

# Multiple pathways contribute to nuclear import of core histones

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**Nuclear import of the four core histones H2A, H2B, H3 and H4 is one of the main nuclear import activities during S-phase of the cell cycle. However, the molecular machinery facilitating nuclear import of core histones has not been elucidated. Here, we investigated the pathways by which histone import can occur. First, we show that core histone import can be competed by the BIB ( $\beta$ -like import receptor binding) domain of ribosomal protein L23a suggesting that histone import is an importin mediated process. Secondly, affinity chromatography on immobilized core histones revealed that several members of the importin  $\beta$  family of transport receptors are able to interact with core histones. Finally, we demonstrate that at least four known and one novel importin, importin 9, can mediate nuclear import of core histones into the nuclei of permeabilized cells. Our results suggest that multiple pathways of import exist to provide efficient nuclear uptake of these abundant, essential proteins.**

## INTRODUCTION

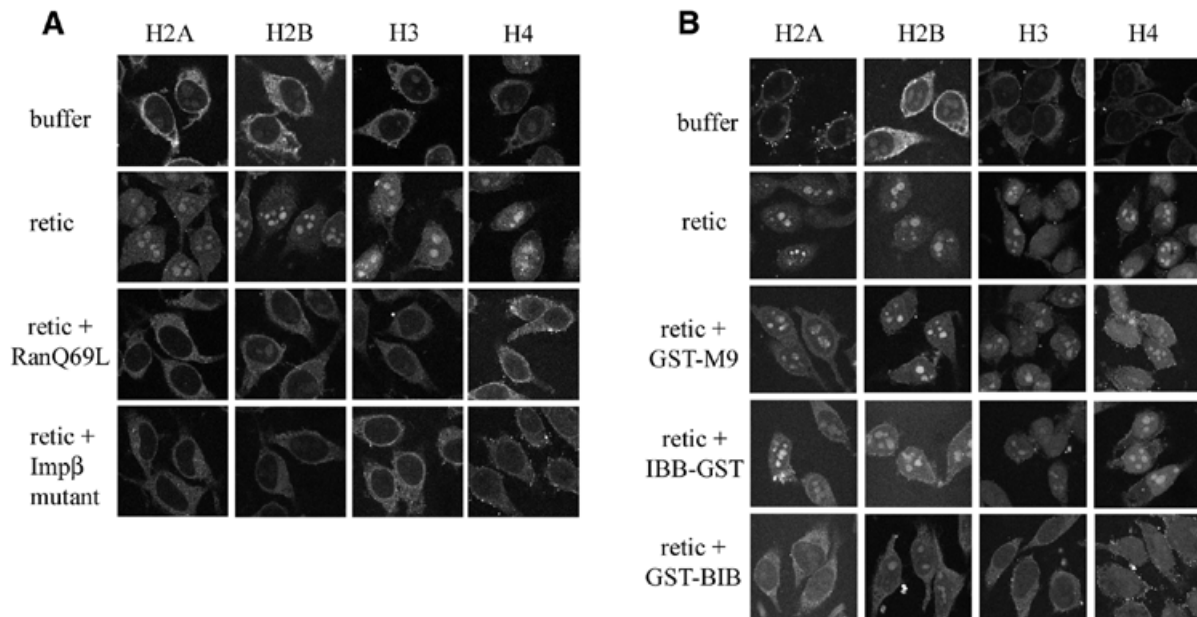
During S-phase of the cell cycle, DNA is replicated and *de novo* chromatin assembly takes place. This requires nuclear uptake of newly synthesized core histones and their deposition onto DNA to form the regularly repeated unit of eukaryotic chromatin, the nucleosome (for review see Verreault, 2000). Although a number of factors have been identified aiding the process of nucleosome assembly, the molecular mechanism by which core histones are transported into the nucleus is unknown.

Transport of proteins into and out of the nucleus is a signal dependent, receptor-mediated process that proceeds through nuclear pore complexes. Nuclear transport is largely mediated by members of a family of nuclear transport receptors referred to as importins/exportins or karyopherins. A defining feature of these receptors is their ability to interact with the small GTPase

Ran in its GTP-bound form. This interaction is instrumental in controlling the compartment specific loading and unloading of the transport substrates and hence the directionality of nucleocytoplasmic transport. While binding of RanGTP to importins facilitates import substrate release, exportins need to bind RanGTP for efficient loading of the export cargo. Both events are specific to the nuclear compartment where the chromatin-associated nucleotide exchange factor RCC1 generates RanGTP. In the cytoplasm, however, the RanGTP concentration is kept low due to the presence of the RanGTPase activating protein (RanGAP1) allowing the formation of import complexes and the disassembly of export complexes (for review see Mattaj and Englmeier, 1998; Görlich and Kutay, 1999).

A number of signals in nuclear proteins have been identified which allow their selective access to the transport machinery. The first signal to be characterized was the classical basic nuclear localization signal (NLS) which is recognized by a complex consisting of the adapter molecule importin  $\alpha$  (Imp $\alpha$ ) and the transport receptor importin  $\beta$  (Imp $\beta$ ) (for review see Görlich and Kutay, 1999). Other signals are directly recognized by import receptors: the M9 domain of hnRNPA1 confers binding to transportin (Siomi and Dreyfuss, 1995; Pollard *et al.*, 1996; Fridell *et al.*, 1997), the RS domain of SR proteins binds to transportin SR (Kataoka *et al.*, 1999), and the BIB domain of rpL23a allows its direct interaction with at least four different importins namely Imp $\beta$ , transportin, importin 5 (Imp5) and importin 7 (Imp7) (Jäkel and Görlich, 1998). Imp $\beta$  and transportin are exceptional in that they can recognize diverse signals. For example, transportin, through separable binding sites, interacts with signals as different as the basic BIB domain and the serine/glycine rich M9 domain. Similarly, Imp $\beta$  uses distinct sites to recognize the IBB (Imp $\beta$  binding) domain of Imp $\alpha$  and BIB. In addition, Imp $\beta$  can associate with Imp7 to form a

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**Fig. 1.** Nuclear import of core histones shows characteristics of an importin-mediated process. **(A)** Nuclear import of core histones is inhibited by dominant-negative Ran and Imp $\beta$  mutants. HeLa cells were grown on coverslips and permeabilized. Nuclear import of 1  $\mu$ M of each fluorescein labelled core histone was performed in the presence of Ran mix, an energy-regenerating system and, where indicated, rabbit reticulocyte lysate (retic) for 20 min at 23°C. Reactions were stopped by fixation and the samples analysed by confocal microscopy. Addition of 5  $\mu$ M RanQ69L(GTP) to the import reactions or preincubation of the permeabilized cells with 1  $\mu$ M of Imp $\beta$  (45–462) abolished nuclear uptake of the four core histones. **(B)** Core histone import is competed by BIB. Nuclear import reactions were performed in the presence of retic, Ran and energy mix and supplemented with either 10  $\mu$ M IBB–GST, GST–M9 or GST–BIB as indicated. Note, that only GST–BIB inhibits the nuclear uptake of the four histones. Complete inhibition is obtained at a concentration of 20  $\mu$ M GST–BIB (not shown).

heterodimer which mediates nuclear import of the linker histone H1 *in vitro* (Jäkel *et al.*, 1999).

Recent studies have analysed sequence motifs involved in nuclear targeting of core histones. It has been demonstrated that both the basic N-terminal domains and the central globular domains of all four core histones can serve as nuclear targeting signals (Baake *et al.*, 2001). However, the transport receptors mediating core histone import have not been identified. Here, we show that multiple import pathways can contribute to nuclear import of core histones. At least five different importins can directly interact with individual core histones and support their nuclear import into the nuclei of permeabilized cells.

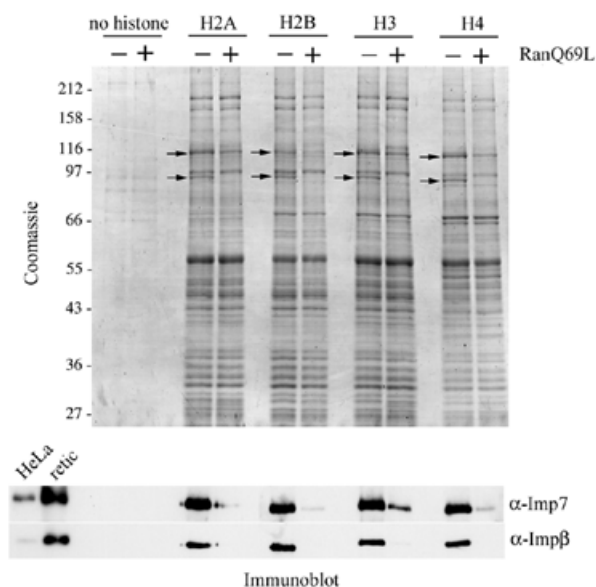
## RESULTS

### Nuclear import of core histones is an importin-mediated process

To identify transport receptors involved in nuclear uptake of core histones we first characterized this process in *in vitro* nuclear transport experiments. Rabbit reticulocyte lysate had been shown to support nuclear accumulation of single core histones into the nuclei of permeabilized cells (Baake *et al.*, 2001). To extend this study further, we analysed the effect of known inhibitors of importin-mediated transport on nuclear uptake of fluorescently labelled core histones. Import of all four core histones was stimulated by the addition of reticulocyte lysate (Figure 1A). Core histones are smaller than the diffusion

limit of the nuclear pore. To exclude that core histones enter the nuclei by diffusion, we further tested whether their nuclear accumulation could be abolished by pre-incubation of the nuclei with a dominant-negative mutant of Imp $\beta$  which prevents receptor-mediated transport through the NPC (Kutay *et al.*, 1997b). The import of all four histones was blocked under these conditions. Biochemical studies had suggested that core histones are able to interact with Imp $\beta$  (Johnson-Saliba *et al.*, 2000). If the interaction between histones and Imp $\beta$  or related factors was required for core histone import, the import reaction should be sensitive to the addition of RanGTP which dissociates most importin/substrate complexes (Rexach and Blobel, 1995; Görlich *et al.*, 1997). Import of all four histones was indeed sensitive to the addition of RanQ69L(GTP), a Ran mutant which is unable to hydrolyse the bound nucleotide. These results suggested that members of the importin  $\beta$  family mediate core histone import.

To define more specifically the pathways involved in nuclear import of histones, we performed the *in vitro* transport assays in the presence of known competitors of different nuclear import pathways (Figure 1B). Overall, the import of all four histones was not prevented by the addition of excess unlabelled IBB–GST or GST–M9 which target the Imp $\alpha$  binding site of Imp $\beta$  and the hnRNP A1 binding site of transportin, respectively. In contrast, the addition of GST–BIB reduced the import of core histones. The competition by BIB argues that at least one of the factors which directly bind and import BIB-containing cargo (i.e. Imp $\beta$ , transportin, Imp5 and Imp7) is required for histone import.



**Fig. 2.** Identification of potential core histone import receptors. Biotinylated histones were immobilized on streptavidin–agarose and used to bind interacting proteins from reticulocyte lysate. Where indicated, 1  $\mu$ M RanQ69L(GTP) had also been added. After washing of the beads bound proteins were eluted and analysed by SDS–PAGE followed by Coomassie staining. The indicated proteins (arrows) of 95 and 116 kD molecular weight were excised and subjected to tryptic digest. The tryptic peptides were analysed by mass spectrometry. The identified peptides in the 95 kD sample were derived from Imp $\beta$  (e.g. VLANPGNSQVAR, WLAIANAR, NYVLQTLGTETYSR, SNELTAIHQGMR, VAALQNLVK) and those in the 116 kD sample were derived from Imp5 (ITFLQAIR, LCDIAAELAR, FLFDSVSSQNFGLR, SLVEIADTVPK, ALLIPYLDNLVK, EGFVEYTEQVVK), Imp7 (AFAVGVQVLLK, EYNEFAEVFLK, AFAVGVQVLLK, ETENDDLTNVIQK) and Imp9 (acAAAAAAGAASGLPGVAQGLK, TSEFTAAVGR). Bold letters indicate deviations of the rabbit peptides from the human Imp5 sequence. The immunoblot confirms that Imp $\beta$  and Imp7 were efficiently bound to all histones in the absence of RanQ69L(GTP). The load in the bound fractions corresponds to 15 times the starting material.

Control experiments revealed that each of the competitors was functional and specific for its respective pathway (see Supplementary figure 1 at *EMBO reports* Online).

### Identification of importins as histone binding proteins

The foregoing results suggested that core histones can be imported into nuclei by one or more members of the importin  $\beta$  family. To identify these transport receptors, we employed affinity chromatography on immobilized histones. The individual histones were biotinylated, immobilized on streptavidin–agarose and binding of proteins from rabbit reticulocyte lysate to the histones was compared in the absence and presence of RanQ69L(GTP). Proteins recovered on the histones in the absence of RanQ69L but whose binding was reduced in its presence were candidate import receptors. Analysis of proteins in the bound fractions showed very similar patterns of binding partners for all four histones (Figure 2). Proteins which displayed

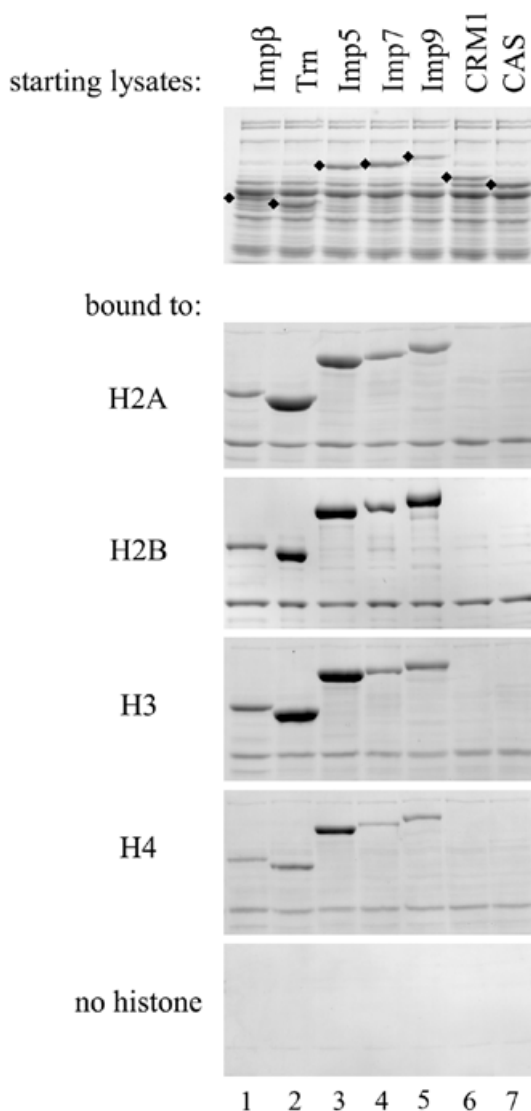
reduced interaction in the presence of RanQ69L were identified by mass spectrometry as Imp $\beta$ , Imp5 and Imp7. In addition, two peptides were found which matched a novel mammalian transport receptor which we had cloned recently based on its homology to the yeast karyopherin Kap114p (for identification and cloning see Methods). We will refer to this novel protein as importin 9 (Imp9). Immunoblotting confirmed that Imp $\beta$  and Imp7 were specifically enriched on the histones in the absence of RanQ69L. We could neither confirm binding of Imp5 nor test other transport receptors such as transportin by immunoblotting as the available antibodies did not detect these proteins in the reticulocyte lysate. Hence we cannot exclude that additional importins had bound to the histones.

Binding of the identified importins could have been either direct or mediated by association with another protein present in the reticulocyte lysate. It was therefore crucial to test whether these importins were able to interact directly with the histones. For this purpose, *Escherichia coli* lysates were supplemented with equal amounts of purified importins and subjected to binding to immobilized histones (Figure 3). Besides Imp $\beta$ , Imp5, Imp7 and Imp9, we tested whether transportin would also bind to the histones as it had been shown before to interact with BIB. All of the tested importins were able to interact directly with the histones. Two other Imp $\beta$  related transport receptors, the exportins CAS and CRM1, neither bound to the histones in the absence (Figure 3) nor in the presence of RanGTP (not shown). As an additional control, with transportin as an example, we analysed whether the binding of this importin was reduced in the presence of RanGTP, which was indeed the case (see Supplementary figure 2 at *EMBO reports* Online).

### Different importins can mediate nuclear import of core histones

Having established that different importins can directly interact with individual core histones, it remained to be determined whether the observed interactions are relevant to nuclear import. All of the tested importins supported nuclear uptake of fluorescently labelled histones (Figure 4). Although all the importins were able to directly interact with the core histones (see Figure 3) we observed differences in their import efficiencies towards the individual histones. Imp5 and transportin strongly supported import of all core histones. Also Imp $\beta$  and Imp7 were able to mediate import of core histones and when added as heterodimer, no significant increase in import efficiency was observed. The only core histone for which we could observe strong nuclear accumulation by the Imp $\alpha$ /Imp $\beta$  complex was H2B. In contrast, Imp $\beta$  mediated import of H2A, H3 and H4 was decreased by the simultaneous presence of Imp $\alpha$ . The novel transport receptor Imp9 appeared to be most productive in H2B import.

As confirmation that the observed effects were specific to core histones, we found that histone H1 was not transported by the individual importins used in our study (Figure 4). Rather, histone H1 is efficiently imported only by an Imp $\beta$ /Imp7 heterodimer (Jäkel *et al.*, 1999). As expected, export receptors like CAS (not shown) or CRM1 did not support nuclear import of core histones.



**Fig. 3.** Direct interaction of nuclear transport receptors with core histones. Immobilized core histones were subjected to binding of recombinant Impβ (1), transportin (2), Imp5 (3), Imp7 (4), Imp9 (5), CRM1 (6) and CAS (7) out of *E. coli* lysates that had been supplemented with the transport receptors to about equal concentrations. As a control, binding to streptavidin–agarose without immobilized histones was performed. Transport receptors bound with slightly increased efficiency in the absence of the bacterial lysate (not shown).

## DISCUSSION

We have shown that nuclear import of single core histones can be mediated by at least four known members of the Impβ superfamily and the novel Imp9. We, for the first time, describe a function for this novel mammalian transport receptor. Imp9 is related to the *Saccharomyces cerevisiae* karyopherin Kap114p, which had been previously characterized as an import receptor for TATA-binding protein (TBP) (Morehouse *et al.*, 1999; Pemberton *et al.*, 1999). Like all other importins, Imp9 is a RanGTP binding protein (not shown). Although mouse Imp9 and Kap114p display 21% overall identity and 40% homology it

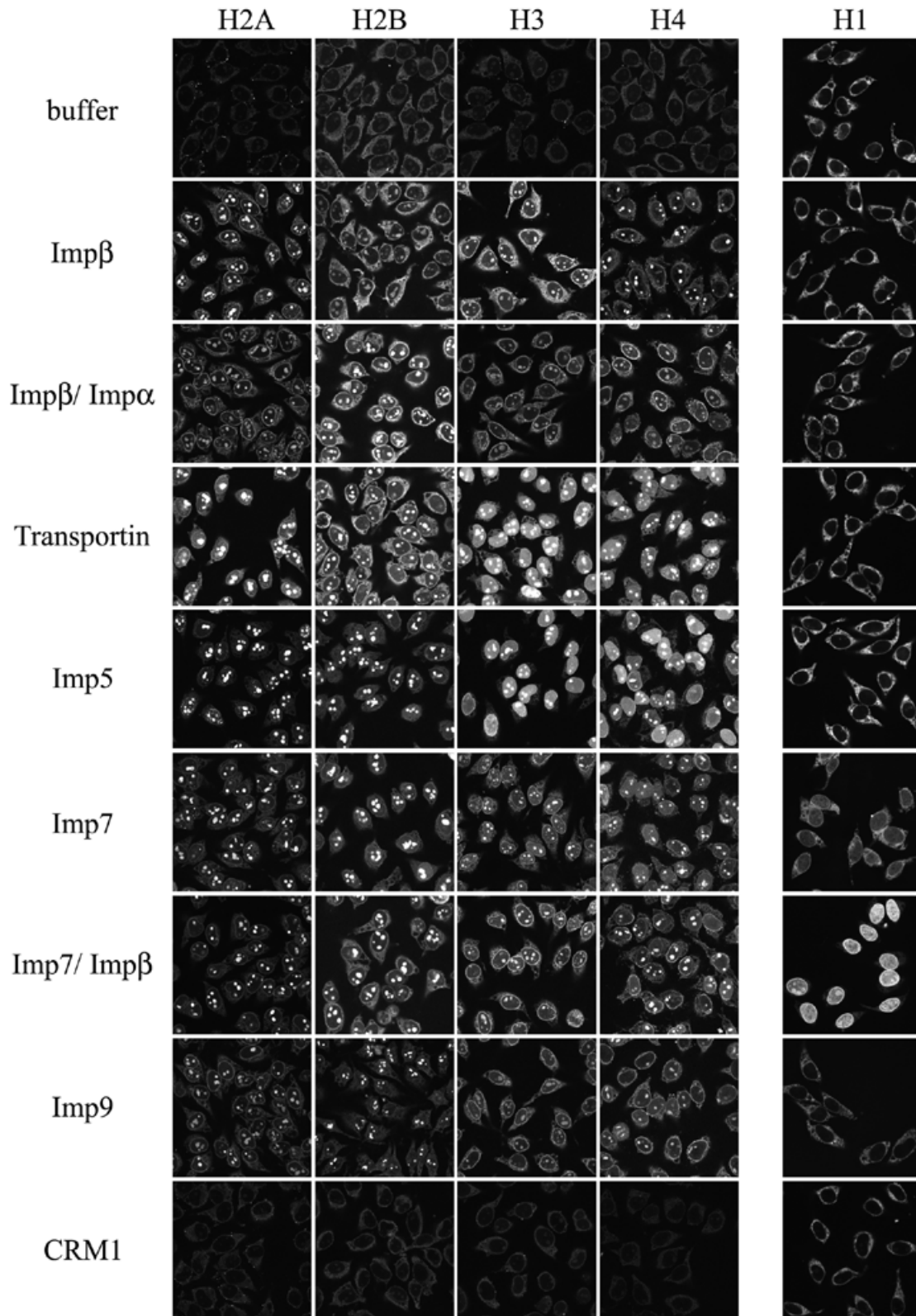
remains to be seen if Imp9 mediates nuclear import of TBP in higher eukaryotic cells.

Besides Imp9, also Impβ, transportin, Imp5 and Imp7 directly interacted with core histones and all of these factors supported core histone import *in vitro*, although to differing extents. Impβ is an exceptional transport receptor in that it not only binds transport substrates directly but also forms heterodimers with import adapters such as Impα or the import receptor Imp7. In contrast to published data (Langer, 2000), we demonstrate that H2B import can be mediated by the Impα/Impβ heterodimer *in vitro*. One explanation for this discrepancy is that Langer used nucleoplasmin core in the import buffer; in our hands, nucleoplasmin core blocks H2B import. The heterodimer of Impβ–Imp7 has been demonstrated to be required for the efficient import of the linker histone H1 (Jäkel *et al.*, 1999). Both Impβ and Imp7 are enriched on the histones from reticulocyte lysate. Unlike H1 import, core histones can be imported by either of the two importins on their own and we could not observe cooperativity in their binding and import of core histones. In this respect, core histone import resembles nuclear import of rpL23a which does not require cooperativity between Impβ and Imp7. Rather, nuclear import of rpL23a occurs along redundant pathways (Jäkel and Görlich, 1998).

Multiple import pathways of core histones may exist to ensure that these essential proteins can be efficiently delivered to their site of action. Core histones as well as many other nuclear proteins are highly basic in nature and often basic patches are presented as import signals. Strikingly, most characterized nuclear import receptors recognize basic import signals and to some extent can substitute for each other. This is reflected in the observation that eight of the ten yeast karyopherins known to be involved in nuclear import are encoded by non-essential genes yet many of these receptors import substrates with essential nuclear functions. It is however difficult to decide which of the four importins defines the predominant pathway *in vivo*. There may even exist additional importins in higher eukaryotes that may either be dedicated to or serve as back-up import receptors for core histones. Indeed, while our study was under review, a comprehensive analysis of nuclear import of H2A and H2B in yeast was published (Mosammaparast *et al.*, 2001), demonstrating that the import of H2A and H2B occurs along redundant pathways *in vivo*.

To assemble into nucleosomes, core histones must first form H2A–H2B heterodimers and (H3–H4)<sub>2</sub> tetramers. The question of whether the formation of histone heteromers takes place in the cytoplasm and/or in the nucleus is still not completely solved. Although in cytosolic cell extracts the existence of histone heteromers has been demonstrated (Chang *et al.*, 1997), it is known that cytoplasmic extracts often contain proteins that are localized to the nucleus *in vivo*. Our data demonstrate that single core histones can be imported into the nucleus *in vitro*. The same holds true *in vivo* as shown for the nuclear import of H2A and H2B deletion mutants that are unable to form heterodimers (Mosammaparast *et al.*, 2001). However, when binding of transport receptors to H2A and H2B monomers was compared with that to H2A–H2B heterodimers the same efficiency was observed (P. Mühlhäusser and U. Kutay, unpublished data). This suggests that the histone import signals are still accessible to importin binding when H2A and H2B are dimerized. Therefore

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**Fig. 4.** Core histones can be imported into the nucleus by multiple pathways. Nuclear import of each 1  $\mu$ M fluorescein labelled H2A, H2B, H3, H4 and H1 was performed in the presence of Ran mix, an energy-regenerating system and either buffer or one of the following: Imp $\beta$  (1  $\mu$ M), Imp $\alpha$  (2  $\mu$ M)/Imp $\beta$  (1  $\mu$ M) heterodimer, transportin (1  $\mu$ M), Imp5 (1  $\mu$ M), Imp7 (1  $\mu$ M), Imp $\beta$  (0.5  $\mu$ M)/Imp7 (0.5  $\mu$ M) heterodimer, Imp9 (1  $\mu$ M) or CRM1 (1  $\mu$ M). After 20 min of import, samples were fixed and analysed by confocal microscopy.

our data does not exclude the possibility that core histones may be imported as heteromers *in vivo*.

Histones are very sticky, basic proteins which presumably have to be shielded from undesired interactions on their way from the ribosome to their final destination. Channelling the histones from one binding partner to the next is a likely mechanism to achieve this. On their way to the site of DNA replication histones are known to interact with histone chaperones, the acetylation machinery, chromatin assembly factors and, as we show here, nuclear transport receptors which can be considered as histone chaperones during nuclear pore passage. It will be interesting to see whether other histone chaperones play an additional role during nuclear transport of core histones.

## METHODS

**Preparation of modified histones.** Histones H1, H2A, H2B, H3 and H4 were purchased from Roche. Each histone was dissolved in 50 mM HEPES pH 7.5 and incubated for 1 h on ice with stoichiometric amounts of either FLUOS or biotin-aminocaproic acid *N*-hydroxy succinimide ester (Roche) for fluorescence labelling or biotinylation of the histones, respectively. Reaction mixtures were then passed over NAP5 columns (Pharmacia) equilibrated in 50 mM HEPES pH 7.5 to remove unincorporated modifying agents.

**Molecular cloning.** Database searches revealed the existence of mouse (AC016814.5) and human (NM\_018085/FLJ10402) sequence tags homologous to *S. cerevisiae* KAP114. The information obtained from the mouse genomic sequence and additional ESTs (AI317597.1 and BE625765.1) comprising the 5' and 3' ends of the coding region, respectively, allowed us to design primers for amplification of the complete coding region. Oligonucleotides (GCCTCCCGGGATGGCGGCAGCGCGGCAG; CCGTCCCGG-GTTAGATGCCGATAGTCTGTAGGACCC) were used to amplify the coding region of Imp9 by PCR from NIH 3T3 cell cDNA. The PCR product was then inserted into the *Xma*I site of pQE32 (Qiagen) to allow the expression of the protein with an N-terminal His<sub>6</sub>-tag in *E. coli*. Several clones were analysed to determine the consensus sequence. The novel sequence data reported in this manuscript have been submitted to the DDBJ/EMBL/GenBank database under accession number AJ309238.

The IBB–GST expression construct was obtained by insertion of the GST coding region as an *Nco*I–*Bam*HI fragment into pQE60IBB (1–63 aa of Rch1).

**Recombinant protein expression and purification.** RanQ69L, Imp $\alpha$ , Imp $\beta$ , Imp $\beta$  (45–462), transportin, Imp5, Imp7 and CAS were purified as previously described (Görlich *et al.*, 1996; Kutay *et al.*, 1997a,b; Jäkel and Görlich, 1998). Untagged CRM1 was expressed from pET3a (Paraskeva *et al.*, 1999) in BL21 DE3 at 23°C with 0.5 mM IPTG. CRM1 was purified after precipitation of the bacterial lysate with 35% ammonium sulfate and buffer exchange to 50 mM Tris pH 7.5, 100 mM potassium acetate (KOAc) on MonoQ followed by gel filtration on Superdex 200 (Pharmacia). Imp9 was expressed in XL1 blue and purified on Ni-agarose, followed by precipitation with 37% ammonium sulfate and gel filtration.

IBB–GST was expressed in BLR(pREP4), purified on Ni-agarose and the buffer exchanged to 50 mM HEPES pH 7.5, 250 mM KOAc, 2 mM magnesium acetate [Mg(OAc)<sub>2</sub>], and 250 mM sucrose.

**Solution binding experiments.** For each binding reaction, 10  $\mu$ l of streptavidin–agarose beads were presaturated with the respective histone. Binding from rabbit reticulocyte lysate (Promega) was performed such that 100  $\mu$ l lysate was diluted into binding buffer (50 mM HEPES pH 7.5, 50 mM KOAc, 2 mM MgCl<sub>2</sub>) to yield a final sample volume of 600  $\mu$ l.

A 100  $\mu$ l aliquot of *E. coli* lysate was supplemented with the corresponding transport receptor to a concentration of 2  $\mu$ M. Binding was performed in a final volume of 600  $\mu$ l in 50 mM Tris pH 7.5, 300 mM NaCl and 4 mM MgCl<sub>2</sub>.

Subsequent binding reactions were carried out at 4°C for 4 h. Beads were then washed three times in binding buffer and bound proteins were eluted with SDS sample buffer and analysed by SDS–PAGE.

**Protein identification by mass spectrometry and databank searching.** The tryptic peptide mixture was desalted by ZipTip C18 (Millipore Corporation, Bedford, MA) and analysed by nano-electrospray mass spectrometry. A 5  $\mu$ l aliquot of the peptide mixture were lyophilized and dissolved in 5  $\mu$ l methanol/1% formic acid (1:1). The MS/MS measurement was performed with a nano-electrospray hybrid quadrupole mass spectrometer Q-ToF (Micromass, Manchester, UK). The collision gas was argon at a pressure of  $6.0 \times 10^{-5}$  mbar in the collision cell.

To identify the respective proteins we used the sequence tag program, which combines partial manual spectrum interpretation of about three aminoacids (sequence tag) with the residual mass N- and C-terminals of the interpreted region and the peptide mass to search in a nonredundant translated nucleotide database.

**Antibodies.** The anti-Imp $\beta$  and anti-Imp7 antibodies were a kind gift of Dr Dirk Görlich.

**Permeabilized cell assay.** *In vitro* transport reactions were performed essentially as described previously (Kutay *et al.*, 1997a; Jäkel *et al.*, 1999). For core histone import with recombinant transport receptors the following buffer was used: 50 mM HEPES pH 7.5, 150 mM KOAc, 1 mM Mg(OAc)<sub>2</sub>, 0.5 mM EGTA and 250 mM sucrose. Further details are given in the figure legends.

**Supplementary data.** Supplementary data are available at *EMBO reports* Online.

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