attias, O., Huegel, S., Bader, M.,

Roux, P. P., and Kristof, A. S. (2012) Regulation of karyopherin alpha1 and nuclear import by mammalian target of rapamycin. *J. Biol. Chem.* **287,** 14325–14335

24. Gabriel, G., Klingel, K., Otte, A., Thiele, S., Hudjetz, B., Arman-Kalcek, G., Sauter, M., Shmidt, T., Rother, F., Baumgarte, S., Keiner, B., Hartmann, E., Bader, M., Brownlee, G. G., Fodor, E., and Klenk, H. D. (2011) Differential use of importin-alpha isoforms governs cell tropism and host adaptation of influenza virus. *Nat. Commun.* **2,** 156

25. King, M. C., Lusk, C. P., and Blobel, G. (2006) Karyopherin-mediated import of integral inner nuclear membrane proteins. *Nature* **442,** 1003–1007

26. Lusk, C. P., Blobel, G., and King, M. C. (2007) Highway to the inner nuclear membrane: rules for the road. *Nat. Rev. Mol. Cell Biol.* **8,** 414–420

27. Lindsay, M. E., Plafker, K., Smith, A. E., Clurman, B. E., and Macara, I. G. (2002) Npap60/Nup50 is a tri-stable switch that stimulates importin-alpha:beta-mediated nuclear protein import. *Cell* **110,** 349–360

28. Matsuura, Y., and Stewart, M. (2005) Nup50/Npap60 function in nuclear protein import complex disassembly and importin recycling. *EMBO J.* **24,** 3681–3689

29. Gilchrist, D., Mykytka, B., and Rexach, M. (2002) Accelerating the rate of disassembly of karyopherin-cargo complexes. *J. Biol. Chem.* **277,** 18161–18172

30. Fukumoto, M., Sekimoto, T., and Yoneda, Y. (2011) Proteomic analysis of importin alpha-interacting proteins in adult mouse brain. *Cell Struct. Funct.* **36,** 57–67