# Nuclear import of histone fold motif containing heterodimers by importin 13

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*Patrick Walker*
aus Frankfurt am Main

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I would like to use the term 'We' instead of 'I' because during a PhD thesis one is usually supported by other persons, in particular the supervisors and lab members. However, the presented work in this dissertation and the writing were essentially obtained and evaluated by myself.

Parts of the data presented in this dissertation were electronically published (Epub ahead of print) in February 2009 in the Journal of Biological Chemistry with the titel: '*Importin 13 mediates nuclear import of histone fold containing CHRAC heterodimers*' (Walker, P., Doenecke, D. and Kahle, J.).

*Für Kerstin*

*In der Wissenschaft gleichen wir allen nur den Kindern, die am Rand des Wissens hier und da einen Kiesel aufheben, während sich der weite Ozean des Unbekannten vor unseren Augen erstreckt.*

Sir Isaac Newton (1643-1727)

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# Abbreviations

| | |
|---|---|
| **Å** | Ångström |
| aa | amino acid |
| ACF1 | ATP-utilizing chromatin assembly and remodeling factor 1 |
| amp | ampicillin |
| APS | ammonium peroxodisulfate |
| ARM | armadillo |
| ATP | adenosine triphosphate |
| **B**IB | beta-like import receptor binding |
| bp | base pairs |
| BSA | bovine serum albumin |
| **C** | cytosine |
| CHRAC | chromatin accessibility complex |
| cNLS | classical NLSs |
| CRM | chromosome region maintenance |
| CIP | Calf intestinal alkaline phosphatase |
| **d** | distilled |
| Da | Dalton |
| DAPI | 4'-6-Diamino-2-phenylindol-dihydrochlorid |
| ddNTP | dideoxy nucleoside triphosphate (ddATP, ddCTP, ddGTP, ddTTP) |
| DMSO | dimethyl sulfoxide |
| DNA | deoxyribonucleic acid |
| DNase | deoxyribonuclease |
| dNTP | deoxyribonucleoside triphosphate (dATP, dCTP, dGTP, dTTP) |
| ds | double strand |
| DTT | dithiothreitol |
| ***E**. coli* | *Escherichia coli* |
| ECL | Enhanced chemiluminescence |
| EDTA | ethylenediaminetetraacetate |
| EEG | EGFP-EGFP-GST |
| EGFP | enhanced green fluorescent protein |
| EGTA | ethylene-bis (oxyethylenenitrilo) tetraacetic acid |
| eIF1A | eukaryotic translation initiation factor 1A |

| | |
|---|---|
| **F**BS | fetal bovine serum |
| Fig. | figure |
| **G** | guanine |
| GDP | guanosine-diphoshate |
| GR | glucocorticoid receptor |
| GST | glutathione S-transferase |
| GTP | guanosine triphosphate |
| **H**EAT | Huntingtin, Elongation factor 3, 'A' subunit of protein phosphatase A, and TOR1 lipid kinase |
| HEPES | *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid |
| HFM | histone fold motif |
| HIV | human immunodeficiency virus |
| hnRNP | heterogenous nuclear ribonucleoprotein |
| HRP | horseradish peroxidase |
| **I**BB | importin β binding |
| IgG | immunoglobulin G |
| imp | importin |
| IPTG | isopropylthio-ß-D-galactoside |
| **K**ap | karyopherin |
| kDa | kilodalton |
| **L**B | Luria Bertani |
| LMB | Leptomycin B |
| LSE | low salt extract |
| **M**CS | multiple cloning site |
| MDa | megadalton |
| mRNA | messenger RNA |
| MW | molecular weight |
| **n**cNLS | non-classical NLS |
| NCP | nucleosome core particle |
| NC2 | negative cofactor 2 |
| NE | nuclear envelope |
| NES | nuclear export signal |
| NLS | nuclear localization signal |
| NPC | nuclear pore complex |

| | |
|---|---|
| NTF2 | nuclear transport factor 2 |
| **O**D | optical density |
| **p** | plasmid |
| PAGE | polyacrylamide gelelectrophoresis |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| PGC | primordial germ cells |
| PTHrP | parathyroid hormone-related protein |
| **R**an | Ras related nuclear protein |
| RanBP | Ran binding protein |
| RanGAP | Ran GTPase activating protein |
| RanGEF | Ran guanine nucleotide exchange factor |
| RCC1 | regulator of chromosome condensation 1 |
| RFP | red fluorescent protein |
| retic | reticulocyte lysate |
| RNA | ribonucleic acid |
| RNase | ribonuclease |
| RNPs | ribonucleoprotein particles |
| rpm | rounds per minute |
| rRNA | ribosomal RNA |
| ***S**. cerevisiae* | *Saccharomyces cerevisiae* |
| SDS | sodium dodecyl sulphate |
| SNF2H | sucrose nonfermenting-2 homolog |
| ss | single strand |
| SUMO | small ubiquitin-related modifier/modification |
| SV40 | simian virus 40 |
| **T** | tymine |
| TAF | TBP-associated factor |
| TBE | Tris-boric acid-EDTA |
| TBP | TATA-binding protein |
| TBS | Tris buffered saline |
| TBST | Tris buffered saline containing Tween 20 |
| TE | Tris-EDTA buffer |
| TELT | Tris-EDTA-LiCl-Triton |

| | |
|---|---|
| TEMED | tetramethylethylenediamin |
| TFIIA | transcription factor II A |
| TRIS | tris (hydroxymethyl) aminomethane |
| **U**V | ultraviolet |
| **W**AKZ | WSTF/ACF1/KIAA0314/ZK783.4 |
| wt | wild type |

One letter code for amino acids:

| | | | |
|---|---|---|---|
| A | alanine | N | asparagine |
| C | cysteine | P | proline |
| D | aspartic acid | Q | glutamine |
| E | glutamic acid | R | arginine |
| F | phenylalanine | S | serine |
| G | glycine | T | threonine |
| H | histidine | V | valine |
| I | isoleucine | W | tryptophan |
| K | lysine | Y | tyrosine |
| L | leucine | X | any amino acid |
| M | methionine | φ | hydrophobic amino acid |

# 1 Introduction

> *'I could exceeding plainly perceive it to be all perforated and porous, much like a Honey-comb, but that the pores of it were not regular;…these pores, or cells, were not very deep, but consisted of a great many little Boxes, separated out of one continued long pore, by certain Diaphragms.'*

Cited above is nothing less than the first report and with that the definition of the term *cell* as the basic unit of life. It was established by Robert Hooke (18 July 1635 – 3 March 1703), an English polymath, and published in his book *Micrographia* in 1665. Hooke, 'the father of microscopy', came to this appellation because the boxlike cell walls in the cork tissue he was looking at reminded him of the cells of a monastery. However, it is noticeable that even in this very first report about cells, the separation was observed as a fact. As science and microscopic techniques advanced, it became clear that not only cells are separated from each other, but that each cell contains enclosed structures like a body contains organs. Hence, it was the German zoologist Karl August Möbius (7 February 1825 – 26 April 1908), although his definition was limited to unicellular organisms, was the first who used the term *organelle,* the diminutive form of the word organ, to define differently formed parts within a cell (Möbius, 1884). In the eukaryotic cell, one of the organelles separated from other compartments is the nucleus. It is embedded in the cytoplasmic compartment and enclosed with a double lipid bilayer membrane called nuclear envelope (NE) that is continuous with the endoplasmatic reticulum. The enclosure of the genetic information in one compartment and thus, the separation of nuclear DNA replication and transcription from cytoplasmic protein synthesis require the steady exchange and distinct transport mechanisms for messenger RNA, transfer RNA, ribosomal RNA and proteins between those two compartments. The advantage of the formation of compartments for the eukaryotic cell is genetic stability and the possibility of gene expression control as well as giving the cells a separated environment for processing of primary transcripts before conveying them to translation. On the other hand, this compartmentalization necessitates a whole orchestra of different proteins to facilitate nucleocytoplasmic exchange.

## 1.1 Nucleocytoplasmic transport - An overview

The central site of exchange between the cytoplasmic and the nuclear compartment is the nuclear pore complex (NPC). The NPC is composed of proteins collectively referred to as nucleoporins (Cronshaw et al., 2002; Rout et al., 2000). The complex structure of each NPC represents one of the largest supramolecular assemblies in the eukaryotic cell (Reichelt et al., 1990) in which one NPC facilitates the passage of 1000 molecules per second. Thus, at least 10 molecules simultaneously get through each NPC (Ribbeck and Görlich, 2001; Yang et al., 2004). In a eukaryotic interphase cell, the NPC is embedded in the NE and spans its complete width, providing a central aqueous channel for the exchange of molecules. Because the NPC remains open for diffusion of ions, molecules and other small molecules, but at the same time represents a distinct barrier for all proteins larger than ~ 40 kDa, it is as a consequence responsible for the selective exchange of macromolecules between the nucleus and the cytoplasm (for review, see Cook et al., 2007; Fried and Kutay, 2003). Proteins that have to enter or to leave the nucleus therefore are transported as a cargo of soluble transport receptors, collectively called karyopherins or more specific as importins and exportins, respectively, in an energy-dependent process. Since the active translocation through the NPC provides a much more efficient and more regulated transport mechanism, molecules which potentially may passively diffuse through the NPC are nevertheless transported in a karyopherin-dependent manner (for review, see Conti and Izaurralde, 2001; Mattaj and Englmeier, 1998; Rout and Aitchison, 2001; Weis, 2003).

The transport receptors are represented by the importin β-like superfamily. Depending on the direction of transport, the receptors are classified as importins or exportins. To ensure a permanent availability, the karyopherins must constantly circulate between the cytoplasm and the nucleus (for review, see Görlich and Kutay, 1999; Stewart, 2007). Thus, after crossing the NPC and the dissociation of transport receptor and cargo, karyopherins have to be recycled. The assembly and disassembly of a karyopherin-cargo complex is dependent on the nucleotide-bound state of the small GTPase Ran which can switch between a guanine triphosphate (GTP) and a guanine diphosphate (GDP) state (Scheffzek et al., 1995; Vetter et al., 1999b). Whereas the RanGTP-bound state is catalyzed by the Ran guanine nucleotide exchange factor RanGEF, the RanGDP form is dependent on RanGAP, the RanGTPase-activating protein and other associated regulating proteins.

Importins bind their cargo in general only in the presence of RanGDP, whereas exportins interact with substrates exclusively in the presence of RanGTP. The directionality of these transport processes is maintained by a steep gradient of RanGTP across the NE with high levels in the nucleus and low levels in the cytoplasm (Görlich et al., 1996b; Izaurralde et al., 1997). Since

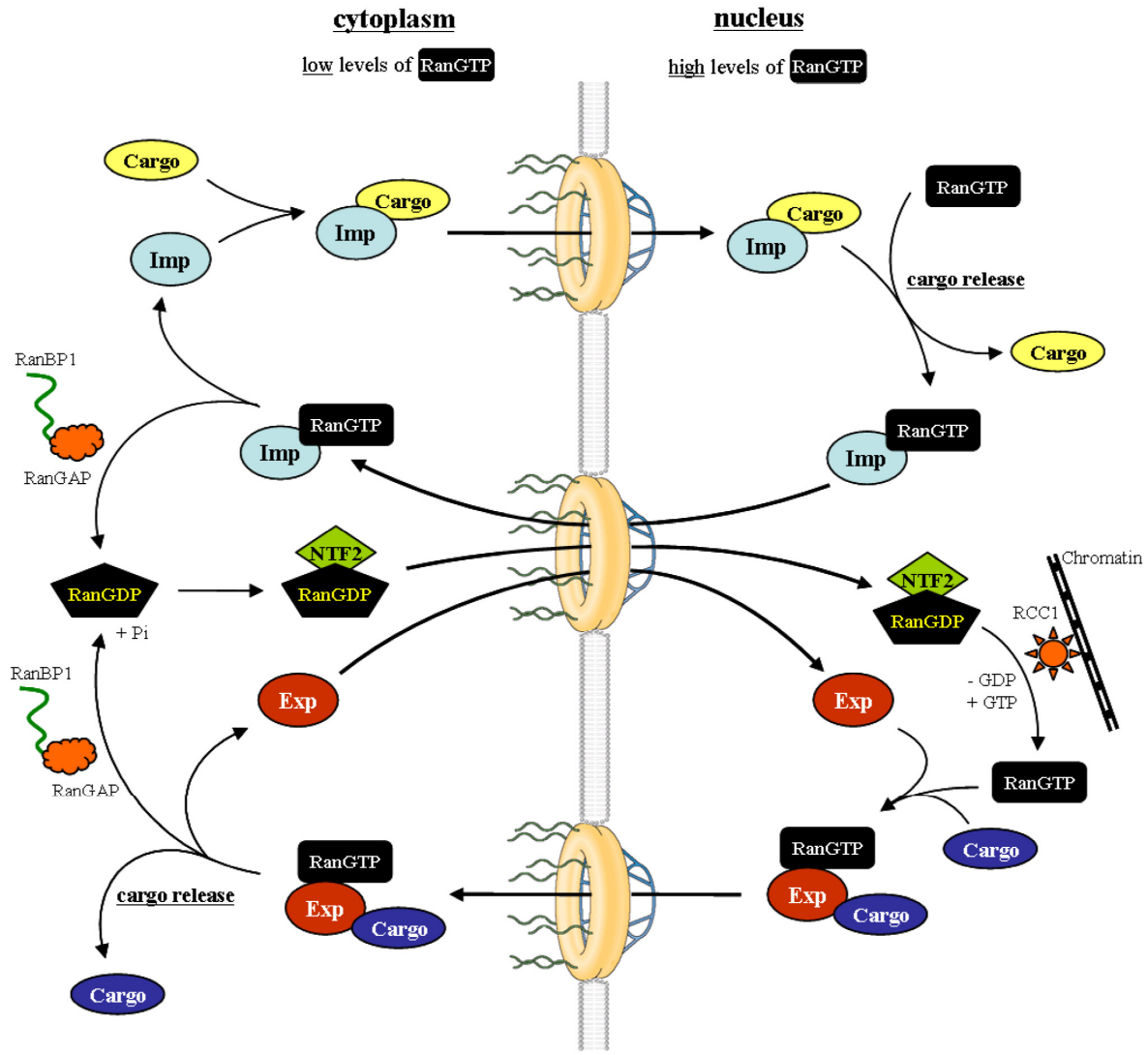


**FIG. 1: Illustration of nucleocytoplasmic transport processes.** Nuclear import and nuclear export pathways are controlled by a steep gradient of RanGTP across the NE with low concentrations in the cytoplasm nucleus and high concentrations in the nucleus. In the cytoplasm, import receptors (Imp) bind to non-classical NLS-containing cargoes in the absence of RanGTP. After the translocation through the NPC, mediated by interaction of the transport factors with FG-repeats of various Nucleoporins (Nups), the cargo dissociates from the nuclear import receptor under the influence of RanGTP. The RanGTP-bound importins are recycled into the cytoplasm, where RanGTP is hydrolyzed from RanGAP and the associated factors RanBP1 (and RanBP2). This leads to a dissociation of the import factor and the anew coupling with a cargo. In contrast, exportins (Exp) bind their cargo in the nucleus in the presence of RanGTP, forming stable trimeric complexes. Upon the action of RanGAP in the cytoplasm, the complex dissociates and the cargo is released. The cytoplasmic RanGDP is transported back into nucleus via its import receptor NTF2. There, nucleotide exchange from GDP to GTP is mediated by the chromatin associated guanine exchange factor (RanGEF) RCC1. (This figure was partially adapted from a figure created by Görlich and Kutay (Görlich and Kutay, 1999)).

RanGTP leaves the nucleus once bound to its export cargo and is hydrolyzed in the cytoplasm, the GDP-bound form of Ran has to be reimported into the nucleus. This step is mediated by the NTF2-like transport receptors, a group of transport factors specialized for the import of RanGDP (for review, see Stewart, 2000).

The recognition between the transport factor and the protein is mediated by specific sequence elements within the cargo protein. Proteins carrying a so-called nuclear localization signal (NLS) are imported into the nucleus whereas those carrying a nuclear export signal (NES) are exported from the nucleus. Because NLSs can be further differentiated into a classical (cNLS) and a non-classical (ncNLS) NLS another import factor, the adapter protein importin α, comes into play. Whereas proteins containing a ncNLS are recognized directly by the transport factor, cargoes with a cNLS are recognized by importin α that operates as an adapter for the binding to the karyopherin importin β (Conti et al., 1998; Görlich and Mattaj, 1996; Nigg, 1997).

In summary, the nucleocytoplasmic transport cycle can be differentiated into four major categories. Firstly, the assembly of a karyopherin-cargo complex, mediated by the binding of the transport signal containing substrate to the karyopherin. This happens in the cytoplasm for nuclear import processes as well as in the nucleus for export processes. In some cases an adapter is needed. Secondly, the translocation through the NPC is mediated by weak interactions between the nucleoporins and the karyopherins. The third step is the disassembly of the transport complex. Thus, RanGTP is responsible for the dissociation of import complexes in the nucleus whereas the hydrolysis of RanGTP to RanGDP results in the dissociation of export complexes in the cytoplasm. The fourth and last step then is the recycling of the transport receptor. Import receptors like importin β are actively exported from the nucleus in a RanGTP bound fashion, whereas importin α transport is dependent on its RanGTP-bound export factor CAS. Cytoplasmically located exportins, however, reenter the nucleus on their own. (For a schematic model see Fig. 1.)

In the following paragraphs, the individual components of importin and exportin mediated nucleocytoplasmic transport of proteins and their functional interactions will be described in detail.

## 1.2 The nuclear pore complex (NPC)

NPC research began with the observation of pores in the structure of the nuclear envelope (NE) by electron microscopy in 1950 (Callan and Tomlin, 1950). Nowadays, electron tomography and X-ray crystallography have fathomed the NPC down to the ultrastructural and atomic level and thereby widened the understanding of the NPC as a selective dynamic barrier.

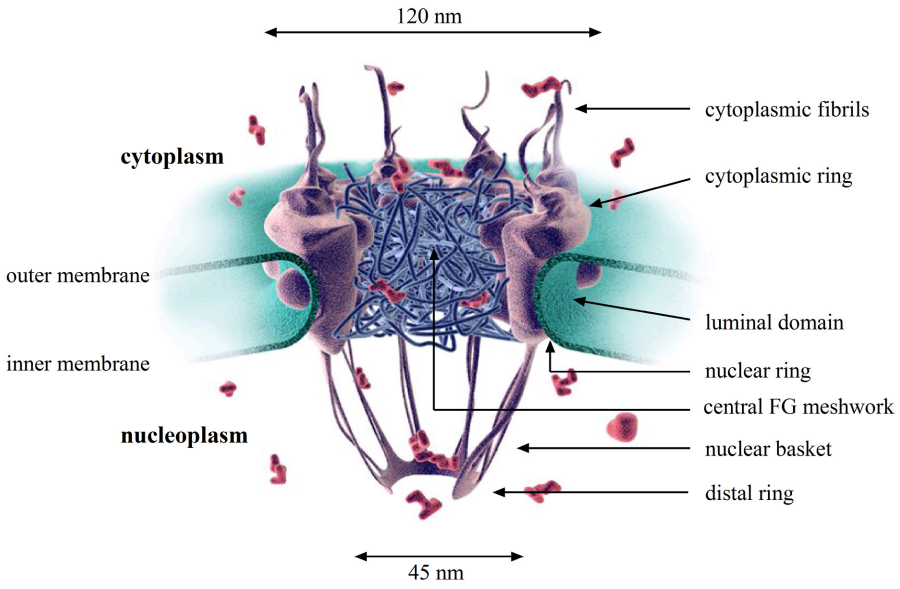


**FIG. 2: The nuclear pore complex (NPC) - a selective dynamic barrier.** Three-dimensional model of a NPC. Each complex spans the phospholipid bilayer of the nuclear envelope through a pore, formed by the fusion of the outer and the inner nuclear membrane. The central part of the NPC is formed in a cylindrical eight spoke symmetry resulting in a 'doughnut'-like structure, sandwiched between the nuclear and the cytoplasmic ring. These rings are anchoring sites for the cytoplasmic filaments on the one site and for the nuclear filaments on the other site. Whereas the cytoplasmic filaments are flexible, the nuclear filaments are bundled by the distal ring, forming the so-called nuclear basket. The pore of the NPC is filled with unstructured FG-repeat rich Nucleoporins (Nups), connected through hydrophobic interactions between the different FG-repeats (Frey et al., 2007; Patel et al., 2007; Ribbeck and Görlich, 2001; Ribbeck and Görlich, 2002) and resulting in the central meshwork. This system avoids the uncontrolled flux of molecules in and out of the nucleus. (The picture used in this figure was created by Samir S. Patel; http://sspatel.googlepages.com/nuclearporecomplex2)

Each NPC is a multiprotein complex of at least 456 individual proteins with a calculated mass of 44 MDa in yeast (Rout et al., 2000), whereas previous calculations had determined a mass between 55 and 72 MDa (Rout and Blobel, 1993; Yang et al., 1998). For vertebrates, a size of 60 MDa has been measured (Cronshaw et al., 2002). A calculation of 125 MDa for vertebrate NPCs proved to be wrong because of the mild isolation conditions chosen in this study (Reichelt et al., 1990). NPCs are spanning the nuclear envelope through a pore, where a lumen is formed by the continuity of the outer and inner nuclear membrane, consequently connecting the cytoplasm with the nucleoplasm. Despite the huge mass, NPCs have a simple protein composition of only ~ 30 distinct proteins with an architectural structure conserved from yeast to mammals (Cronshaw et al., 2002; Rout et al., 2000). Multiple copies of these proteins, collectively called nucleoporins or

Nups, are arranged in a morphologically similar eight 'spoke' formation that is cylindrically arranged around the pore (Hsia et al., 2007). The resulting 'doughnut'-like structure has a height of ~ 35 nm and an outer diameter of ~ 120 nm. Originating from the central framework, eight filaments, integral parts of the NPC, are projecting towards the cytoplasm and the nucleus, respectively. Whereas the eight cytoplasmic filaments with a length of ~ 50 nm are unstructured, the nuclear filaments are assembled in a distal ring, forming the nuclear basket pointing ~ 60 nm into the nuclear interior (Alber et al., 2007; Beck et al., 2004; Beck et al., 2007; Stoffler et al., 2003; Yang et al., 1998). The central pore has a diameter of 45-50 nm at its narrowest position (Fahrenkrog and Aebi, 2003), and it has been shown that it allows the passage of macromolecules up to 50 MDa with a diameter of 40 nm (Pante and Kann, 2002). (For a three-dimensional model see Fig. 2.)

However, the answer to the key question, namely how NPCs control the access from and to the nucleus, lies in the composition of the nucleoporins. Referring to their architecture, Nups can be grouped in three classes: the transmembrane group, the central framework group and the peripheral group (Devos et al., 2006; Schwartz, 2005). From exceeding interest is the peripheral group because besides small structured anchoring sites it contains large unstructured and flexible regions with repeated, characteristic Phenylalanine-Glycine residues also known as FG-repeats, which are lining the lumen of the NPC (Denning et al., 2003; Denning and Rexach, 2007). According to the sequence of FG-repeats, they can be classified as FG-, FXFG- or GLFG-domains, where X is any residue (Suntharalingam and Wente, 2003). The unfolded nature of these FG domains is the basis for selective nuclear transport. These regions mediate the interaction with transport receptor-cargo complexes but also prevent undefined entering or exiting of proteins by passive diffusion (Ben-Efraim and Gerace, 2001; Ribbeck and Görlich, 2002). The details of the gating mechanism at the NPC though are still a matter of debate, and several models have been proposed. The Brownian affinity or *virtual gating* model (Rout et al., 2003; Rout et al., 2000) considers the NPC as an aqueous tunnel with FG-repeat containing Nups as entropic barrier. According to this model, interaction between FG-Nups and transport receptors lowers the activation energy used for cargo translocation. Thus, the NPC acts as a catalyst, providing kinetic advantages for transport receptor-cargo complexes. The *'oily-spaghetti'* model (Macara, 2001) postulates that unstructured and non-interacting FG-Nups, normally occluding the NPC channel in a repulsive bristle-like conformation, can be pushed aside by receptor-cargo complexes. The flux of collapsing and restructuring FG-Nups promotes the translocation of cargo complexes and simultaneously maintains the entropic barrier for other proteins (Lim et al., 2007; Lim et al., 2006). In contrast, the *selective phase* model (Ribbeck and

Görlich, 2001; Ribbeck and Görlich, 2002) predicts the interaction of FG residues via hydrophobic interaction, resulting in a sieve-like hydrogel. Upon contact with the nuclear transport receptors, the hydrophobic FG-cluster disengages, now interacting with the transport complex. This bindings allows a 'melting' through the meshwork before it reseals behind the transport complex (Frey and Görlich, 2007; Frey et al., 2006). In addition, it has been shown that hydrogel formation is not restricted to one FG-domain and that the barrier function against inert molecules remains intact even during the influx of transport-complexes (Frey, 2008). Based on FG-motif interaction studies, a fourth mechanism has emerged. The *two-gate* model (Patel et al., 2007) combines the selective phase and the virtual gate model by identifying weak cohesive interactions between GLFG-domains in the center of the NPC and non-interacting FxFG-Nups on both cytoplasmic and nuclear peripheries acting like repulsive bristles.

## 1.3 Nuclear transport receptors

As long as proteins cannot directly interact with a NPC, or pass the NPC by passive diffusion, their active transition in and out of the nucleus is mediated by soluble nucleocytoplasmic transport receptors, constantly shuttling between the nucleus and the cytoplasm. The largest class of transport factors belongs to the superfamily of importin β-like proteins and accounts for the majority of cargo transport through the NPC (Radu et al., 1995). The name derives from importin β (Kap95 in yeast), the first identified import receptor (Görlich et al., 1995). The β-like family of soluble transport receptors includes at least 14 members in yeast and more than 20 different members in human (for review, see Fried and Kutay, 2003; Görlich and Kutay, 1999; Macara, 2001; Ström and Weis, 2001, and also appendix, table III). According to their ability to transport proteins in or out of the nucleus, transport receptors are divided into importins and exportins. Generally, importins bind substrates in the absence of Ran in the cytoplasm and release their cargo upon RanGTP binding in the nucleus (Görlich et al., 1996b; Izaurralde et al., 1997; Rexach and Blobel, 1995). In contrast, exportins load cargo proteins only in the presence of RanGTP within the nucleus. The release of cargoes is accomplished in the cytoplasm when the Ran-bound GTP is hydrolyzed to GDP (Bischoff and Görlich, 1997; Fornerod et al., 1997; Kutay et al., 1997a). All import receptors contain tandem repeats of ~ 40 amino acids, named HEAT repeats after the non-family members Huntington, elongation factor 3, PR65/A subunit of protein phosphatase 2A and the TOR lipid kinase (Andrade and Bork, 1995). Each HEAT repeat consists of a pair of α-helices, known as A helices and B helices, connected by a short loop. The modular design of 19 to 20 HEAT repeats per molecule results in an overall spiral-like, superhelical architecture, and implies an intrinsic flexibility within the protein (Chook and

Blobel, 1999; Conti et al., 2006; Vetter et al., 1999a). Until now, just three crystal structures of known karyopherins, namely importin β, transportin and the export factor Cse1, have been solved (Cingolani et al., 1999; Imasaki et al., 2007; Matsuura and Stewart, 2004). However, studies with known importin β cargoes, such as the IBB domain from importin α, the transcription factor SREBP-2 and the parathyroid hormone-related cargo protein (PTHrP), have identified different interaction sites at the inner concave surface of importin β formed by the B helices (Cingolani et al., 2002; Cingolani et al., 1999; Lee et al., 2003, and see also Fig. 3). Recent studies with crystal structures of transportin in a cargo-free form as well as in complex with the ncNLS of hnRNP D (Suzuki et al., 2005), JKTBP (Kawamura et al., 2002) and TAP (Truant et al., 1999), have revealed that transportin harbors a high affinity NLS-binding site A and one low affinity site B essential for cargo interaction (Imasaki et al., 2007). This suggests that karyopherins harbor multiple binding sites for the recognition of various cargo proteins. In addition, karyopherins also show cargo-dependent conformational variability (Fukuhara et al., 2004).

Binding sites at the concave surface are also provided for the small GTPase Ran. The amino-terminus of RanGTP binds to importin β at three separate sites, with interaction sites in HEAT repeat 1-3, 12-15 and at the conserved acidic loop within HEAT repeat 8 (Lee et al., 2005; Vetter et al., 1999a, and see also Fig. 3). In contrast, structural studies in the yeast exportin Cse1p (CAS in human) have identified a RanGTP binding site at the carboxy-terminus of HEAT repeat 1-3 and at the amino-terminus at HEAT repeat 13-14 and 19 (Matsuura and Stewart, 2004). In addition, importins and exportins show different affinities for RanGTP binding. Whereas exportins bind to RanGTP with high affinity only when complexed with a cargo protein (Fornerod et al., 1997; Kutay et al., 1997a), importins display an unrestricted high affinity for RanGTP thus providing a permanent accessibility (Görlich et al., 1997).

Separated from the interaction sites for RanGTP and the cargo molecules at the inner surface of nucleoporins, the convex outer surface, formed by the A helices, is responsible for low affinity interaction with the FG-Nups of the NPC (Bayliss et al., 2000; Ribbeck and Görlich, 2001). For importin β, it has been shown that phenylalanine containing side chains of FG-Nups protrude into a hydrophobic pocket on the surface of the transport receptor at HEAT repeat 5 and 6 and between the A helices of HEAT repeat 6 and 7 (Bayliss et al., 2000), but also between HEAT repeat 14-15 and 15-16 (Bednenko et al., 2003). A more recent study has also identified a pocket within HEAT 7-8 (Liu and Stewart, 2005). The different importin β binding sites for FG-Nups at the outer surface and cargo proteins at the inner surface (see Fig. 3) explain how transport factors

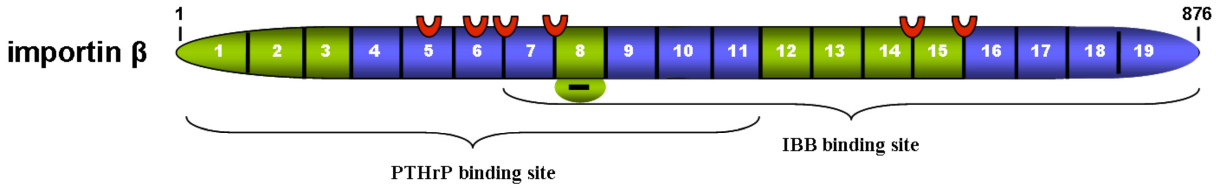


**FIG. 3: Schematic domain organization of importin β.** Importin β (1-876aa) consists of 19 HEAT repeats. Each HEAT repeat is assembled by a pair of α-helices, known as A and B helices, which are connected by a short loop. In green, the binding sites for RanGTP at the inner surface of importin β, including the acidic loop (-) at HEAT repeat 8, are indicated (Lee et al., 2005; Vetter et al., 1999a). Hydrophobic pockets mediating the interaction between the nuclear transport receptor and FG-Nups at the NPC are indicated in red at the outer surface of importin β (Bayliss et al.,2000; Bednenko et al., 2003; Liu and Stewart, 2005). The binding sites for the IBB domain (Cingolani et al., 1999) and PTHrP (Cingolani et al., 2002) at the inner surface of importin β are indicated by arrows.

can shuttle through the NPC mediating the interactions with the nucleoporins independently from their complexation with or without a cargo.

In contrast, the recognition between a transport receptor and a cargo protein is mediated by specific transport signals within the amino acid sequence of the cargo. Unlike many other cargo proteins with a non-classical NLS (ncNLS), those containing a so-called classical nuclear localisation signal (cNLS) (see chapter 1.5.1) are not recognized by transport receptors of the importin β-like family directly, but by the transport adapter importin α. The structure of importin α is composed of ten super helices forming tandem armadillo (ARM) repeats, first discovered in the ARM protein in *Drosophila* (Peifer et al., 1994), and the flexible amino-terminal importin β-binding (IBB) domain (Conti et al., 1998; Herold et al., 1998; Kobe, 1999). ARM repeats, structurally related to HEAT repeats (Andrade et al., 2001), consist of ~ 40 amino acids and are formed by the α-helices H1, H2, and H3. These helices are the building blocks of importin α thus resulting in an elongated protein with a superhelical twist. Much like the B helices in importin β, the H3 helices form a concave inner surface responsible for various electrostatic and hydrophobic contacts of ARM repeat 2-4 and 7-9 with a given cNLS (Conti and Kuriyan, 2000; Fontes et al., 2003; Fontes et al., 2000). In contrast to the karyopherin β-like import receptors that reenter the cytoplasm solely in a RanGTP bound state, recycling of importin α back into the cytoplasm is mediated by the tenth ARM repeat, which serves as the binding site for the mammalian importin α export receptor CAS (Kutay et al., 1997a). Because binding of CAS, which binds predominantly to cargo-free importin α, is also dependent on nuclear RanGTP, a

ternary export complex of CAS, RanGTP and importin α is formed. The flexible IBB domain of importin α, however, has a dual function. It can act *in trans* by binding to the inner concave surface of importin β or *in cis* by binding to itself at the cNLS-binding site. Thus, in the nucleus in the absence of a cargo the IBB domain mimics a cNLS and interacts as a competitive inhibitor with the cNLS-binding pocket, whereas in the cytoplasm in the presence of a cNLS-containing cargo a ternary import complex of cargo, importin α and importin β is formed (Görlich et al., 1996a; Weis et al., 1996b).

## 1.4 The small GTPase Ran and its function in nuclear transport

### 1.4.1 Ran and the nucleotides GDP and GTP

Ran stands for Ras-related-Nuclear Protein and belongs to the Ras superfamily of small GTPases. It is a 24 kDa protein with the characteristic basic guanine nucleotide-binding domain (G domain). The G domain is responsible for binding to the inner concave surface of transport receptors and possesses a two loop region, referred to as switch I and switch II (Vetter et al., 1999a; Vetter and Wittinghofer, 2001). Upon switching between a GTP- and a GDP-bound form, this domain undergoes marked conformational changes (Bischoff and Ponstingl, 1991; Drivas et al., 1990; Melchior et al., 1993; Moore and Blobel, 1993). This enables RanGTP to bind to karyopherins whereas the carboxy-terminal helix of RanGDP provides a steric barrier that prevents binding (Nilsson et al., 2002; Scheffzek et al., 1995). The state of binding is thereby catalyzed by two regulatory proteins, the Ran guanine nucleotide exchange factor RanGEF (Bischoff and Ponstingl, 1991; Ohtsubo et al., 1987), also known as RCC1 (regulator of chromosome condensation 1), and the RanGTPase-activating protein RanGAP1 (Bischoff et al., 1994) in combination with the Ran binding proteins RanBP1 (Beddow et al., 1995; Bischoff et al., 1995a; Coutavas et al., 1993) and RanBP2 (Wu et al., 1995; Yokoyama et al., 1995).
RanGAP is mainly present at the cytoplasmic NPC periphery where it is bound to RanBP1 that contains a single Ran-binding domain (Coutavas et al., 1993). In higher eukaryotes, RanGAP is via its SUMO-modified carboxy-termini additionally associated with the nucleoporin RanBP2, also known as Nup358 and component of the cytoplasmic fibrils (Mahajan et al., 1997; Matunis et al., 1996). RanBP2 itself contains four Ran-binding sites (Delphin et al., 1997). RanBP1 and RanBP2 stimulate RanGAP-mediated hydrolysis and the dissociation of RanGTP from the transport receptor (Bischoff and Görlich, 1997; Bischoff et al., 1995b). GTPase activation is facilitated by sequestering the carboxy-terminus of Ran which otherwise seems to be inaccessible for RanGAP. Crystallographic data suggest that this access is mediated by RanBP1

(Vetter et al., 1999b), leading to a 10 fold stimulated GTP-hydrolysis, and in fact, an intermediate complex of importin β, RanGTP and RanBP1 has been observed (Chi et al., 1996; Görlich et al., 1996b; Lounsbury and Macara, 1997). In yeast, however, binding of the importin β homolog, Kap95p to Ran GDP which is abrogated upon cargo binding, has also been demonstrated (Forwood et al., 2008).

Export complexes as well as the recycling of importin α or other import receptors are dependent on RanGTP. Because of the constant efflux of RanGTP from the nucleus, Ran, in its GDP-bound form, has to be reimported into the nucleus. This transport process is mediated by the specific RanGDP import receptor NTF2 (Ribbeck et al., 1998; Smith et al., 1998). In contrast to RanGTP which would sterically collide with NTF2 (Vetter et al., 1999b), RanGDP is specifically recognized at the switch II region (Moore and Blobel, 1994; Paschal and Gerace, 1995; Stewart et al., 1998; Weis et al., 1996a). NTF2 itself is a homodimeric protein that shows low-affinity interactions with the FG-containing Nups upon NPC translocation (Bayliss et al., 2002), although it is not related to karyopherins. After nuclear import, NTF2 and RanGDP dissociate; NTF2 returns to the cytoplasm and the Ran-bound GDP is exchanged for GTP.

This nucleotide exchange is mediated by the exclusively nuclear protein RanGEF, also named RCC1. It is found at the periphery of the nucleus where it is associated with chromatin via interaction with the core histones H2A and H2B (Nemergut et al., 2001) and with double stranded DNA (Chen et al., 2007). RCC1 catalyzes the nucleotide replacement of RanGDP by RanGTP thus, increasing the low intrinsic rate of nucleotide dissociation by several orders of magnitude (Vetter and Wittinghofer, 2001). Data from crystallographic and biochemical approaches reveal that, under the action of RCC1, a loop, called β-wedge, is inserted into Ran. This loop induces dissociation and the uptake of nucleotides, and GTP loading is favored *in vivo* due to the high GTP concentration in the nucleus (Renault et al., 2001). In addition, RCC1 also stabilizes the nucleotide-free form of Ran to assure a proper nucleotide binding which is probably assisted by the zinc finger-containing Ran-binding Nup153 (Schrader et al., 2008). To sum up, the compartment specific separation of RanGEF and RanGAP leads to a nuclear concentration of RanGTP that is about 1000 times greater than the concentration of cytoplasmic RanGTP (Görlich et al., 2003). On the other hand, it yields high levels of the GDP bound form of Ran in the cytoplasm (for review, see Dasso, 2002; Weis, 2003). This different nucleotide distribution is also the basis for the specific cargo dissociation processes in import- and export-complexes.

### 1.4.2 Ran and Cargo release

In the nucleus, switch I and II of RanGTP are able to bind to the import-complex via interactions with importin β at HEAT repeat 1-3, 12-15 and via electrostatic interaction at the conserved acidic loop within HEAT repeat 8. It has been shown that cargo and RanGTP binding is mutually exclusive, because the binding sites are largely overlapping (Lee et al., 2005; Vetter et al., 1999a). Thus, disassembly of the cargo protein is mediated by steric interference upon RanGTP binding. In addition, importin β appears to undergo conformational changes. A tightly packed conformation when bound to the IBB domain switches to a more loosened conformation by the binding of RanGTP, creating an incompatible conformation for proper cargo binding (Cingolani et al., 1999). In contrast to importin β, RanGTP binds to transportin by interactions with HEAT repeat 1-3 and the comparatively long acidic loop of HEAT repeat 8 (Chook and Blobel, 1999). Besides different RanGTP binding sites, the mechanism of cargo dissociation in importin β and transportin differs as well. Upon binding of RanGTP, the acidic loop of transportin blocks the NLS-binding site, whereas in the absence of RanGTP the loop has no contact to the negatively charged binding residues. Interaction between the acidic HEAT repeat 8 and the basic surface of Ran alters the electrostatic potential of the whole loop region. Thus, cargo release depends more on the alteration of the electrostatic potential within transportin than on actual changes in the conformational arrangement.

The direct binding of RanGTP to an importin β-like transport receptor might explain the dissociation of the cargo from the import complex. But how is cargo release in an adaptor-mediated nuclear import complex achieved when the cargo is associated with the ARM domain of importin α? Analogous to the import of a ncNLS-containing cargo, RanGTP binds to importin β upon reaching the nucleoplasm. This leads to conformational changes in importin β under which the IBB domain of importin α is released (Cingolani et al., 1999). The dissociation of the cargo from importin α is now mediated by the autoinhibitory influence of the IBB domain in which the affinity to the substrate is abrogated (for review, see Goldfarb et al., 2004). This is mediated by a nine-residue segment of the IBB domain that interacts with the cNLS-cargo binding site (Kobe, 1999). Another factor that influences cargo release is the nuclear localized nucleoporin Nup50 (Nup2 in yeast), which binds the ternary import complex at several sites (Booth et al., 1999; Hood et al., 2000; Lindsay et al., 2002; Solsbacher et al., 2000). It actively affects the disassembly by binding with its amino-terminal domain to distinctive regions of the importin α ARM domains. This leads to steric interference with the cargo in the binding pocket and finally to the release of the substrate. Nup2 itself is then removed from importin α in a two-step cooperation between CAS and the IBB domain (Matsuura and Stewart, 2005).

Unlike importins, exportins bind their cargo in the nucleus in the presence of RanGTP. Subsequently, a ternary export complex enters the cytoplasm and the cargo is released upon GTP hydrolysis. At present, Cse1, the yeast homolog of the human importin α export factor CAS, is the only exportin whose structure in both cargo-bound and unbound form has been determined (Cook et al., 2005; Matsuura and Stewart, 2005). Cse1 has two distinct binding sites for RanGTP interaction (Kutay et al., 1997a; Solsbacher et al., 1998). One binding residue is provided amino-terminally at HEAT repeats 1-3, the second carboxy-terminally at HEAT repeats 13-14 and 19. Importin α interacts with an insertion at the Cse1 HEAT repeat 8 which is, in contrast to the import factors, non-acidic and folds into two α-helices instead of forming a loop. Upon binding to Cse1, the IBB domain of importin α is folded *in cis*, capturing the autoinhibiting conformation to ensure that importin α recycling only occurs when no cargo is loaded. In comparison to this ternary complex, the unbound form of Cse1 possesses a much more closed conformation with intramolecular HEAT repeat interactions, and is unable to bind any cargo molecules. Under the influence of RanGTP, however, a more open conformation is induced. In this process, RanGTP binds weakly to the amino-terminus of the exportin. Then, in the presence of the cargo, Cse1 is induced into a higher-energy state that allows the additional binding of RanGTP to the carboxy-terminus so that both binding sites are engaged (Matsuura and Stewart, 2004). This higher-energy conformation, referred to as spring-loaded, is important for the dissociation in the cytoplasm, because hydrolysis of RanGTP to RanGDP would initiate the release of the stored energy resulting in the dissociation of the ternary complex. Studies of human and yeast Crm1, also named exportin 1, one of the most studied export factors, showed that the unbound, closed superhelical conformation and the more open structure upon cargo binding is likely to be a general feature of exportins (Fukuhara et al., 2004; Petosa et al., 2004). On the other hand, binding of a cargo and RanGTP evokes distinct conformational changes, suggesting that there is no general conformational regulation and that the mechanisms may be unique for each export receptor. Furthermore, there also seemed to be no general rule that distinguishes between import and export karyopherins, since certain transport factors like importin 13 and Kap142/Msn5 can function as import as well as export receptors (Mingot et al., 2001; Yoshida and Blobel, 2001).

## 1.5 Localization signals - The NLS and the NES

Directional nuclear import or nuclear export is of course dependent on the transport receptors and their regulators. This whole machinery, however, would be worthless if the active translocation cycles were not based on specific recognition processes between the transport

receptor and the cargo protein. For this purpose, cargo proteins contain specific signals within their sequence that are classified as nuclear export signals (NES) or nuclear localization signals (NLS). In contrast to other signal elements, NESs and NLSs are not cleaved from the protein but are integral parts of the protein and thus can, if required, be used several times. Moreover, transport signals do not contain a well defined consensus. Usually, features like hydrophobicity, length or charge are important, but a prediction of a general consensus motif (Dingwall and Laskey, 1991) at present appears to be impossible.

### 1.5.1 The nuclear localization signal (NLS)

As mentioned above, nuclear localization signals (NLS) can principally be divided into two different groups, the classical NLSs (cNLS) and the non-classical NLS (ncNLS). The existence of a great number of cNLS-containing proteins has been demonstrated. In fact, a recent study empirically determined that 45 % of the proteins in the cell have the potential to be transported via the cNLS pathway (Lange et al., 2007). These cNLS-containing proteins are recognized by importin α and imported into the nucleus as a ternary complex together with importin β (for review, see Görlich and Mattaj, 1996; Melchior and Gerace, 1995; Nigg, 1997). In contrast, substrates with a ncNLS are recognized directly by a member of the importin β-family.

The first transport signal to be identified was the cNLS of the *Xenopus* protein nucleoplasmin (Dingwall et al., 1982). In this case, the NLS consists of two short clusters of basic amino acids, essentially lysines (K) or arginines (R), separated by a spacer of 10 amino acids ($^{155}$KRPAATKKAGQAKKKK$^{170}$). With that peptide motif, the nucleoplasmin-NLS represents the prototype of a bipartite cNLS with the loose consensus sequence $(K/R)_2X_{10\text{-}12}(K/R)_3$ (Dingwall and Laskey, 1991; Robbins et al., 1991). Besides the bipartite signal, a cNLS can also be monopartite, with only a single stretch of positively charged amino acids, as in the simian virus 40 (SV40) large-T antigen (Kalderon et al., 1984; Lanford and Butel, 1984). The SV40-NLS is based on five basic amino acids ($^{126}$PKKKRKV$^{132}$) resulting in the loose consensus K(K/R)X(K/R) where X represents any amino acid. Regardless which motif is present, the cNLS binds to the major and minor NLS-binding pocket of importin α. Both pockets are generated by exposed, conserved tryptophan residues together with a set of asparagines located four residues downstream (Conti et al., 1998; Kobe, 1999). Upon binding, this leads to a conformation where the key lysine residues of the cNLS lie anti-parallel to the direction of the stacked, hydrophobic, and tryptophan-mediated importin α chain. This characteristic binding pattern also explains the autoinhibitory effect of the KRR-containing IBB domain of importin α (Harreman et al., 2003a; Harreman et al., 2003b).

Besides the indirect binding of import substrates via importin α, members of the karyopherin β-family can also directly interact with their cargo. This is the case for ncNLS-containing cargoes. Because ncNLSs can be relatively large, defining of even a loose consensus sequence is not possible. The occurrence of versatile ncNLS like the largely non-basic ~ 38 amino acid long, glycine and aromatic residue comprising M9 domain in hnRNP A1 (Bogerd et al., 1999; Pollard et al., 1996) or the extremely basic 43 amino acid containing beta-like importin receptor binding (BIB) domain of the ribosomal protein L23a (rpL23a) (Jäkel and Görlich, 1998) raises the possibility that primarily the three-dimensional structure of the ncNLS-containing cargo is crucial for the recognition process (Rosenblum et al., 1998).
In addition, although import receptors share only minimal sequence identity, some ncNLS containing cargoes are subject to redundant transport pathways. This is the case for the above mentioned rpL23a that is imported by importin β, importin 5, importin 7, and transportin (Jäkel and Görlich, 1998), but also for the core histones H2A, H2B, H3 and H4 that are at least transported by importin β, importin 5, importin 7, importin 9, and transportin (Baake et al., 2001a; Mühlhäusser et al., 2001). However, although only a few ncNLS motifs have been identified in detail, the list of known ncNLS cargoes is constantly growing (for review, see Pemberton and Paschal, 2005).

### 1.5.2 The nuclear export signal (NES)

Proteins, RNA molecules and ribonucleoprotein particles (RNPs) exiting the nucleus are actively transported via distinctive export factors. Besides the aforementioned export of the adapter protein importin α (see chapter 1.3) by CAS (Kutay et al., 1997a), several other export pathways have been identified e. g. the export of ribosomal subunits, signal recognition particles (SRPs) and certain cellular RNAs and RNPs by CRM1 (Ciufo and Brown, 2000; Fridell et al., 1996; Johnson et al., 2002; Murdoch et al., 2002; Paraskeva et al., 1999; Popa et al., 2002), mRNA export by exportin 5 and exportin t (Arts et al., 1998; Kim, 2004; Kutay et al., 1998), exportin 5 mediated export of tRNAs (Calado et al., 2002) and microRNA precursors (Lund et al., 2004), exportin 6 mediated reentering of actin-profilin complexes into the cytoplasm (Stüven et al., 2003) and several export pathways for translation factors mediated by exportin 4, exportin 5 and astonishingly by the import factor importin 13 (Bohnsack et al., 2002; Calado et al., 2002; Lipowsky et al., 2000; Mingot et al., 2001). Altogether, most export factors seem to possess specificity for a small group of cargoes or even for a single substrate. In contrast, for the export factor CRM1, also known as XPO1 and in the following referred to as exportin 1 (Fornerod et al., 1997; Stade et al., 1997), a broad substrate specificity has been demonstrated including

transcription factors, cell-cycle regulators or RNA binding proteins. Since exportin 1 cannot directly bind to RNA, it requires the assistance of a distinct adapter protein like the NES-containing PHAX (phosphorylated adaptor for RNA export), for snRNA export (Ohno, 1998; Ohno et al., 2000). However, adapter proteins are also suggested to specify an individual pathway since NMD3 is needed for the exportin 1-dependent export of pre-60S ribosomal subunits (Johnson et al., 2002; Thomas and Kutay, 2003; Trotta et al., 2003).

Two of the first and best characterized cargoes are the cAMP-dependent protein kinase A inhibitor (Wen et al., 1995) and the viral HIV 1 Rev protein (Fischer et al., 1995) which functions as an adapter protein for the export of unspliced HIV-RNAs. Both proteins are recognized by exportin 1 via their hydrophobic nuclear export signal (NES) (Fornerod et al., 1997; Fukuda et al., 1997; Ossareh-Nazari et al., 1997; Stade et al., 1997). The short, leucine-rich NES is a loosely conserved motif with three to four hydrophobic residues and the consensus $LX_{2-3}(L/I/V/F/M)X_{2-3}LX(L/I/V)$ where X can be any amino acid (Bogerd et al., 1999). Because of the individual character of each NES, different cargoes bind to exportin 1 with different affinities (Henderson and Eleftheriou, 2000). The fact that the group of exportin 1 dependent NES-comprising proteins is rather large was shown by a study of la Cour et al. (2003) in which already 75 different proteins were identified.

As for the export of importin α, the export of NES containing cargoes also requires the binding of RanGTP to exportin 1 (Fornerod et al., 1997). However, exportin 1 dependent export requires another cofactor, namely the RanBP3 protein (Mueller et al., 1998). Similar to the Nup50 cofactor in the classical nuclear import, nuclear RanBP3 binds directly and exclusively to exportin 1. This leads to a stabilization of the export-cargo interaction and to the formation of a quaternary complex consisting of exportin 1, the cargo, RanGTP and RanBP3 (Englmeier et al., 2001; Lindsay et al., 2001).

A large cargo recognition spectrum has not only been found for exportin 1, but has also been shown for exportin 7 with at least 12 different substrates (Mingot et al., 2004). In the same study it has been shown that transport processes like the export of the 14-3-3σ protein are not mediated by exportin 1 as previously suggested (Brunet et al., 2002; van Hemert et al., 2004), but are accomplished by the direct binding to exportin 7. To carry it to extremes, it has also been shown that export signals recognized by exportin 7 fundamentally differ from those recognized by exportin 1. Exportin 7 binding is not dependent on hydrophobic residues but rather on the folding and even on positively charged patches within the recognition motif.

## 1.6 Histones - Being part of something bigger

### 1.6.1 The core, the linker, and the chromatin

Every eukaryotic cell stores its genetic information in the nucleus as DNA molecules. The DNA is hierarchically packed and compacted into chromatin, where it is closely associated with a number of highly conserved proteins known as histones (for review, see Kornberg and Lorch, 1999; Marino-Ramirez et al., 2005; Widom, 1998). The basic level of chromatin organization is represented by the nucleosome core particle (NCP), composed of a disc-shaped octamer of the core histone proteins H2A, H2B, H3 and H4 around which 146-147 base pairs (bp) of DNA are wrapped (Luger et al., 1997b; Noll and Kornberg, 1977). The NCP is considered as the universally repeating unit in chromatin. It is formed by the association of two H3-H4 dimers into a tetramer and two H2A/H2B heterodimers on each side (Adams and Kamakaka, 1999; Arents and Moudrianakis, 1993). DNA is around this octamer in a 1.67 left-handed superhelical turn (Finch et al., 1977; Luger et al., 1997a). The resulting core particles are connected by linker DNA of variable length, from 10-60 bp (Kornberg and Thomas, 1974), forming the so called 10-nm-fiber, also known as beads-on-a-string array (Olins and Olins, 1974). The linker DNA region is bound by the H1 linker histone (Wolffe, 1997), leading to a stabilization of higher order chromatin structures (for review, see Happel and Doenecke, 2008). The complex of core particles and H1 associated to the linker DNA is referred to as a nucleosome (Oudet et al., 1975). However, chains of nucleosomes are further compacted into higher order structures of increasing complexity of a yet largely unknown architecture (Luger and Hansen, 2005).

### 1.6.2 Structure, dimerization, and the *histone fold motif*

Histones are among the most conserved proteins in eukaryotes (Mardian and Isenberg, 1978). Core histones contain a high proportion of the basic amino acids lysine and arginine, responsible for 14 ionic interactions between the histone octamer with the negative backbone of the DNA. In addition, core histones show striking similarities with each other concerning their three-dimensional structure. They generally consist of a hydrophobic globular domain and disordered basic amino- and carboxy-termini, also referred to as the histone tails, extending from both sides of the globular domain. These tails protrude from the center of the nucleosome core to stabilize higher order structures through internucleosomal interactions (Allan et al., 1982; Garcia-Ramirez et al., 1992; Schwarz et al., 1996). The positively charged amino-terminal tail is subject to extensive post-translational modifications like acetylation, methylation, phosphorylation or ubiquitylation. These modifications influence the structure of chromatin and, thus, are implicated

in DNA replication, transcriptional activation, silencing, and chromatin assembly. It also provides a direct link between the epigenetic regulation and the modification pattern of the core histones (for review, see Imhof, 2006; Peterson and Laniel, 2004).

The globular domain contains the *histone fold motif* (HFM), another structural feature common to all core histones. The HFM is an ancient structural element and is evolutionarily conserved from archaebacteria to mammals. It is composed of three, sometimes four α-helices connected by short loops (Arents and Moudrianakis, 1995; Baxevanis et al., 1995), and emerges as a fundamental protein dimerization motif. Dimerization between core histones or other histone fold motif containing proteins occurs by a head-to-tail association and the resultant formation of a so-called *handshake* conformation (Arents et al., 1991). Similarly as histone binding to the DNA organizes packaging in a NCP (Luger et al., 1997a), non-histone proteins carrying the HFM are also engaged in protein-DNA interactions.

### 1.6.3 Nuclear import of histones

Histones represent the fundamental structural unit of eukaryotic chromatin. For the *de novo* formation of nucleosomes during DNA replication in S-phase, huge amounts of newly synthesized histones are needed. Thus, histones are among the most abundant substrates for nuclear import. According to their size between 11 and 15 kDa for core histones and about 22 kDa for the linker histone H1, histones were previously expected to enter the nucleus by passive diffusion, since molecules smaller than 40 kDa are subject to enter the nucleus without the help of import factors (Feldherr et al., 1984). In fact, it has been shown that nuclear transport of histones is signal dependent and receptor-mediated (Baake et al., 2001b; Breeuwer and Goldfarb, 1990; Imamoto et al., 1995; Jäkel et al., 1999; Kurz et al., 1997; Schwamborn et al., 1998). Furthermore, it became evident that histones are imported via multiple pathways (Baake et al., 2001a; Mosammaparast et al., 2002; Mosammaparast et al., 2001; Mühlhäusser et al., 2001) and that nuclear uptake of core histones and linker histones differs fundamentally (Bäuerle et al., 2002; Jäkel et al., 1999).

The H1 linker histone is imported via an astonishing energy-dependent mechanism mediated by an importin β-importin 7 heterodimer (Jäkel et al., 1999; Wohlwend et al., 2007). Nuclear import of the core histones H2A, H2B, H3 and H4, however, is mediated by at least five different importins of the importin β like family, namely importin β, importin 5, importin 7, importin 9, and transportin (Baake et al., 2001a; Mühlhäusser et al., 2001), whereas both, the amino-terminal tail and the central globular domain, contain a nuclear targeting signal responsible for nuclear uptake (Baake et al., 2001b). Contrary to this mechanisms in human, the yeast histones H2A and

H2B are imported by the importin 9 homolog Kap114p as primary import factor (Mosammaparast et al., 2001), and import of H3 and H4 is mediated by Kap123p, the human importin 4 homolog (Mosammaparast et al., 2002). In addition, Kap121p, the importin 5 homolog, was identified as an universal import factor for all four yeast core histones.

## 1.7 Chromatin remodeling factors - How dynamic can nucleosomes be?

### 1.7.1 Function and classification

For a long time, nucleosomes have been considered as immobile and static particles. With the increased understanding of chromatin and molecular processes like DNA transcription, repair and replication, the need for DNA accessibility for different factors became obvious. In other words, regulatory protein complexes must be able to reach highly packed DNA sequence elements. This requires sequential changes in the chromatin structure achieved by covalent post-transcriptional modifications of histones, which are subsequently recognized by transcriptional regulators (Jenuwein and Allis, 2001; Strahl and Allis, 2000) or by the action of ATP-dependent chromatin remodeling complexes which displace, mobilize and restructure nucleosomes noncovalently *in trans* to regulate the access to the DNA (for review, see Becker and Horz, 2002; Johnson et al., 2005; Lusser and Kadonaga, 2003; Owen-Hughes, 2003; Saha et al., 2006). Thus, chromatin remodeling complexes confer the stable and immobile chromatin its dynamic character, leading to different phenomena, ranging from simple shifting of the nucleosome position (Belikov et al., 2001; Fazzio and Tsukiyama, 2003; Goldmark et al., 2000; Kent et al., 2001; Lomvardas and Thanos, 2001), and the complete absence of nucleosomes at regulatory sites (Reinke and Horz, 2003), up to increasing the access ability of the DNA on the surface of positioned nucleosomes (Truss et al., 1995) to the whole exchange of H2A variants (Krogan et al., 2003; Mizuguchi et al., 2004). The energy to perform these processes comes from the hydrolysis of ATP which consequently requires an ATPase in each chromatin remodeling complex.

Eukaryotic cells contain at least five different families of chromatin remodelers, namely SWI/SNF, ISWI, NURD/Mi-2/CHD, INO80 and SWR1 (Bao and Shen, 2007; Denslow and Wade, 2007; Dirscherl and Krebs, 2004; Martens and Winston, 2003; van Attikum et al., 2007). Sometimes also relatives of the RAD54 protein family of DNA translocases are described as chromatin remodelers (Alexeev et al., 2003; Jaskelioff et al., 2003). The classification is based on the identity of the ATPase subunit, whereas associated proteins determine the specificity of each remodeling complex. One of the largest groups and best characterized nucleosome

remodelers is represented by the ISWI family (imitation switch) which has been originally identified in *Drosophila melanogaster* (Elfring et al., 1994), but since then also has been found in yeast, *Xenopus laevis*, *Arabidopsis thaliana*, and mammals. Generally, ISWI complexes exhibit central roles in nucleosome assembly after DNA replication (Corona and Tamkun, 2004) and the promoting of orderly and regularly spaced nucleosomes along the DNA which results in transcriptional repression (Deuring et al., 2000; Ito et al., 1999; Varga-Weisz et al., 1997). However it has also been shown that ISWI complexes are involved in DNA and chromatin replication (Neves-Costa and Varga-Weisz, 2006). In human, ISWI is encoded by the homologous genes *SNF2L* (Tsuchiya et al., 1992) and *SNFSH* (Aihara et al., 1998). Currently, only one SNF2L containing complex, namely NURF (Barak et al., 2003), but six different SNF2H-containing remodeling complexes have been described, among them the remodeling and spacing factor RSF (LeRoy et al., 1998; Loyola et al., 2003), the WSTF-related chromatin remodeling factor ACR/WCRF (Bochar et al., 2000; LeRoy et al., 2000), the WICH complex (Bozhenok et al., 2002), the nucleolar remodeling complex NoRC (Strohner et al., 2001) and the chromatin accessibility complex CHRAC (Poot et al., 2000).

## 1.8 The chromatin accessibility complex

The human chromatin accessibility complex (HuCHRAC) is suggested to play a role in the formation of regular nucleosome arrays in compact heterochromatin after DNA replication. Because of its composition, CHRAC is able to facilitate efficient nucleosome sliding under conditions that are not favorable to other remodeling complexes, leading to the maintenance of higher-order chromatin structures (Kukimoto et al., 2004). The human CHRAC consists of four subunits, the ATPase SNF2H (sucrose nonfermenting-2 homolog), ACF1 (ATP-utilizing chromatin assembly and remodeling factor 1) and the histone fold motif containing subunits CHRAC-17 and CHRAC-15. The initially reported copurification of topoisomerase II with the *Drosophila* CHRAC (Varga-Weisz et al., 1997) could neither be confirmed for the CHRAC complex of *Xenopus laevis* (Guschin et al., 2000) nor for human CHRAC (Poot et al., 2000) nor for *Drosophila* CHRAC itself (Eberharter et al., 2001).

SNF2H, an ISWI homolog of the SWI2/SNF2 superfamily of ATPases (Eisen et al., 1995), is considered as core ATPase and, thus, as the ‘motor’ of the complex (for review, see Corona and Tamkun, 2004; Längst and Becker, 2001). Since it is able to react with a nucleosomal substrate with increased ATPase activity, SNF2H can by itself be considered as a remodeling factor, although it has never been purified from a native source on its own (Corona et al., 1999). Remodeling activity is, however, only stimulated by nucleosomes and not by free DNA or free

histones. This is remarkable, because the carboxy-termini of ISWI ATPases contain two domains, known as SANT and SLIDE (SANT-like ISWI domain), which are responsible for DNA and histone-tail binding (Aasland et al., 1996; Grune et al., 2003). In fact, SNF2H is known to associate with ACF1, forming a heterodimeric chromatin remodeling complex that has been named ACF (Bochar et al., 2000; Ito et al., 1999; LeRoy et al., 2000). In addition, this heterodimeric ACF complex has also been identified as a component of CHRAC (Poot et al., 2000). Human ACF1, a 185 kDa protein, is the largest subunit of CHRAC and is a member of the WAL (WSTF-, ACF1-like) protein family. Family members like the 'Williams syndrome transcription factor' WSTF (Lu et al., 1998; Peoples et al., 1998) and the 'bromodomain adjacent to zinc finger domain' proteins BAZ2A and BAZ2B (Jones et al., 2000) are characterized by a conserved WAKZ (WSTF/ACF1/KIAA0314/ZK783.4) motif (Ito et al., 1999) followed directly by a PHD finger motif (Aasland et al., 1995) and a bromodomain at their carboxy-terminus (for review, see Winston and Allis, 1999). The bromodomain, normally common in ATPases of the SWI2/SNF2 family (Hassan et al., 2002), but absent in SNF2H, enables ACF1 to facilitate binding to acetylated lysine residues in histone tails (Jacobson et al., 2000; Owen et al., 2000). While ACF1 alone is inactive for nucleosome mobility, it enhances the SNF2H-induced nucleosome sliding by an order of magnitude. Remarkably, it also modulates the nucleosome mobilization by altering the directionality of the sliding process (Eberharter et al., 2001). This mechanism strictly depends on the tail of histone H4, since the deletion of the H4 amino-termini completely abolishes nucleosome movement (Clapier et al., 2001). In the formation of CHRAC, however, ACF1 serves as a scaffold. Whereas the carboxy-terminal part of ACF1 binds at least to histone H4, binding to SNF2H is mediated via the central region (Collins et al., 2002). On the other hand, the amino-terminally located WAC containing region binds to the CHRAC-15 subunit which is complexed with CHRAC-17 (frequently abbreviated as p15 and p17). CHRAC-15 and CHRAC-17 both contain a histone fold motif and thus are able to form a heterodimer in the typical handshake structure. In addition, both proteins contain carboxy-terminal regions adjacent to their histone fold motif that are rich in charged residues (Poot et al., 2000). While the carboxy-terminal 'tail' of CHRAC-15 is essential for the interaction with ACF1, the carboxy-terminus of CHRAC-17 facilitates DNA binding (Kukimoto et al., 2004). Since the CHRAC-15/CHRAC-17 heterodimer (hereafter referred to as CHRAC-15/17), in comparison to SNF2H and ACF1 alone, enhances nucleosome sliding of the complex, both proteins are functional components and crucial for human CHRAC function. A recent study has shown that the CHRAC-15/17 heterodimer recruits the ACF1-SNF2H-complex to the nucleosomes. The DNA-binding capacity of the CHRAC-15/17 dimer seemed to increase

the substrate availability, thus bridging the interaction between ACF and DNA and helping to overcome the limiting step of the reaction (Kukimoto et al., 2004).

### 1.8.1 The CHRAC-15/17 heterodimer - A closer look

Both CHRAC-15 and CHRAC-17, like their *Drosophila* homologues CHRAC-16 and CHRAC-14 (Corona et al., 2000; Varga-Weisz et al., 1997) and their yeast homolog Dls1p and Dpb4p in *Saccharomyces cerevisiae* (McConnell et al., 2004), contain a histone fold motif at their amino-terminus (Fig. 4). CHRAC-15 consists of 131 amino acids and CHRAC-17 of 148 amino acids, generating a 15 and a 17 kDa protein respectively. X-ray crystallography at 2.4 Å resolution has determined the structure of the *Drosophila* CHRAC-16/14 complex (Hartlepp et al., 2005). The core histone fold motif of CHRAC-16 is formed by a helix α1-loop L1-helix α2-loop L2-helix α3 structure and is extended by a fourth helix αC at the carboxy-terminus. Like CHRAC-16, CHRAC-14 also contains a rather long fourth helix following helix α3. The histone fold core domains for CHRAC-16 and CHRAC-14 are packed head-to-tail against each other generating a heterodimer of the H2A/H2B type, where CHRAC-16 (HuCHRAC-15) is related to H2A and CHRAC-14 (HuCHRAC-17) to H2B. Heterodimerization via the histone fold motif then allows binding to the DNA, since key residues involved in H2A/H2B interactions with DNA in the nucleosome are identical or at least conservatively substituted (Corona et al., 2000).

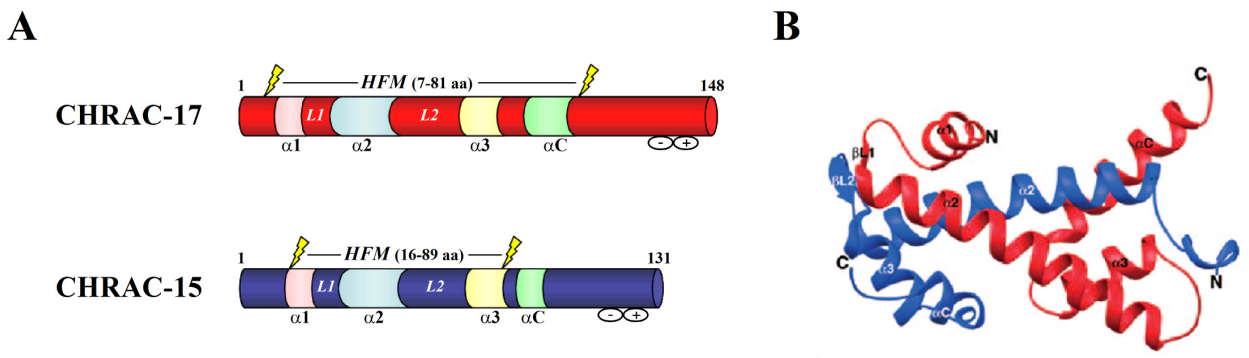


**FIG. 4: Histone fold motif (HFM) proteins CHRAC-15 and CHRAC-17.** *(A)* Schematic representation of CHRAC-15 (blue) and CHRAC-17 (red) with their domain structure. The core HFM of CHRAC-15 and CHRAC-17 is indicated and consists of a helix α1-loop L1-helix α2-loop L2-helix α3 structure. In addition, both proteins exhibit a fourth helix αC. The carboxy terminal 'tails' in CHRAC-15 and CHRAC-17 essential for the interaction with ACF1 and the DNA, respectively are rich in charged residues (Kukimoto et al., 2004). *(B)* Ribbon representation of the CHRAC-15/17 homologous histone fold pair CHRAC-16/14 from *Drosophila melanogaster* (Hartlepp et al., 2005). CHRAC-16 (blue), the homologue of CHRAC-15, and CHRAC-14 (red), the homologue of CHRAC-17, are packed head to tail against each other resulting in the typical handshake structure.

In addition, the histone motifs in the CHRAC-15/17 heterodimer are closely related to those found in the subunits of NF-YB and NF-YC of the transcription factor NF-Y (for review, see Maity and de Crombrugghe, 1998; Mantovani, 1998; Romier et al., 2003) and the NC2α/NC2β heterodimer of the negative transcriptional cofactor NC2 (Goppelt et al., 1996; Kamada et al., 2001). Surprisingly, CHRAC-17 is not only part of the CHRAC complex, but was also discovered as a subunit of the human DNA polymerase ε where it is complexed with the histone fold motif containing protein p12 (Li et al., 2000).

## 1.9 DNA polymerase ε

DNA polymerase ε represents one of eight different mammalian template-directed DNA polymerases and possesses an intrinsic proof reading 3'→5' exonuclease activity (Syvaoja et al., 1990). It is implicated in DNA recombination, repair and, as it is located at the replication fork, it serves as the leading strand synthesizing enzyme in chromosomal DNA replication (Fukui et al., 2004; Pursell et al., 2007). Together with polymerase δ, polymerase ε performs the bulk DNA synthesis (Pospiech and Syvaoja, 2003). Therefore both polymerases are suggested to proofread opposite DNA stands (Karthikeyan et al., 2000) according to the 'one DNA polymerase per strand' hypothesis. Although these data seemed to be contradictory (Zeng et al., 1994), it has also been concluded that DNA polymerase ε is involved in the nucleotide excision-repair mechanism upon DNA damage (Nishida et al., 1988; Shivji et al., 1995). Besides its isolation out of HeLa cells (Syvaoja and Linn, 1989), polymerase ε has been isolated from various organisms as a conserved four-subunit complex, among them *Saccharomyces cerevisiae* (Morrison et al., 1990), *Schizosaccharomyces pombe* and *Drosophila melanogaster* (Aoyagi et al., 1997). The human DNA polymerase ε is composed of the 261 kDa catalytic subunit (p261), a 59 kDa accessory protein (p59) and two histone fold motif containing subunits which are named p12 and CHRAC-17 (p17), corresponding to their size of 12 and 17 kDa. The amino-terminal domain of p261 harbors the sites for the polymerase activity as well as the site for the exonuclease activity. Similarly essential, although not required for polymerase activity (Morrison et al., 1990), is the nonenzymatic carboxy-terminal domain because it not only mediates the interaction with the three smaller regulatory subunits, but also links the replication machinery to the S-phase checkpoint (Navas et al., 1995; Pospiech and Syvaoja, 2003). Thus, it was shown that Dpb2p, the p59 homolog in *Saccharomyces cerevisiae*, is phosphorylated by cdc28 in a cell cycle dependent manner (Araki et al., 1991a; Kesti et al., 2004).

Unfortunately, the structure of the human DNA polymerase ε has not been solved yet. However, the structure of the multisubunit yeast DNA polymerase was determined by Asturias and

colleagues (2006) by cryo-electron microscopy at a 20 Å resolution. A flexible link between the catalytic subunit Pol2 and the tail consisting of Dpb2p and Dpb3p/Dpb4p, the homolog of the p12/CHRAC-17 heterodimer, enables the polymerase to interact with single- and double-stranded DNA. After DNA entry into the polymerase complex, a switch of the flexible tail encloses the nucleic acids leading to a close binding. This covalent binding mode also explains the involvement of the DNA double-strand binding complex Dpb4p/Dpb3p and the occasionally PCNA-independent action of polymerase ε (Fuss and Linn, 2002).

### 1.9.1 The p12/CHRAC-17 heterodimer - A closer look

Whereas CHRAC-17 dimerizes with CHRAC-15 in the chromatin remodeling complex, CHRAC-17 additionally interacts with the histone fold motif protein p12 (Li et al., 2000). The gene of p12 codes for a 117 amino acids protein with a predicted molecular mass of 12.2 kDa. Like CHRAC-17, the p12 histone fold motif comprises the typical helix-strand-helix motif. p12 and its yeast homolog Dpb3p (Araki et al., 1991b; Asturias et al., 2006) are related to H2A and exhibit sequence similarity to CHRAC-15 within the histone fold motif but lack the adjacent acidic carboxy-terminal tail (Poot et al., 2000).

Heterodimerization of p12 and CHRAC-17 in the characteristic handshake formation is a prerequisite for the interaction with and binding to both subunits of polymerase ε. In addition, the heterodimer has substantial affinity to double-stranded DNA (Tsubota et al., 2003). Consistent with this data, mutation of Dpb3 indicates that the subunit is important for stable formation of the polymerase complex. Thus, *dpb3*Δ strains showed an increased rate of spontaneous mutations upon DNA replication (Araki et al., 1991b). Anyhow, Dpb3 is dispensable for cell growth.

Like Dpb3, Dpb4, the homolog of CHRAC-17 in the budding yeast *Saccharomyces cerevisiae*, is not essential for cell viability, but mutation of Dpb4p influences the replication of chromosomal DNA by a delay of S-phase progression (Ohya et al., 2000). Thus, Dpb3 and Dpb4 play crucial roles in the structural maintenance of the DNA polymerase ε as in the binding of double stranded DNA and other proteins during the replication process.

In addition, the human p12/CHRAC-17 heterodimer, which is not part of the chromatin accessibility complex and did not interact with ACF1 at all, also efficiently enhanced the nucleosome assembly like the related CHRAC-15/17 complex (Kukimoto et al., 2004). The reason is still not understood.

## 1.10 The aim of this study

Due to the function of CHRAC-15/17 as part of the human chromatin remodeling complex CHRAC (Poot et al., 2000) and p12/CHRAC-17 as integral component of the human polymerase ε (Li et al., 2000), both heterodimers are located in the nuclear compartment. In addition, the subunits of both complexes exhibit a *histone fold motif* (HFM) (Arents and Moudrianakis, 1995; Baxevanis et al., 1995), the structural feature that allows heterodimerization analogous to the core histones H2A and H2B (Goppelt et al., 1996). Previous reports have proven that core histones, including H2A and H2B, are imported by at least five different members of the importin β-like superfamily (Baake et al., 2001a; Mühlhäusser et al., 2001). In contrast, recent studies have shown that the H2A/H2B related heterodimeric histone fold motif containing complexes NF-YB/NF-YC of the transcriptional activator NF-Y and NC2α/NC2β of the negative cofactor NC2 are both imported as a complex at least by the import karyopherin importin 13 (Kahle et al., 2005; Kahle et al., 2009). Thus, the aim of this study was the determination of the nuclear transport pathway of the CHRAC-15/17 and the p12/CHRAC-17 complexes. Another question to be investigated was whether the subunits are recognized independently by transport receptors or imported as a complex. Furthermore, it should be ascertained if a general importin 13 dependent import pathway for HFM-containing heterodimers of the H2A/H2B type exists and if specific residues within the structure of the heterodimeric complexes can be identified that facilitate the interaction with a given import factor.

For that purpose and to identify potential nuclear transport receptors, *in vitro* nuclear import assays with digitonin permeabilized cells and *in vitro* binding studies with purified import receptors were applied. To identify amino acid residues essential for nuclear accumulation, *in vivo* transfection experiments with various fluorescently labelled wild-type and mutated HFM-containing heterodimers were performed. In addition, RNAi experiments were done in order to determine the localization of the HFM-heterodimers in cells that are depleted in specific import receptors.

# 2 Materials and Methods

## 2.1 Materials

### 2.1.1 Organisms

#### 2.1.1.1 Cell lines

HeLa cells were obtained from the German Collection of Microorganisms and Cell Culture (Deutsche Sammlung von Mikoorganismen und Zellkulturen, Braunschweig; DSMZ No: ACC57). HeLa cells are human cervix carcinoma cells. They grow epithelial-like in monolayers.

HeLa P4-R5 MAGI cells (referred to as HeLa P4) were obtained from the NIH AIDS Research and Reference Reagent Program (catalog #3580). The cell line stably expresses the human receptors CCR-5 and CD4. Additionally, the cells harbor the β-galactosidase gene under the control of HIV-1 LTR (Charneau et al., 1994).

#### 2.1.1.2 Bacterial strains

The following *Escherichia coli* (*E. coli*) strains have been used in this study:

##### 2.1.1.2.1 Cloning strains

DH5α (Clontech, Heidelberg)

The DH5α *E. coli* strain contains *rec*A1 and *end*A1 mutations to increase insert stability and improve the quality of prepared plasmid DNA (Hanahan, 1983).

Genotype: F-, Φ 80d*lac*ZΔM15, Δ(*lac*ZYA-*arg*F)U169, *deo*R, *rec*A1, *end*A1, *hsd*R17 ($r_K^-$, $m_K^+$), phoA, *sup*E44, λ-, *thi*-1, *gyr*A96, *rel*A1

TOP10 (Invitrogen, Karlsruhe)

TOP10 *E. coli* allows stable replication of high-copy number plasmids and thus is ideal for high-efficiency cloning and plasmid propagation. For efficient transformation of unmethylated DNA the genotype of TOP10 cells contains a *hsd*R mutation (Yanisch-Perron et al., 1985).

Genotype: F-, *mcr*A, Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15, Δ*lac*X74, *rec*A1, *ara*Δ139, Δ(*ara-leu*)7697, *gal*U, *gal*K, *rps*L (Str$^R$), *end*A1, *nup*G

#### 2.1.1.2.2 Protein expression strains

BL21 (DE3) (Stratagene, Heidelberg)

For high-level expression the BL21 (DE3) cells contain the T7 promoter expression system with the T7 RNA polymerase gene under the control of the *lac*UV5 promoter. Furthermore, the DE3 strain is deficient in the two key proteases *lon* (lon-Protease) and *Omp* (Outer Membrane Protease) and thus is capable of producing more protein than other expression hosts.

Genotype: F-, *omp*T, *hsd*S($r_B^-$, $m_B^+$), *gal*, *dcm*, λDE3 (*lac*I, *lac*UV5-T7 gene 1, *ind*1, *sam*7, *nin*5)

JM109 (Stratagene, Heidelberg)

The JM109 stain harbors a mutation in the lacI gene (*lac*$I^q$) leading to an overexpression of the lac repressor. Thus, transcription is blocked efficiently and the expression of proteins is repressed until induction with IPTG (Studier and Moffatt, 1986).

Genotype: *rec*A1, *end*A1, *gyr*A96, *thi*, *hsd*R17($r_K^-m_K^+$), *sup*E44, *rel*A1, Δ(lac-*pro*AB)/F' [*tra*D36, *pro*$AB^+$, *lac*$I^q$, *lac*ZΔM15]

### 2.1.2 Chemicals

β-mercaptoethanol (Serva, Heidelberg)

**A**cetic acid (Roth, Karlsruhe)

Acetone (Roth, Karlsruhe)

Acrylamide solution [30 % (w/v) acrylamide, 0.8 % (w/v) bisacrylamide] (Roth, Karlsruhe)

Adenine (Sigma, Steinheim)

Adenosine-5'-diphosphate (ADP) (Sigma, Steinheim)

Adenosine-5'-triphosphate (ATP) (Sigma, Steinheim)

Agarose peqGold Universal (Peqlab, Erlangen)

Ammonium acetate (Merck, Darmstadt)

Ammonium chloride (Merck, Darmstadt)

Ammonium peroxodisulfate (APS) (Merck, Darmstadt)

**B**acto-agar (AppliChem, Darmstadt)

Bacto-peptone (DIFCO, Detroit)

Bacto-tryptone (AppliChem, Darmstadt)

Bacto-yeast extract (AppliChem, Darmstadt)

Boric acid (Roth, Karlsruhe)

Bovine serum albumin (BSA) (Sigma, Steinheim)

Bromophenol blue (Serva, Heidelberg)

**C**aCl$_2$ (Merck, Darmstadt)

Coomassie Brilliant Blue G-250 (Merck, Darmstadt)

Coomassie Brilliant Blue R-250 (Merck, Darmstadt)

Creatine phosphokinase (Sigma, Steinheim)

**D**eoxyribonucleoside triphosphates (dNTPs) (Roche, Mannheim)

4',6-diamidino-2'-phenylindole (DAPI) (Sigma, Steinheim)

Digitonin (Calbiochem, Bad Soden)

Dithiothreitol (DTT) (Biomol, Hamburg)

Dimethylsulfoxide (DMSO) (Sigma, Steinheim)

**E**DTA (disodium ethylenediaminetetraacetate) (Serva, Heidelberg)

EGTA (ethylene-bis(oxyethylenenitrilo)tetraacetic acid) (Serva, Heidelberg)

Ethanol (Merck, Darmstadt)

Ethidium bromide (Sigma, Steinheim)

**F**etal bovine serum (FBS) (Biochrom, Berlin)

**G**lycerol (Serva, Heidelberg)

Glycine (Serva, Heidelberg)

Guanosine-5'-diphoshate (GDP) (Sigma, Steinheim)

Guanosine-5'-triphoshate (GTP) (Sigma, Steinheim)

**H**$_2$O (HPLC purity) (Merck, Darmstadt)

H$_2$SO$_4$ (Merck, Darmstadt)

HCl (Merck, Darmstadt)

HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (Serva, Heidelberg)

HistoGel (Linaris, Wertheim)

Hoechst33258 (Invitrogen, Karlsruhe)

**I**GEPAL CA-630 (Sigma, Steinheim)

Imidazole (Roth, Karlsruhe)

IPTG (isopropylthio-ß-D-galactoside) (Peqlab, Erlangen)

Isopropanol (Merck, Darmstadt)

**K**$_2$HPO$_4$ (Merck, Darmstadt)

KCl (Merck, Darmstadt)

KH$_2$PO$_4$ (Merck, Darmstadt)

**L**iCl (Serva, Heidelberg)

**M**agnesium acetate (Serva, Heidelberg)

Maltose (Serva, Heidelberg)

Methanol (Serva, Heidelberg)

$MgCl_2$ (Merck, Darmstadt)

Milk powder (Roth, Karlsruhe)

**N**$a_2HPO_4$ (AppliChem, Darmstadt)

$NaH_2PO_4$ (Merck, Darmstadt)

$NaHCO_3$ (Merck, Darmstadt)

NaOH (Merck, Darmstadt)

**O**ligofectamine™ Reagent (Invitrogen, Karlsruhe)

**P**araformaldehyde (Serva, Heidelberg)

Phosphocreatine (Sigma, Steinheim)

Phosphoric acid (Merck, Darmstadt)

Ponceau S solution (AppliChem, Darmstadt)

Potassium acetate (Serva, Heidelberg)

**R**eticulocyte lysate (Promega, Mannheim)

**S**DS (sodium dodecyl sulfate) pellets (Roth, Karlsruhe)

Sodium acetate (Merck, Darmstadt)

Sodium azide ($NaN_3$) (Roche, Mannheim)

Sucrose (Serva, Heidelberg)

**T**EMED (N,N,N',N'-tetramethylethylenediamine) (Serva, Heidelberg)

Trichloroacetic acid (TCA) (Merck, Darmstadt)

Tris [Tris(hydroxymethyl)aminomethane] (Roth, Karlsruhe)

Triton X-100 (Merck, Darmstadt)

Tween-20 (Sigma, Steinheim)

**V**ectashield® (Mounting medium with DAPI) (Vector Laboratories, Burlingame, USA)

**Y**east nitrogen base (DIFCO, Detroit)

### 2.1.3 Liquid media and solutions

All solutions used in this study were prepared with deionized water and sterilized by autoclaving at 121°C for 20 minutes Solutions with heat-sensitive substances were sterilized by filtration through a 0.2 µm micron filter.

### 2.1.3.1 Frequently used solutions and buffers

DNA gel loading buffer (5 x)

40 % (w/v) sucrose (in water), 0.1 % (w/v) bromophenol blue, 0.1 M EDTA (pH 7.5)

Glutathione Elution buffer (10 x)

500 mM Tris-HCl (pH 8.0), 1.5 M NaCl, 100 mM glutathione

GST-Pulldown binding buffer

50 mM Tris-HCl, (pH 7.5), 100 mM NaCl, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol

'Korn' buffer

20 mM HEPES-NaOH (pH 7.4), 100 mM NaCl, 1 mM EDTA, 10 % glycerol and 5 mM DTT

'Laemmli' buffer (5 x)

0.25 M Tris, 10 mM EDTA, 1.9 M glycine (pH 8.7), 0.5 % (w/v) SDS

PBS (phosphate-buffered saline) (10 x)

1.4 M NaCl, 27 mM KCl, 15 mM $KH_2PO_4$, 90 mM $Na_2HPO_4$ (pH 7.4)

SDS-PAGE loading buffer (3 x)

('Sample buffer'): 150 mM Tris-HCl (pH 6.8), 3 % (w/v) SDS, 3 % (v/v) β-mercaptoethanol, 30 % (w/v), glycerol, 0.003 % (w/v) bromophenol blue

Stripping buffer

62.5 mM Tris-HCl (pH 6.7), 2 % SDS (w/v), 100 mM β-mercaptoethanol

TBE buffer (10 x)

0.9 M Tris base (pH 8.3), 0.9 M boric acid, 0.025 M EDTA

TBS (10 x)

200 mM Tris-HCl (pH 7.6), 140 mM NaCl

TBST (10 x)

200 mM Tris-HCl (pH 7.6), 140 mM NaCl, 0.1 % Tween 20

TE buffer

10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)

TELT buffer

50 mM Tris-HCl (pH 7.5), 62.5 mM EDTA, 2.5 M LiCl, 4 % (v/v) Triton X-100

Transport buffer

20 mM HEPES-KOH (pH 7.4), 110 mM KAc, 5 mM $MgAc_2$, 1 mM, EGTA, 250 mM Sucrose, 2 mM DTT

### 2.1.3.2 Liquid growth media and agar plates

Media for bacterial cultivation

| | |
|---|---|
| Luria-Bertani (LB) medium: | 1 % (w/v) bacto-tryptone |
| | 1 % (w/v) NaCl (pH 7.0) |
| | 0.5 % (w/v) bacto-yeast extract |
| | 0.2 % (w/v) maltose |
| LB agar plates: | 1.5 % (w/v) bacto-agar were added to the LB-medium |
| 2YT medium: | 1.6 % (w/v) bacto-tryptone |
| | 1 % (w/v) bactoyeast extract |
| | 0.5 % (w/v) NaCl |

Medium for eukaryotic cultivation

| | |
|---|---|
| DMEM: | Dulbecco's MEM liquid media (Invitrogen, Karlsruhe) |
| | 2mM glutamine |
| | 10 % FBS |

The media were sterilized by filtration and stored at 4°C.

### 2.1.4 Antibiotics

added to media for prokaryotes

| | |
|---|---|
| Ampicillin (Ratiopharm, Ulm): | Stock solution: 100 mg/ml in $H_2O$ |
| | Working concentration: 100 µg/ml |
| Kanamycin sulfate (Roth, Karlsruhe): | Stock solution: 50 mg/ml in $H_2O$ |
| | Working concentration: 50 µg/ml |

added to media for eukaryotes

| | |
|---|---|
| Gentamycin (PAA, Cölbe): | Stock solution: 10 mg/ml in $H_2O$ |
| | Working concentration: 50 µg/ml |
| Penicillin-Streptomycin (Biochrom, Berlin): | Stock solution: 10 mg/ml in $H_2O$ |
| | Working concentration: 100 µg/ml |

### 2.1.5 Antibodies

The following antibodies were used for protein detection in Western blotting (WB) and Immunofluorescence (IF):

Primary antibodies:

| Antibody | Organism | Company | Application | Dilution |
|---|---|---|---|---|
| anti-Actin | rabbit | Sigma | WB | 1:200 |
| anti-CHRAC-15 | goat | Santa Cruz | IF | 1:100 |
| anti-FLAG | rabbit | Sigma | WB | 1:1000 |
| anti-GFP | rabbit | Santa Cruz | WB | 1:200 |
| anti-GST | rabbit | Santa Cruz | IF | 1:200 |
| anti-His | mouse | Novagen | WB | 1:1000 |
| anti-RFP | rabbit | MoBiTec | WB | 1:1000 |
| anti-Stella | rabbit | Santa Cruz | WB, IF | 1:100 |

Secondary antibodies:

| Antibody | Organism | Company | Application | Dilution |
|---|---|---|---|---|
| anti-goat-IgG | rabbit | Sigma | WB | 1:5000 |
| anti-mouse-IgG | goat | Sigma | WB | 1:12500 |
| anti-rabbit-IgG | goat | Sigma | WB | 1:100000 |
| AlexaFluor488-anti-mouse | goat | MoBiTec | IF | 1:1000 |
| AlexaFluor555-anti-mouse | goat | MoBiTec | IF | 1:1000 |
| AlexaFluor488-anti-rabbit | goat | MoBiTec | IF | 1:1000 |
| AlexaFluor555-anti-rabbit | goat | MoBiTec | IF | 1:1000 |

**Table Ia and Ib: Schedule of the applied primary (top table) and secondary (lower table) antibodies used in this study.**

### 2.1.6 Enzymes

Alkaline Phosphatase from calf intestinal (CIP) (1 U/µl) (Roche, Mannheim)
Lysozyme (Muramidase), stock solution 50mg/ml $H_2O$ (Sigma, Steinheim)
RedTaq™-DNA Polymerase (*Thermus aquaticus*) (1U/µl; Sigma, Steinheim)
Restriction enzymes (New England Biolabs, Schwalbach; MBI Fermentas, St. Leon-Rot)
RNase A, stock solution 10mg/ml (Sigma, Steinheim)
T4 DNA Ligase (1U/µl; MBI-Fermentas, St. Leon-Rot)
Trypsin (Biochrom, Berlin)
$Vent_R$®-DNA Polymerase (*Thermococcus litoralis*) (2 U/µl; New England Biolabs, Schwalbach)

### 2.1.7 Inhibitors

| | |
|---|---|
| Aphidicolin (Alexis, Lörrach): | Stock solution: 8,61 mM in methanol |
| | Working concentration: 10 µM |
| Leptomycin B (LMB) (Sigma, Steinheim): | Stock solution: 5µg/ml in 70 % ethanol |
| | Working concentration: 10 ng/ml |
| Sodium butyrate (Sigma, Steinheim): | Stock solution: 100 mg/ml |
| | Working concentration: 5 mM |

### 2.1.8 Protease inhibitors

Complete® (EDTA free) protease inhibitor cocktail tablets (Roche, Mannheim)
Phenylmethylsulfonyl fluoride (PMSF) (Serva, Heidelberg)

### 2.1.9 Affinity matrices

Gamma-Bind™ Plus Sepharose (GE Healthcare, München)
Glutathione Sepharose™ 4 Fast Flow (GE Healthcare, München)
Nickel-NTA Agarose (Qiagen, Hilden)

### 2.1.10 Vectors

pCS2+ (Rupp et al., 1994; Turner and Weintraub, 1994)
pEGFP-C1 (Clonetech, Heidelberg)
pEGFP-N1 (Clonetech, Heidelberg)
pET-21b (Novagen - Merck, Darmstadt)

pET-28b (Novagen - Merck, Darmstadt)
pGEM®-T Easy (Promega, Mannheim)
pGEX 4T-1 (GE Healthcare, München)
pRFP-C1 (Clonetech, Heidelberg)
pRFP-N1 (Clonetech, Heidelberg)

### 2.1.11 Oligonucleotides

The oligonucleotides used in this study were synthesized by MWG-Biotech (Ebersberg) and Sigma (Steinheim).

### 2.1.12 siRNA

Stealth™/siRNA duplex oligoribonucleotides were synthesized by Invitrogen (Karlsruhe). The duplexes were diluted with 1000 µl RNase-free water resulting in a 20 µM solution. After dilution the RNAi was stored at -20°C.

### 2.1.13 Chromatography columns

NAP™-5 column (GE Healthcare, München)
Mono S column (GE Healthcare, München)
HiLoad Q Sepharose Fast Flow column (GE Healthcare, München)
Superdex® 200 HiLoad 16/60 column (GE Healthcare, München)
Superdex® 200 10/300 GL column (GE Healthcare, München)
Superdex® 75 HiLoad 16/60 column (GE Healthcare, München)
Vivaspin (Vivascience; Biofiltronics, Nörten-Hardenberg)

### 2.1.14 Filters and membranes

Folded filters (Ø12.5 cm) (Macherey-Nagel, Düren)
Filters 2668 (Whatman, Dassel)
Syringe filters (0.2 µm) (Roth, Karlsruhe)
OPTITRAN BA-S 83 (nitrocellulose membrane) (Whatman, Dassel)
ZelluTrans (dialysis membrane) (Roth, Karlsruhe)

### 2.1.15 Molecular weight standards

DNA

λ-DNA size markers (digested *Hind*III or *Eco*RI-*Hind*III) (MBI Fermentas, St.Leon-Rot)

Protein

PageRuler™ Prestained Protein Ladder (Fermentas, St.Leon-Rot)

SDS-PAGE Molecular weight standard broad range marker (BioRad, München)

### 2.1.16 Kits

ECL™ Western Blotting Detection Reagents (GE Healthcare, München)

Effectene™ Transfection Reagent (Qiagen, Hilden)

peqGOLD Cyle-Pure Kit (Peqlab, Erlangen)

pGEM®-T Easy Vector System 1 (Promega, Mannheim)

PureYield™ Plasmid Midiprep System (Promega, Mannheim)

QIAEX® II Agarose Gel Extraction Kit (Qiagen, Hilden)

Qiagen PlasmidMidi-Kit (Qiagen, Hilden)

Quick Start™ Bradford Protein Assay Kit 3 (BioRad, München)

Wizard® *Plus* SV Minipreps DNA Purification System (Promega, Mannheim)

Wizard® SV Gel and PCR Clean-up System (Promega, Mannheim)

### 2.1.17 Hardware

Cell Culture

Cell-counter Casy Counter (Schärfe System, Reutlingen)

$CO_2$-Inkubator Heraeus BBD 6220 (Kendro, Langenselbold)

Horizontal laminar flow hood HERAsafe Typ 18/2 (Heraeus, Hanau)

Incubator Shaker G 25 (New Brunswick, Edison, USA)

Incubator Type B5050 (Heraeus, Hanau)

Centrifuges

Biofuge pico (Heraeus, Hanau)

Sorvall RC 5B Plus (Kendro, Langenselbold)

Table centrifuge 5415 (Eppendorf, Hamburg)

Varifuge 3.0 R (Heraeus, Hanau)

Microscope

Fluorescence Microscope Axioskop 20 with AxioCam MRm (Zeiss, Oberkochen)

Inverted Microscope Olympus CK40 (Olympus, Hamburg)

PCR

GeneAmp PCR system 2400 (Perkin Elmer, Freyung)

T3 Thermocycler (Biometra, Göttingen)

Various

Äkta*Purifier*-System (GE Healthcare, München)

Block Heater Thermostat 5320 (Eppendorf, Hamburg)

Digital Sonifier (Branson Sonifier, Schwäbisch Gmünd)

Liquid nitrogen storage container (Air Liquide Kryotechnik, Düsseldorf)

Magnetic stirrer IKAMAG® REO (Janke & Kunkel, Staufen)

Mini-gel-chambers (Biometra, Göttingen; workshop)

Mini Trans-Blot® Cell (BioRad, München)

Nucleofector® II Device (Amaxa, Cologne)

pH-Meter 525 (WTW, Weilheim)

Photometer ULTROSPEC 3000 (GE Healthcare, München)

Power supply Power Pack P25 (Biometra, Göttingen)

Precision Balance (E. Mettler, Zürich, Switzerland)

Refrigerated Incubator Shaker Innova 4230 (New Brunswick, Edison, USA)

Speed Vac SC100 (Savant, Holbrook, NY, USA)

Vortex Genie 2™ (Bender & Hobein, Zürich, Schweiz)

Additional equipment

One way articles were purchased from the following companies: Eppendorf (Hamburg), Falcon (Heidelberg), Schütt (Göttingen), Greiner (Frickenhausen), Heinemann (Duderstadt), Qiagen (Hilden), Sarstedt (Langenhagen).

### 2.1.18 Software

Windows XP, Microsoft Word, Microsoft Excel, Microsoft Power Point, DNA Star, FreeHand, Adobe Photoshop, Adobe Acrobat, Axio Vision, Image J, GelEval, 1Dscan Ex, EndNote, Boxshade, PyMOL

## 2.2 Methods

### 2.2.1 Molecular biological methods

#### 2.2.1.1 Prokaryotic cells

In molecular biology the bacterium *Escherichia coli* (*E. coli)* serves as a versatile host for the production of plasmid DNA and recombinant eukaryotic proteins.

##### 2.2.1.1.1 Cultivation and Storage of *Escherichia coli*

Bacteria were either streaked onto the surface of a LB agar plate or directly added to liquid LB media. Then the culture media, in both cases containing the appropriate antibiotics, were incubated for 12-14 hours at 37°C (for the expression of several import receptors 2YT medium was used). For the preparation of a liquid (pre-) culture a single colony was inoculated in 3-50 ml of LB medium with the appropriate antibiotic followed by an additional incubation at 37°C for 12-14 hours. For protein expression this pre-culture was used to set up the main culture. For storage 0.5 ml of the respective bacterial culture was mixed with 0.5 ml of sterile glycerol. The culture was vortexed, frozen in liquid nitrogen and stored at -80°C. In this way, the bacteria can be stored over several years.

##### 2.2.1.1.2 Counting of *Escherichia coli*

Because of the small size of *E. coli* (about 2 µm long and 0.5 µm in diameter) the bacteria can be ‘counted’ by measuring the optical density (OD) of the cell suspension, using a photometer. The measurement was performed at 600 nm in a plastic cuvette with the width of 1 cm.

#### 2.2.1.2 Preparation of competent cells

To enable bacterial cells to take up exogenous (naked) DNA from the environment and thus to serve as a host for plasmid DNA, *E. coli* cells have to be made ‘competent’. In this study a calcium chloride solution was used to produce competent *E. coli*. Performing this treatment one

takes advantage of the fact that the bacterial cell membrane is permeable to chloride ions, but is non-permeable to calcium ions. As the chloride ions enter the cell, water molecules accompany the charged particle. The influx of water then causes the cell to swell which is necessary for the subsequent uptake of the plasmid DNA.

Competent cells were prepared by picking a single, freshly grown bacterial colony of the respective *E. coli* strain from a LB agar plate. The colony was used to inoculate 50 ml of liquid LB medium (without the addition of any antibiotics). Then, the culture was incubated at 37°C until an $OD_{600}$ of 0.4 was reached. Using a sterile tube, the culture was pelleted by centrifugation at 4000 rpm for 10 minutes at 4°C, resuspended in 10 ml ice-cold sterile 0.1 M calcium chloride and incubated on ice for up to 2 hours, but at least for 30 minutes. After another centrifugation step, the pellet was resuspended in 2 ml 0.1 M calcium chloride. Furthermore, 0.4 ml glycerol was added and 100 µl aliquots of the competent cells were immediately frozen in liquid nitrogen. The cells were stored at -80°C.

#### 2.2.1.3 Bacterial cell transformation

Besides the natural DNA uptake processes like transduction (introduction of DNA by viruses) and conjugation (introduction of DNA by cell-cell contact between different bacteria), transfection provides an effective tool to introduce naked DNA molecules into bacterial cells. Although the mechanism is not fully understood, a combination of calcium chloride treatment with a sufficient heat shock after plasmid DNA addition facilitates the uptake.

Transformation was started by thawing an aliquot of competent *E. coli* cells on ice. After the addition of 50-100 ng of plasmid DNA or an entire DNA ligation reaction, the mixture was further incubated on ice for 30 minutes and then heat shocked at 42°C for 2 minutes. The cells were again incubated on ice for 5 minutes. To help the bacteria to recover from the heat shock treatment and to allow expression of the antibiotic resistance gene, cells were incubated at 37°C in non-selective LB medium. After 1 hour the suspension was transferred and streaked onto an antibiotic-containing LB agar plate. Once the whole fluid was taken up the agar plates were inverted and finally incubated overnight at 37°C.

Resulting transformants of a ligation were generally screened for correct insertion of the coding fragment by restriction enzyme digestion analysis of the plasmid DNA (plasmid purification using the TELT method, chapter 2.2.1.4.2).

#### 2.2.1.4 Plasmid purification

##### 2.2.1.4.1 Preparative plasmid purification

Depending on the amount of plasmid DNA needed for further studies (e.g. transfection of eukaryotic cells, transformation of prokaryotic protein expression strains, DNA sequencing, restriction enzyme digestion) the following kits for isolation of plasmid DNA were used: PureYield™ Plasmid Midiprep System and Wizard® SV Gel (both Promega, Mannheim). The preparations were performed according to the manufacturer's instruction. Although the range from analytic to large scale quantities of plasmid DNA differ, both kits are based on a modified alkaline lysis procedure, followed by binding of the plasmid DNA to an anion-exchange resin under appropriate low-salt and pH conditions. Washing the resin with a medium-salt buffer leads to the removal of RNA, proteins, and low-molecular-weight impurities. Finally the plasmid DNA was eluted with nuclease-free water and stored at -4°C.

##### 2.2.1.4.2 TELT method

As described before (see chapter 2.2.1.3) plasmid purification with the TELT method was used to screen different *E. coli* colonies for correct insertion of a coding fragment into a vector after cloning and transformation, and resulted in the isolation of analytical amounts of plasmid DNA. The name TELT is derived from the ingredients of the used buffer (**T**ris, **E**DTA, **L**iCl and **T**riton X-100).

Inoculation of 3 ml liquid LB containing the appropriate antibiotics with a single colony from an agar plate was followed by incubation over night at 37°C. The next day, 1.5 ml of each culture was centrifuged for 5 minutes at 5000 rpm in a pre-cooled centrifuge. Then, the cells were resuspended in 150 µl of TELT buffer supplemented with 15 µl lysozyme (10 mg/ml in $dH_2O$) and boiled for 2 minutes at 95°C. After a 5 minute incubation on ice the solution was centrifuged for 10 minutes at 14000 rpm in a cooled centrifuge and the cell debris was removed with a toothpick. The plasmid DNA in the remaining solution was precipitated with 100 µl isopropanole and centrifuged for 15 minutes at 14000 rpm. The DNA pellet was washed with 100 µl of 70 % ethanol and afterwards dried with a Speed Vac centrifuge. The resulting DNA pellet was dissolved in 30 µl $dH_2O$. For subsequent analysis by restriction enzyme digestion 10 µl of plasmid DNA were used. When the expected digested products were smaller then 200 bp, bacterial RNA had to be digested by adding 0.5 µl of RNase (10 mg/ml), in order to be able to see the resulting band on the agarose gel after gel electrophoresis in the low molecular weight region of the gel.

### 2.2.1.5 DNA Quantification

DNA concentration was determined spectrophotometrically. The absorption maximum of double stranded DNA is 260 nm. Contaminants such as proteins and single stranded DNA and RNA absorb maximally at 280 nm. Therefore, the ratio between the OD values at 260 nm and 280 nm indicates the purity of DNA. The higher the ratio, the more pure the DNA is present in the sample. A ratio between 1.8 and 2.0 indicates pure DNA.

Another method is estimating the amount of DNA semi-quantitatively by the intensity of fluorescence emitted by ethidium bromide. For that, plasmid DNA and an aliquot of DNA size marker of known concentrations were analyzed by agarose gel electrophoresis (see chapter 2.2.1.10). The fluorescence intensities of the intercalated ethidium bromide were compared and the DNA concentration was assessed.

### 2.2.1.6 Restriction enzyme digestion

Restriction enzymes (restriction endonucleases) cut double stranded DNA at specific recognition nucleotide motifs, also known as restriction sites. These sites are usually 4 to 8 nucleotides in length and often palindromic. Restriction enzymes themselves differ in the way they cut DNA. While some restriction enzymes produce ‘sticky’ ends, other enzyme cleavage results in digested DNA with ‘blunt’ ends.

According to the manufacturer’s instructions the DNA was incubated with the appropriate amount of enzymes and the respective buffer at the optimal temperature. Typically 5-10 µg of DNA were used for preparative digestion and 0.5-2 µg of DNA for analytic digestion were incubated with 5-10 U of restriction enzyme per µg DNA. Usually the mixture of DNA, enzyme and buffer was incubated for 2-3 hours at 37°C. In some cases double digestions were performed. However, prerequisite for a double digestion was that both enzymes were active in the same buffer.

### 2.2.1.7 Dephosphorylation of digested plasmid DNA

To prevent self-ligation of digested and linearized vectors, alkaline phosphatase from calf intestine (calf intestinal phosphatase [CIP]) was used to remove the 5' phosphates from the nucleic acid.

After restriction enzyme digestion, the DNA was incubated with 10 U CIP and $^{1}/_{10}$ of the appropriate dephosphorylation CIP buffer for 30 minutes at 37°C. To achieve inactivation of the alkaline phosphatase, $^{1}/_{10}$ volume of 200 mM EGTA was added and the mixture was

heat-inactivated at 65°C for 10 minutes. However, in most cases ligations were performed without inactivation.

#### 2.2.1.8 Polymerase chain reaction (PCR)

PCR was used to amplify defined DNA segments from a specific template. Two oligonucleotides complementary to the DNA target to be amplified served as the starting point for replication. In addition, the primers contained sequences for restriction enzymes on their 5'-end to allow subsequent cloning of the resulting PCR product. In this study, two different thermostable polymerases were used. In general DNA amplification was performed using the $Vent_R^{®}$-DNA polymerase from *Thermococcus litoralis* (New England Biolabs, Schwalbach) which contains a proof reading exonuclease activity. To amplify fragments with adenine (A) protrusions at the 3' ends of the products for cloning into the pGEM®-T-Easy vector (Promega, Mannheim) amplification was performed with the RedTaq™-DNA Polymerase from *Thermus aquaticus* (Sigma, Steinheim). Typically, a PCR reaction mixture comprised of 100 ng DNA template or 100 ng of genomic DNA, 100 pmol of forward and reverse primer, 5 µl of 2 mM dNTPs, $^1/_{100}$ DMSO (v/v), 1 U of Vent polymerase or 3 U of Redaq polymerase, and $^1/_{10}$ (v/v) of the appropriate 10 x polymerase buffer in a total volume of 100 µl. The PCR was accomplished in a GeneAmp PCR system 2400 (Perkin Elmer, Freyung) or a T3 Thermocycler (Biometra, Göttingen). Apart from minor changes a standard protocol of 30 cycles with 1 minute denaturation at 95°C, 1 minute primer annealing at 55-65°C and an elongation step of 1 minute per 1000 bp of the expected DNA fragment was performed. The final step included an elongation step for 7 minutes at 72°C. To control the amplified PCR products, an aliquot of 5 µl was analyzed by agarose gel electrophoresis. Depending on the availability, the remaining product was purified with the peqGOLD Cyle-Pure Kit (Peqlab, Erlangen) or the Wizard® SV Gel and PCR Clean-up System (Promega, Mannheim). Afterwards the DNA fragments were digested according to their included restriction sites.

#### 2.2.1.9 Site-directed mutagenesis

Nucleotide exchanges were generated using two different methods. When the corresponding amino acids to be mutated were located at the very amino- or carboxy-terminus of the protein (approximately the first or last 20 amino acids) the mutation was introduced by using an antisense amplification primer carrying the desired point mutation. Amino acids not fulfilling

this prerequisite were mutated according to the Quick Change Site-directed Mutagenesis Kit protocol (Stratagene).

To generate the nucleotide exchanges K47A, K92A, R69A, R45A in p12; R25A, K70A, K47A, R23A in CHRAC-15; K100A, R92A, K62A, K86A and S122E in CHRAC-17; K18A, K63A, R40A, R19A in NC2α and R101A, R102A, K95A, K64A, K88A in NC2β, site-directed mutagenesis was performed. For that purpose, two oligonucleotides with the appropriate mutation and complementarily to each other were generated and a PCR reaction was used to introduce site-specific mutations in the double stranded template DNA. Typically a reaction mixture contained 500 ng of template, plasmid DNA, 13 pmol of complementary oligonucleotides, 5 µl of 2 mM dNTPs, 2 mM MgSO4, 5 µl 10x polymerase buffer and 2 U Vent® polymerase (New England Biolabs, Schwalbach) in a total volume of 50 µl. The mixture was splitted into two aliquots. 25 µl were incubated at room temperature, representing the negative control. The remaining 25 µl were used to perform the PCR with the a 28 cycle containing program with 30 seconds of strand denaturation at 95°C, primer annealing for 1 minute at 60-65°C and an elongation step at 72°C followed by the final elongation for 11 minutes. The melting point of the oligonucleotides, needed for calculating the annealing temperature can be determined by the following formula (Meinkoth and Wahl, 1984):

Tm = 81.5°C + 16.6 log[Na+] + 0.41 (% GC) – 0.61 (% for) – 500/N; (N is the number of bases of the oligonucleotide).

However, the annealing step was normally carried out at 60 or 65°C. Elongation depends on the size of the vector with approximately 70 seconds per 1000 bp. After the PCR reaction the restriction enzyme 0.5 µl *Dpn*I (10 U/µl) was added to both samples and incubated for 2 hours at 37°C. Treatment with *Dpn*I, specifically cleaving methylated DNA led to the digestion of the methylated template DNA, isolated from *E. coli*, whereas the newly synthesized vector DNA carrying the mutation is unmethylated and thus untouched by the enzyme. 5 µl of each reaction batch were use to transform *E. coli* DH5α. The resulting clones were analyzed by DNA sequencing.

In this study the following complementary oligonucleotides were used:

5’-CTGCCTCTATCCCGCATCGCCGTCATCATGAAGAGCTCC-3’ (sense) and 5’-GGAGCTCTTCATGATGACGGCGATGCGGGATAGAGGCAG-3’ (antisense) for R25A, 5’-GGCAGTGGAAAGGAAAAGGCCGTACTGACTTACAGTGAT-3’ (sense) and 5’-ATCACTGTAAGTCAGTACGGCCTTTTCCTTTCACTGCC-3’ (antisense) for K70A, 5’-GCGTTGGTGCTCACGGCCGCCGCCACGGAGCTCTTTGTT-3’ (sense) and 5’-AACAAAGAGCTCCGTGGCGGCGGCCGTGAGCACCAACGC-3’ (antisense) for K47A

and 5’-ATCTCGCTGCCTCTATCCGCCATCGCCGTCATCATGAAG-3’ (sense) and 5’-CTTCATGATGACGGCGATGGCGGATAGAGGCAGCGAGAT-3’ (antisense) for R23A in CHRAC-15.

5’-CGGGAGCAGAAAGGCAAGGCCGAGGCCTCAGAGCAAAAG-3’ (sense) and 5’-CTTTTGCTCTGAGGCCTCGGCCTTGCCTTTCTGCTCCCG-3’ (antisense) for K100A, 5’-GAAGCTCTGGAAGCATATGCCCGGGAGCAGAAAGGCAAG-3’ (sense) and 5’-CTTGCCTTTCTGCTCCCGGGCATATGCTTCCAGAGCTTC-3’ (antisense) for R93A, 5’-GCAATGAAAGGAAAGCGGGCCACGCTGAATGCCAGTGAT-3’ (sense) and 5’-ATCACTGGCATTCAGCGTGGCCCGCTTTCCTTTCATTGC-3’ (antisense) for K62A, 5’-CGGTTCGTTATCCCATTGGCCGAAGCTTGGAAGCATAT-3’ (sense) and 5’-ATATGCTTCCAGAGCTTCGGCCAATGGGATAACGAACCG-3’ (antisense) for K86A and 5’-TCGGAAGAGCAAGACAAGGAAAGGGATGAGGACAATGAT-3’ (sense) and 5’-ATCATTGTCCTCATCCCTTTCCTTGTCTTGCTCTTCCGA-3’ (antisense) for S122E in CHRAC-17.

5’-TTGCCTCTGGCGCGAGTGGCAGCCTTGGTGAAGGCAGAT-3’ (sense) and 5’-ATCTGCCTTCACCAAGGCTGCCACTCGCGCCAGAGGCAA-3’ (antisense) for K47A, 5’-GCTCAGCAGGGAAAAAGGGCAACCCTTCAGAGGAGAGAC-3’ (sense) and 5’-GTCTCTCCTCTGAAGGGTTGCCCTTTTTCCCTGCTGAGC-3’ (antisense) for K92A, 5’-GCCATCTTCATTCTGGCAGCAGCCGCGGAACTGTTTGTG-3’ (sense) and 5’- CACAAACAGTTCCGCGGCTGCTGCCAGAATGAAGATGGC-3’ (antisense) for R69A and 5’-TCGAGGTTGCCTCTGGCGGCAGTGGCAGCCTTGGTGAAG-3’ (sense) and 5’-CTTCACCAAGGCTGCCACTGCCGCCAGAGGCAACCTCGA-3’ (antisense) for R45A in p12.

5’-TTCCCGCCGGCGCGGATCGCAAAGATCATGCAGACGGAC-3’ (sense) and 5’ GTCCGT CTGCATGATCTTTGCGATCCGCGCCGGCGGGAA-3’ (antisense) for K18A, 5’-ACCCAGTCG CGGAACGCGGCAACCATGACCACATCCCAC-3’ (sense) and 5’-GTGGGATGTGGTCATGGTTGCCGCGTTCCGCGACTGGGT-3’ (antisense) for K63A, 5’-CCTGTCATCATCTCCGCAGCGCTCGAGCTCTTC-3’ (sense) and 5’-GAAGAGCTCGAGCGCTGCGGAGATGATGACAGG-3’ (antisense) for R40A and 5’-CGGTTCCCGCCGGCGGCAATCGCGAAGATCATG-3’ (sense) and 5’-CATGATCTTCGCGATTGCCGCCGGCGGGAACCG-3’ (antisense) for R16A in NC2α.

5’-ACAGTAGCATTAAAAGCAGCAAAGGCCAGTTCTCGTTTG-3’ (sense) and 5’-CAAACGAGAACTGGCCTTTGCTGCTTTTAATGCTACTGT-3’ (antisense) for R102A, 5’-GAAGTCTTGCAAGAGTGTGCAACAGTAGCATTAAAAGCA-3’ (sense) and

5’-TGCTTTTAATGCTACTGTTGCACACTCTTGCAAGACTTC-3’ (antisense) for K95A, 5’-TGTAACAAATCGGAAAAGGCGACCATCTCACCAGAGCAT-3’ (sense) and 5’-ATGCTCTGGTGAGATGGTCGCCTTTTCCGATTTGTTACA-3’ (antisense) for K64A and 5’-TCTTACATCAGTGAAGTAGCAGAAGTCTTGCAAGAGTGT-3’ (sense) and 5’-ACACTCTTGCAAGACTTCTGCTACTTCACTGATGTAAGA-3’ (antisense) for K88A in NC2β.

Nucleotide exchanges S131/124E in CHRAC-15 and K5A in NC2α were inserted using the antisense amplification primers: 5’-AAGGATCCTTATTCGTCAGCTTCATCATGGTC TTCTTCATTATCATT-3’ for CHRAC-15 and 5’-TGCAGTCGACATGCCCTCCAAG GCAAAAAGTACAATGCC-3’for NC2α.

### 2.2.1.10 Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate DNA by size, to analyze or identify PCR products and to purify digested DNA fragments. Depending on the size of the expected DNA fragments agarose gels from 0.8 to 2 % (w/v) in TBE buffer (90 mM Tris base (pH 8.3), 90 mM boric acid, 2.5 mM EDTA) were used. The respective DNA samples were loaded with $^1/_5$ of DNA gel loading buffer (40 % (w/v) sucrose in water, 0.1 % (w/v) bromophenol blue, 0.1 M EDTA) and loaded onto the agarose gel. In addition a DNA size marker was coelectrophoresed. The gel electrophoresis was performed at 100-120 mA at room temperature. After the DNA had reached the desired separation (deduced from the position of the bromophenol blue dye front) the DNA bands were made visible with the fluorescent and intercalating agent ethidium bromide (Sharp et al., 1973). For that purpose, the agarose gel was incubated in an ethidium bromide solution (3 µg ethidium bromide [10 mg/ml] in 600 ml $H_2O$) for 15 minutes and visualized under UV light (302 nm). When the fragments should be recovered from the gel for further working steps, a UV lamp with a radiation of 366 nm was used to avoid DNA damage.

### 2.2.1.11 DNA elution after agarose gel electrophoresis

Elution of DNA fragments from an agarose gel was performed using the QIAEX® II Agarose Gel Extraction Kit (Qiagen, Hilden) according to the manufacturer’s instructions. After excision of the corresponding DNA band, the gel slice was transferred into a microcentrifuge tube (at that stage the DNA containing gel slice can be stored at -20°C). By adding three volumes of QX1 solution the gel was melted at 50°C. Subsequently, 15 µl of glass bead matrix was added and the sample was incubated for further 10 minutes. Glass bead bound DNA was centrifuged for

1 minute at 13000 rpm, washed once with QX1 solution and then twice with buffer PE. The beads were air-dried and finally eluted with 30 µl $dH_20$.

### 2.2.1.12 DNA ligation

The T4 DNA Ligase catalyzes the formation of covalent phosphodiester bonds between adjacent 3' hydroxyl ends of one nucleotide with the 5' phosphate end of another. It thus can be used for ligating a DNA fragment into a cloning vector. The DNA ends generated by cleavage with restriction enzymes can be 'sticky' or 'blunt'. For the ligation reaction the enzyme requires ATP as an energy source. A typical ligation reaction was performed using 100 ng of linearized vector in combination with insert DNA in a ratio of 1:5. In the presence of T4 ligation buffer and 0.5 U of T4 DNA ligase the reaction batch was incubated for 2 hours at 25°C or overnight at 4°C. To check for background clones resulting from self-ligation of inefficiently cleaved or dephosphorylated vectors a control ligation in the absence of insert DNA was performed.

### 2.2.1.13 DNA sequencing

DNA sequencing was performed by using the dideoxy sequencing or chain termination method (Sanger et al., 1977). The method is based on the use of 2',3'-dideoxynucleotide 5'-triphosphates (ddNTPs) in addition to the normal 2'-deoxynucleotide 5'-triphosphates (dNTPs). These nucleotides differ insofar, as the ddNTPs contain a hydrogen group on the 3' carbon instead of a hydroxyl group. These modified nucleotides lead to a termination of chain elongation when incorporated into a growing DNA strand because phosphodiester bonds cannot form between dideoxynucleotides and the succeeding nucleotide. In addition to the dNTPs and the ddNTPs the DNA sequencing mixture contains the DNA polymerase, combined in the Dye Terminator Ready Mix (Big Dye™ Terminator Sequencing Kit, Applied Biosystems). Specific primers constructed in a way that their 3' end is located next to the DNA sequence of interest were used. As the DNA template of interest is synthesized and nucleotides are added the polymerization products differ in length depending on the place where a ddNTP was incorporated. Theoretically this incorporation should take place at least once per each base on the particular DNA template. The generated DNA fragments can therefore be separated by size in a capillary electrophoresis. In addition, the ddNTPs (ddATP, ddGTP, ddCTP and ddTTP) are tagged with four different fluorescent dyes. This allows the detection of the sequencing products by laser scanning in an ABI sequencer (model 373A, Applied Biosystems). This capillary electrophoresis analysis was

done by Andreas Nolte in the Department of Developmental Biochemistry (University of Göttingen).

A sequence reaction contained 400 ng DNA, 8 pmol of specific primer, 1.5 µl sequence buffer and 1.5 µl Dye Terminator Ready Mix. $dH_20$ was added to a final volume of 10 µl. Generally the PCR protocol consisted of 25 cycles including denaturation at 96°C for 30 seconds, primer annealing at 55°C for 15 seconds and chain elongation at 60°C for 4 minutes. After the amplification the fluorescently labeled DNA fragments were purified. For that, 1 µl 3 M sodium acetate, 1 µl 125 mM EDTA and 50 µl 100 % ethanol were added to the PCR reaction batch to precipitate the DNA. After centrifugation the supernatant was removed and the DNA was washed with 70 µl of 70 % ethanol. The supernatant was discarded, the DNA pellet dried, using a Speed Vac and finally dissolved in 30 µl $H_2O$ (HPLC quality).

#### 2.2.1.14 Expression constructs

The coding regions of the respective genes were amplified from plasmid DNA or genomic DNA using two specific oligonucleotide primers complementary to the sequences flanking the coding region of interest with appropriate restriction sites. The resulting constructs were verified by DNA sequencing.

##### 2.2.1.14.1 Bacterial expression constructs

To produce **GST**-fusion proteins expressed in the *E. coli* strain BL21 cloning was performed as follows: the coding regions of human full length p12 and CHRAC-15 as *Eco*RI/*Not*I and the coding region of CHRAC-17 as *Bam*HI/*Xho*I fragment (kindly provided by Patrick Varga-Weisz) into the respective sites of pGEX 4T-1 (GE Healthcare).

The coding region for **His**-tagged proteins were cloned as follows: the coding region of human CHRAC-17 as *Nhe*I/*Eco*RI fragment into the respective sites of pET-28b(+) (Novagen). Respective PCR fragments of human full length p12 and CHRAC-15 were cloned ligation independent in the pET-30 Ek/LIC Vector. Both constructs were kind gifts of Patrick Varga-Weisz (The Babraham Institute, Cambridge, United Kingdom).

##### 2.2.1.14.2 Eukaryotic expression construct

Constructs with an enhanced green fluorescent protein (**EGFP**) tag were generated using the coding regions of human p12, CHRAC-15 and CHRAC-17 as *Eco*RI/*Bam*HI fragments into the

respective sites of pEGFP-C1 (Clontech) or into the respective sites of pEGFP- N1 (Clontech). Human NC2α was cloned into pEGFP-C1 (Clontech) as *Sal*I/*Bam*HI fragment.

The cloning of expression constructs with a red fluorescent protein (**RFP**) tag were cloned as follows: *Eco*RI/*Bam*HI fragments of human p12, CHRAC-15 and CHRAC-17 and *Nhe*I/*Bgl*II fragment of NC2β into the respective sites of pPW1 (modified pEGFP-C1 in which EGFP was replaced by RFP using the restriction sites *Nhe*I/*Bgl*II) or into pRFP-N1, (modified pEGFP-N1 in which EGFP was replaced by mono RFP using the restriction sites *Age*I/*Not*I).

The coding regions for the **EGFP-EGFP-GST** fusion proteins were cloned as follows: gene fragments of the ribosomal protein L23 (more precisely the BiB domain from amino acid 33-74) or full length L23, CHRAC-15 and CHRAC-17 as *Eco*RI/*Bam*HI fragments into the respective sites of pEGFP-EGFP-GST-C1 (modified pEGFP-C1 vector (Clontech) in which a second EGFP as *Nhe*I fragment and a GST-tag as *Bgl*II/*Xho*I fragment were inserted amino-terminally of the MCS). The pEGFP-EGFP-GST-C1 vector was a kind gift of Saskia Hutten and Ralf Kehlenbach (Institute for Biochemistry and Molecular Cellbiology, Department Biochemistry I, University of Göttingen).

**FLAG**-tagged fusion proteins were cloned as follows: the coding region and gene fragments of importin 13 as *EcoR*I/*Xho*I fragments into the respective sites of pCS2flag (modified pCS2plus (Rupp et al., 1994; Turner and Weintraub, 1994) in which a flag tag was inserted amino-terminally of the multiple cloning site as *Nco*I/*EcoR*I fragment); the coding regions of human importin β, *Xenopus* importin 7, and murine importin 9 as *Nru*I fragments into the *Stu*I sites of pCS2flag; the coding region of human importin 5 as *Stu*I/*Xho*I fragment into the respective sites of pCS2flag; the coding region of HSP70 from *Rattus norvegicus* and murine PGC7/Stella as *EcoR*I/*Xba*I fragment into the respective sites of pCS2flag. Full length human hACF1 was cloned into pcDNA3.1 and was kindly provided by Patrick Varga-Weisz (The Babraham Institute, Cambridge, United Kingdom).

All constructs were checked by DNA sequencing.

### 2.2.2 Biochemical methods

#### 2.2.2.1 SDS-PAGE

The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) separates proteins according to their electrophoretic mobility. The migration is thereby not determined by the intrinsic electric charge of the polypeptides, but by the molecular weight of the polypeptide chain (Weber and Osborn, 1969). SDS itself is a strong anionic detergent which denatures secondary and non-disulfide-linked tertiary structures. This is achieved by wrapping the hydrophobic tail around the backbone of the polypeptide. SDS thereby confers a negative charge to each protein proportional to its mass. Specific molecular weight standard range markers allow the assessment of the molecular weight. To attain a complete unfolding of the proteins reducing agents like β-mercaptoethanol must be used.

SDS-PAGE was performed in a discontinuous buffer system including two layers of gel, namely stacking and resolving gel (Laemmli, 1970). The stacking gel is a large pore polyacrylamide gel composed of 5 % acrylamide/bisacrylamide (30:0.8) with a pH of 6.8 (about 2 pH units lower than the electrophoresis buffer). In contrast to the Tris-HCl used for the preparation of the stacking gel, the buffer contains Tris/Glycine. This provides the environment for 'Kohlrausch' reactions determining molar conductivity, which lead to a concentration of the proteins at a thin starting zone before the separation in the resolving gel takes place. However, the stacking gel provides smaller pores with 10-15 % of acrylamide/bisacrylamide (30:0.8) using a Tris-HCl buffer with a pH of 8.9. Here, macromolecules are separated according to their size. In addition, sharpening of the band by the stacking gel results in a higher resolution of the proteins. The different moving properties in the gels are due to the predominant pH values. While chloride ions in the 3 x SDS-PAGE loading buffer (150 mM Tris-HCl (pH 6.8), 3 % (w/v) SDS, 3 % (v/v) β-mercaptoethanol, 30 % (w/v), glycerol, 0.003 % (w/v) bromophenol blue) and the stacking gel and the glycine molecules in the 'Laemmli' buffer (0.25 M Tris, 10 mM EDTA, 1.9 M glycine (pH 8.7), 0.5 % (w/v) SDS) form a moving boundary that carries the SDS treated samples across the stacking gel, when electric current is applied, the higher pH values of the resolving gel results in a ionization of the glycine molecules. Hence, SDS becomes the heading ion, leading to a separation of the proteins.

The different sieving-like properties of the gels are determined by the concentration of the acrylamide/bisacrylamide. However, polymerization only occurs in the presence of free radicals provided by ammonium persulfate (APS) and a cross-linking agent like TEMED (N,N,N',N'-tetramethylethylenediamine).

SDS-PAGE was performed in vertical gels using mini-gel-chambers (Biometra). The composition of the different buffers and gels was as follows: stacking gel buffer: 0.5 M Tris-HCl (pH 6.8), 8 mM EDTA; resolving gel buffer: 1.5 M Tris-HCl (pH 8.9), 8 mM EDTA.

| | **resolving gel** | | | **stacking gel** |
|---|---|---|---|---|
| | 10 % | 12.5 % | 15 % | 5 % |
| $dH_2O$ | 8.45 ml | 6.14 ml | 3.825 ml | 7.2 ml |
| resolving gel buffer | 7 ml | 7 ml | 7 ml | – |
| stacking gel buffer | – | – | – | 3.75 ml |
| acrylamide/bisacrylamide (30:0.8) | 9.25 ml | 11.56 ml | 13.875 ml | 2.4 ml |
| SDS (10 %) | 0.275 ml | 0.275 ml | 0.275 ml | 0.15 ml |
| TEMED | 25 µl | 25 µl | 25 µl | 15 µl |
| APS (1.5 %) | 2.75 | 2.75 | 2.75 | 1.5 ml |

**Table II: Composition of SDS-polyacrylamide gels** (sufficient for 5 'minigels')

Proteins dissolved in SDS-PAGE loading buffer were heated at 95°C for 5 minutes. In addition to the samples a molecular weight marker was loaded onto the gel to estimate the molecular weight of the proteins in the samples. Usually an electric current of 15 mA for migration through the stacking gel and 15-20 mA for migration through the resolving gel was applied.
After electrophoresis the proteins were detected in the gel or transferred onto a nitrocellulose membrane by Western blotting (see chapter 2.2.2.2). For detection the gel was stained and simultaneously fixed in a Coomassie Brilliant Blue solution (50 % methanol, 10 % acetic acid, 0.15 % Coomassie Brilliant Blue R250). For destaining the gel and thus visualizing the proteins, the gel was soaked in a 'strong' destaining solution (50 % methanol, 10 % acetic acid) followed by a treatment in a 'weak' destaining solution (10 % methanol, 5 % acetic acid).

### 2.2.2.2 Immunoblotting

#### 2.2.2.2.1 Tank Blot

To detect proteins via specific antibodies after SDS-PAGE, they have to be transferred from the gel onto a nitrocellulose membrane (Whatman, Dassel). Thus Western blotting was done using the wet blot or tank blot method. Tank blotting was performed by placing the gel onto the nitrocellulose membrane, sandwiched by one Whatman paper on each side. Before assembling the blot, nitrocellulose membrane and Whatman paper were soaked in blotting buffer (25 mM

Tris, 192 mM glycine, 20 % (v/v) methanol). Thereafter, the blotting cassette was clamped to the Mini Trans-Blot® Cell (BioRad, München) and a thermal pack and ice cold blotting buffer were added. The proteins are transferred vertically between electrodes arranged on the sidewalls for 80 minutes at 350 mA. To check uniform blotting the proteins were stained with Ponceau S solution (AppliChem, Darmstadt) afterwards.

#### 2.2.2.2.2 Antibody detection of immobilized proteins

After the proteins had been transferred to the nitrocellulose membrane, they were probed with specific primary antibodies. Using a second antibody, directed against the Fc fragment-specific portion of the primary antibody then allowed the detection of the blotted proteins. In this study, these secondary antibodies were conjugated to the reporter enzyme horseradish peroxidase (HRP). In a reaction with the agent luminol, HRP catalyzed the oxidation of luminol into a chemiluminescent molecule which could be detected with a photographic film. The produced luminescence is proportional to the amount of protein.

After blotting the nitrocellulose membrane was blocked in blocking solution (5 % milk powder in TBST [20 mM Tris-HCl (pH 7.6), 14 mM NaCl, 0.01 % Tween 20]) for 2 hours at room temperature to avoid unspecific binding of the antibodies. Incubation with the primary antibody (diluted in blocking solution) was carried out for 2 hours at room temperature or overnight at 4°C. Some antibodies were used several times before they were discarded. In this case, 0.02 % sodium azide was added and the antibody was stored at 20°C.

After incubation with the primary antibody, the membrane was washed three times for 10 minutes with TBST. The nitrocellulose membrane was incubated with the secondary antibody, also diluted in blocking solution, for 2 hours at room temperature and then again washed with TBST three times for 10 minutes. Protein detection was performed using the ECL™ Western Blotting Detection Reagent (GE Healthcare) according to the manufacturer's instructions on Amersham Hyperfilm™ ECL.

#### 2.2.2.2.3 Stripping of Western blot membranes

Before reusing a protein-containing nitrocellulose membrane, the bound antibodies had to be removed. Therefore, the membrane was incubated in stripping buffer (62.5 mM Tris-HCl (pH 6.7), 2 % SDS (w/v), 100 mM β-mercaptoethanol) at 65°C for 15 minutes and subsequently washed in TBST three times for at least 10 minutes. After washing the membrane was blocked again and incubated with the respective primary and secondary antibody.

#### 2.2.2.3 Recombinant protein expression and purification

In general, recombinant proteins were expressed in the *E. coli* expression strain BL21 (DE3) or JM109. A 50 ml preculture of the appropriate clone was grown in LB media overnight at 37°C. The culture was scaled up to 1 l and further grown until an optical density of 0.8 was reached. Expression of the recombinant proteins was induced by the addition of 0.2 to 1 mM of isoprypylthio-β-D-galactoside (IPTG). Binding of IPTG inactivates the lac repressor and transcription of the downstream region can occur. The expression temperature and time as well as the IPTG concentration depended on the protein to be expressed. After expression, the culture was cooled on ice and 5 ml of 2 mM phenylmethylsulfonyl fluoride (PMSF) dissolved in 100 % ethanol was added. Cells were collected by centrifugation at 4°C for 10 minutes at 6000 rpm (GSA rotor, Sorvall®), resuspended in the suitable buffer, containing 1 % Complete® protease inhibitor solution and then frozen in liquid nitrogen (frozen suspensions can be stored at -80°C for several months). After thawing, the cells were lysed by sonication in an ice-water bath for 3x 40 seconds (amplitude 42 %) with a break of 10 seconds after each pulse. The lysate was cleared by centrifugation for 30 minutes at 15000 rpm (SS34 rotor, Sorvall®) and the resulting supernatant was subjected to affinity chromatography.

Proteins containing a 6xHis-tag were bound to nickel nitrilotriacetic acid (NTA) -agarose (Qiagen), whereas proteins or protein complexes carrying a GST-tag (glutathione S-transferase; ~ 27 kDa) were bound to Glutathione Sepharose™ 4 Fast Flow (GE Healthcare) at 4°C. After washing three times, recombinant proteins were eluted in a column procedure. Nickel-NTA Agarose bound proteins were eluted from the matrix (resin) with 250 mM and 400 mM imidazole, respectively, while the proteins or protein complexes bound to GST were eluted with 10 mM reduced glutathione (10 x stock solution: 500 mM Tris-HCl (pH 8.0), 1.5 M NaCl, 100 mM glutathione)

Depending on the protein and the desired purity further purification steps like size exclusion (gel filtration) or ion-exchange chromatography were performed. In gel filtration the proteins are separated based on their size. This is achieved with a column packed with small porous polymer beads. As the protein solution travels through the column, small proteins enter these pores while large particles traverse the column and thus elute faster. Purification using ion exchange chromatography depends on the reversible adsorption of charged molecules to immobilized ion exchange groups of opposite charge. Molecules vary in their interaction with the ion exchanger due to differences in their charges and distribution of charge on their surface.

Before freezing, the purified proteins were dialysed in transport buffer or supplemented with 250 mM sucrose.

#### 2.2.2.3.1 Expression and purification of CHRAC subunits

**GST-CHRAC-15** and **GST-CHRAC-17** were expressed in *Escherichia coli* BL21 (DE3) in LB medium for 3 hours at 30°C with 0.5 mM isopropyl-β-D-thiogalactopyranoside. The cells were centrifuged at 4°C and resuspended in buffer A (50 mM Tris-HCl (pH 7.5), 500 mM NaCl, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol) completed with 1 % Complete® protease inhibitor solution and lysed by sonication. The recombinant proteins were bound to glutathione-Sepharose (500 µl). After washing three times with buffer A, the immobilized proteins were used for GST-Pulldown experiments or the GST-fused proteins were eluted using reduced glutathione in 50 mM Tris (pH 8.0), 150 mM NaCl.

#### 2.2.2.3.2 Expression, purification and dialysis of nuclear cargo complexes

Epitope-tagged CHRAC-15/17 and p12/CHRAC-17 complexes were generated as follows: CHRAC-15 and CHRAC-17 as well as p12 and CHRAC-17 were coexpressed in *Escherichia coli* BL21 (DE3). The cultures were grown in LB medium at 37°C to an optical density of 0.8 at 600 nm. After shifting the temperature to 25°C bacterial protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside and the culture was grown for 3 hours. The collected bacteria were resuspended in buffer B (20 mM Tris-HCl (pH 7.5), 300 mM NaCl, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol) with 1 % Complete® protease inhibitor solution, lysed by sonication and the recombinant complexes were purified on 500 µl nickel NTA-agarose at 4°C for 3 hours. Afterwards, the protein solution was washed three times with buffer A containing 20 mM imidazole, loaded onto a column and bound proteins were eluted with 3x 1 ml buffer A containing 250 mM imidazole and additionally with 2x 1 ml buffer A containing 400 mM imidazole. Protein complexes destined for *in vitro* import assay were dialyzed three times for a least 3 hours against transport buffer (20 mM HEPES-KOH (pH 7.4), 110 mM KAc, 5 mM $MgAc_2$, 1 mM, EGTA, 250 mM Sucrose, 2 mM DTT) using the QuixSep™ Micro Dialyzer (Roth, Karlsruhe) in combination with the ZelluTrans dialysis membrane (Roth, Karlsruhe)
For complexes to be applied for *in vitro* GST-Pulldown assays an additional purification step on Glutathione Sepharose™ 4 Fast Flow (GE Healthcare) followed directly after elution. For that, GST-CHRAC-15/His-CHRAC-17 and GST-p12/His-CHRAC-17 complexes were pooled and immobilized on 200 µl glutathione-Sepharose. Binding to glutathione-Sepharose was performed in GST-Pulldown binding buffer (50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol) at 4°C overnight. After binding, the matrix was washed three times with GST-Pulldown binding buffer.

#### 2.2.2.3.3 Expression and purification of import receptors and accessory factors

For expression of recombinant import factors, *E. coli* strains carrying a pREP4 repressor or harboring the $lacI^q$ gene on the F-factor episome were used in this study. Otherwise, because expressed import factors are toxic to the cells, 'leaky' expression may result in impaired growth or even death of the bacteria carrying the expression plasmid. To maximize transcription expression 2 % glucose was added to the media. To guarantee activity and stability of the recombinant import factors different Tris buffers were used for purification. Furthermore, before freezing 250 mM sucrose was added.

##### 2.2.2.3.3.1 Importin α

Carboxy-terminally His-tagged importin α1 (referred to as importin α) from *Xenopus laevis* was expressed in *E. coli* JM109 as described (Görlich et al., 1994). It was cloned as a *Sph*I/*Bam*HI fragment in pQE70. The expression plasmid was kindly provided by Dirk Görlich (Max Planck Institute for Biophysical Chemistry, Göttingen). For expression, JM109 cells were freshly transformed and grown overnight. The next day, the resulting colonies were rinsed with LB und used for inoculation of a pre-culture consisting of LB medium with 2 % glucose. This pre-culture was incubated at 37°C overnight. Then the culture was scaled up to 2 l of LB medium and grown to an $OD_{600}$ of 0.9. 2 mM IPTG was used to start expression for 3 hours at 26°C. The bacterial cells were pelleted, resuspended in buffer C (50 mM Tris-HCl (pH 7.5), 500 mM NaCl, 5 % glycerol, and 2 mM β-mercaptoethanol), freeze-thawed and lysed by sonication. The expressed proteins were centrifuged and bound to nickel NTA-agarose overnight at 4°C. The matrix was washed three times with buffer C containing 20 mM imidazole. In a column approach, importin α was eluted, using 100 mM imidazole in 50 mM Tris-HCl (pH 7.5) and 300 mM NaCl and dialyzed against buffer D (50 mM Tris-HCl (pH 7.5), 200 mM NaCl, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol).

##### 2.2.2.3.3.2 Importin β

The importin β expression vector was a kind gift from Dirk Görlich (Max Planck Institute for Biophysical Chemistry, Göttingen). Human importin β was cloned in pQE60 containing a carboxy-terminal His-tag and expressed in *E. coli* M15 [pREP4] as described in the literature (Kutay et al., 1997b). Briefly, starting from a single colony a 50 ml pre-culture was grown overnight in 200 ml LB medium containing 2 % glucose, ampicillin, and kanamycin. This culture was used to inoculate 600 ml of the same medium. The bacteria were grown at 37°C to an $OD_{600}$ of 1 and then 800 ml of ice-cold LB medium with 4 % ethanol was added. The

temperature was now shifted to 21°C. When the medium had reached this temperature 0.5 mM IPTG was added and incubated for 4 more hours. Afterwards, cells were collected, resuspended in buffer C, freeze-thawed, and lysed by sonication. Purification was performed using nickel NTA-agarose and eluted with 200 and 400 mM imidazole. Further purification was performed by chromatography on Superdex 200, equilibrated with buffer D.

#### 2.2.2.3.3.3 Importin 5

The expression plasmid for amino-terminally 6xHis-tagged importin 5 was a kind gift of Dirk Görlich (Max Planck Institute for Biophysical Chemistry, Göttingen). Human importin 5 was expressed in *E. coli* JM109 from pQE32 as described (Jäkel and Görlich, 1998). A pre-culture was grown overnight in 100 ml 2YT medium with 30 mM $K_2HPO_4$ (pH 7.0) and 2 % glucose and used to inoculate 900 ml of the same medium but lacking the glucose. After an $OD_{600}$ of 1 was reached, 1 l of cold medium containing 4 % ethanol was added. Expression took place for 4 hours at 17°C using 0.25 mM IPTG. Purification was performed as described for importin β.

#### 2.2.2.3.3.4 Importin 7

Importin 7 from *Xenopus* was cloned in pQE80, containing a 10xHis-tag. The expression vector was provided by the lab of Dirk Görlich (Max Planck Institute for Biophysical Chemistry, Göttingen). Expression was performed according to Jäkel and Görlich (1998) in *E. coli* M15 [pREP4]. A pre-inoculum was grown in 100 ml 2YT medium containing 30 mM $K_2HPO_4$ and 2 % glucose overnight. Then, 900 ml of the same medium but without glucose were inoculated and grown at 37°C until an $OD_{600}$ of 0.7 was reached. After shifting the temperature to 26°C the culture was further incubated until a density of 2 was reached. Expression was induced with 0.05 mM IPTG. In addition, 1 l of 2YT medium containing 30 mM $K_2HPO_4$ and 4 % ethanol was added and incubated overnight at 13°C. After cooling the culture on ice 1 mM PMSF was added, centrifuged and resuspended in buffer E (50 mM Tris-HCl (pH 7.5), 300 mM NaCl, 5 mM $MgCl_2$, 2 mM β-mercaptoethanol) containing 5 % glycerol. Lysis was performed by sonication and importin 7 was bound to nickel NTA-agarose. The matrix was washed three times with 20 mM Tris-HCl (pH 7.5), 300 mM NaCl and 40 mM imidazole, and the protein was eluted with 200 mM imidazole and dialyzed against buffer D.

#### 2.2.2.3.3.5 Importin 9

Amino-terminally 6xHis-tagged importin 9 was cloned as *Xma*I fragment into pQE32 and was kindly provided by Ulrike Kutay (Institute for Biochmemistry, ETH Zürich). Expression of the murine importin 9 was performed as described (Mühlhäusser et al., 2001) using *E. coli* JM109 strain. A 100 ml 2YT overnight culture containing 30 mM $K_2HPO_4$ (pH 7.0) and 2 % glucose was used to inoculate 900 ml 2YT medium with 30 mM $K_2HPO_4$. The culture was grown to an $OD_{600}$ of 1 before 1 l of cold 2YT medium with 30 mM $K_2HPO_4$ and 2 % ethanol was added. Expression took place at 20°C for 3 hours using 0.5 mM of IPTG. Importin 9 was purified as described for importin β.

#### 2.2.2.3.3.6 Importin 13

The expression plasmid for human importin 13 was kindly provided by the group of Dirk Görlich (Max Planck Institute for Biophysical Chemistry, Göttingen) and expressed in *E. coli* JM109 as described (Mingot et al., 2001). Importin 13 was cloned as *Bam*HI/*Hin*dIII fragment in the MCS of pQE80 with a His-tag at the amino-terminus. For expression, a 50 ml pre-culture of 2YT medium with 30 mM $K_2HPO_4$, 2 % glucose and 100 µg/ml ampicillin as appropriate antibiotic was inoculated with a freshly grown bacterial colony harboring the expression vector and was grown overnight. The culture was scaled up to 700 ml 2YT containing 30 mM $K_2HPO_4$, 100 µg/ml ampicillin and 2 % glycerol. The pre-culture was grown at 37°C until an $OD_{600}$ of 1 was reached. Then, 1.2 l of the same 2YT medium was added and the temperature was shifted to 16°C. Expression of importin 13 was induced with 0.1 mM IPTG for 18 hours at 16°C when an $OD_{600}$ of 0,75 was reached. Cells were centrifuged, resuspended in 50 mM Tris-HCl (pH 7.5), 500 mM NaCl, 10 mM MgAc, 5 mM β-mercaptoethanol, 5 % glycerol, and lysed by sonication. Afterwards, the protein was purified in two steps. First, it was bound on nickel NTA-agarose and eluted with 250 mM imidazole and further purified by gel filtration with Superdex 200. The column was therefore equilibrated with 50 mM Tris-HCl (pH 7.5), 100 mM NaCl and 3 mM MgAc.

#### 2.2.2.3.3.7 NTF2

Expression and purification of the RanGDP specific import factor NTF2 was performed as described (Kutay et al., 1997b; Ribbeck et al., 1998). Briefly, untagged NTF2 was expressed from a pET expression vector in *E. coli* BL21 (DE3) pLysS (Novagen) for 2 hours at 30°C with 1 mM IPTG (Kent et al., 1996). NTF2 was precipitated from the bacterial lysate with ammonium

sulfate (50 % saturation), the pellet was dissolved in 250 ml buffer F (50 mM Tris (pH 8.0), 2 mM DTT) and NTF2 was then bound to Q Sepharose FF (HiLoad Q Sepharose Fast Flow column, GE Healthcare) and equilibrated in buffer F. NTF2 was eluted in the NaCl gradient (buffer G: 50 mM Tris (pH 8.0), 500 mM NaCl, 2 mM DTT) at about 230 mM NaCl. Final purification was performed on Superdex 75 (GE Healthcare) where NTF2 eluted at a position expected for the homodimer.

#### 2.2.2.3.3.8 Ran

Ran was cloned as *Sph*I/*Hin*dIII fragment into pQE32. The resulting amino-terminally His-tagged Ran (wild-type and Q69L) was expressed in *E. coli* JM109 according to Ribbeck et al. (1998) using 2 mM IPTG for 30 hours at 30°C. The bacterial pellet of **wild-type Ran** was resuspended in buffer H (50 mM $K_2HPO_4$ (pH 7.0), 200 mM NaCl, 5 mM $MgCl_2$), sonicated and centrifuged. Then, 1 mM GDP was added and Ran was bound on nickel NTA- Agarose. Elution was performed with 150 mM imidazole in 50 mM $K_2HPO_4$ (pH 7.0), 5 mM $MgCl_2$. The fractions were pooled and diluted 5-fold in 5 mM $K_2HPO_4$ (pH 7.0), 0.5 mM $MgCl_2$, 5 % glycerol. Using a Mono S column (GE Healthcare) Ran was eluted with a linear gradient from buffer I (20 mM $K_2HPO_4$ (pH 7.0), 0.5 mM Mg $Cl_2$, 5 % glycerol) to buffer J (0.5 mM $K_2HPO_4$ (pH 7.0), 0.5 mM Mg $Cl_2$). Thereby, RanGDP elutes earlier than RanGTP.

Purification of **RanQ69L (GTP)** was performed as follows: the bacterial pellet was resuspended in 50 mM HEPES-KOH (pH 7.0), 100 mM NaCl, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol, sonicated, centrifuged and bound to nickel NTA-agarose. Pre-bound nucleotides were removed with 50 mM HEPES-KOH (pH 7.0), 200 mM NaCl, 5 mM β-mercaptoethanol by washing for 1 hour. In the presence of 100 µM GTP RanQ69L was reloaded with GTP in buffer K (50 mM HEPES-KOH (pH 7.0), 200 mM NaCl, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol) for 1 hour. Elution was also performed in buffer K with 250 mM imidazole. The resulting positive RanQ69L fractions were pooled, supplemented with 250 mM sucrose and frozen in liquid nitrogen.

### 2.2.2.4 Bradford protein assay

The Bradford assay is a spectroscopic analytical procedure to measure the protein concentration in a solution (Bradford, 1976). It is based on an absorbance shift of the dye Coomassie Brilliant Blue 250 from 465 nm to 595 nm based on the binding of proteins. The cationic, unbound red form of the Coomassie dye interacts with the proteins via van der Waals forces and furthermore through ionic interactions, leading to the protein bound stable blue form of Coomassie. Since the

amount of protein in the solution is proportional to the color change of Coomassie, observable in an increasing absorbance, the protein concentration can be measured spectroscopically in a photometer. In this study, the Quick Start™ Bradford Protein Assay (BioRad, München) was used according to the manufacturer's instruction. In addition, BSA (1 mg/ml) in the range of 0-0.5 mg/ml served as protein standard. Briefly, standard and unknown protein solutions were pipetted each into a clean microcentrifuge tube and filled up with the same buffer as the proteins are diluted in, to a final volume of 20 µl. 1 ml of 1x Dye Reagent was added, incubated for at least 5 minutes at room temperature and measured at 595 nm.

#### 2.2.2.5 GST-Pulldown assays

##### 2.2.2.5.1 *In vitro* binding studies with bacterially expressed proteins

Recombinant GST-fusion proteins or protein complexes immobilized on Glutathione Sepharose™ 4 Fast Flow (GE Healthcare) were used as affinity matrix for *in vitro* binding experiments. Appropriate amounts of affinity matrix were incubated at 4°C for 4 hours with 0.2 µM recombinant import receptors. Binding experiments were performed in the absence or presence of 2 µM RanQ69L (GTP). After washing three times with ice-cold GST-Pulldown binding buffer and an additional incubation for ten minutes in between, the affinity matrix was boiled in SDS-PAGE loading buffer and the matrix-bound proteins were analyzed by SDS-PAGE followed by Coomassie-staining.

##### 2.2.2.5.2 *In vivo* GST-Pulldown assay of CHRAC subunits in HeLa P4 cells ('Korn-Assay')

To analyze the *in vivo* dimerization of overexpressed CHRAC subunits in HeLa P4 cells a GST-Pulldown assay was performed based on the method described by Korn et al. (2002). For that, HeLa P4 cells were cultured in 10 cm dishes and transiently cotransfected with CHRAC-15 and CHRAC-17 fused to EGFP-EGTP-GST and RFP, respectively. Twenty-four hours post transfection, cells were washed with PBS and harvested in 1 ml PBS using a cell scraper (Sarstedt, Langenhagen), then pelleted by centrifugation at 1000 rpm for 5 minutes and finally resuspended in 1 ml Korn buffer (20 mM HEPES-NaOH (pH 7.4), 100 mM NaCl, 1 mM EDTA with 10 % glycerol and 5 mM DTT freshly supplemented). Cell lysis was performed by sonication (5x 3 seconds, amplitude 45 %) before 0.01 % Triton X-100 was added and incubated end-over-end at 4°C for 5 minutes. Subsequently, the extract was centrifuged for 30 minutes at 14000 rpm and 4 °C. The resulting supernatant was applied on 40 µl glutathione-Sepharose

(equilibrated with Korn buffer) and incubated at 4°C overnight. After washing three times with Korn buffer with an additional end-over-end incubation step of 10 minutes in between, the beads were boiled in SDS-PAGE loading buffer and analyzed by Western blotting.

### 2.2.3 Cell biological methods

#### 2.2.3.1 Eukaryotic cells

In this study, HeLa P4 cells (Charneau et al., 1994) were applied for cell biological approaches.

##### 2.2.3.1.1 Cultivation and Storage

HeLa P4 cells were grown in Dulbecco's modified Eagle's medium (DMEM, GIBCO) additionally containing 10 % (v/v) fetal calf serum (FCS), 2 mM glutamine and 1 % (v/v) Penicillin-Streptomycin (10 mg/ml in $H_2O$) and 0.5 % (v/v) Gentamycin (10 mg/ml in $H_2O$), respectively. Cells were maintained in a humidified cell incubator with 5 % $CO_2$ atmosphere at 37°C.

Having passed the exponential growth phase (log phase) where the cells have the highest metabolic activity and reach a stationary stage (plateau phase) when the cell number remains constant, the cells have to be subcultured. A confluent, adherently growing HeLa P4 culture was splitted every three days about 1:10 to 1:20, respectively. For that purpose, the medium was removed and the cells wee washed with PBS. To detach the cells from the surface (also enforced by shaking) trypsin/EDTA (0.05 % (w/v) trypsin, 0.02 % (w/v) EDTA in PBS) was incubated at 37°C for 3 minutes to allow the proteolytic reaction to take place. With the addition of 5 volumes of DMEM the proteolysis was stopped and the cells were transferred to a 50 ml conical tube, vigorously pipetted up and down and centrifuged at 1000 g for 5 minutes. Afterwards 10 ml of medium were added, the cells were resuspended and finally filled up with DMEM to 20 ml.

For subsequent transfection experiments cells were plated into 12 wells with $1x10^5$ cells/well and in 6 wells for *in vitro* nuclear import assays with $3x10^5$ cells/well. For *in vivo* GST-Pulldown assays, a 10 cm dish with $3x10^6$ cells was used. Cells were plated 24 hours prior to the experiments. For *in vitro* nuclear import assays and fluorescence microscopy after *in vivo* transfection, the cells were grown on glass cover slips (Ø 10 mm).

HeLa P4 cells were stored in liquid nitrogen. To prevent damage because of the formation of ice crystals the cryoprotective agent DMSO, which also lowers the freezing point, was added. $3x10^6$ cells per ml freezing medium (20 % FCS, 10 % DMSO in DMEM) were cooled slowly to -80°C

allowing the water to move out of the cells before freezing to avoid cell damage and stored in a polystyrene box. The next day the cells were transferred onto liquid nitrogen.
For recovery the cells were thawed very quickly in a 37°C water bath. Afterwards, the culture was diluted into pre-warmed DMEM medium. When the cells were attached to the surface, the medium was changed to remove traces of DMSO.

#### 2.2.3.2 Transient transfection of HeLa cells

In this study a chemical method was used to introduce plasmid DNA into eukaryotic HeLa P4 cells. Based on the observation that DNA coated with synthetic cationic lipids can be introduced into cells by fusion, chemical transfection with liposome-like vesicles using the Effectene™ Transfection Regent (Qiagen) was performed. The DNA was condensed using the kit specific Enhancer in a defined buffer system (EC Buffer). Effectene was added to generate Effectene-DNA complexes and, mixed with DMEM, it was directly added to the cells. The medium was changed 6 hours after transfection. Twenty-four hours post transfection the cells were fixed with 3 % paraformaldehyde in PBS for 15 minutes. After washing with PBS the nuclei were stained using Hoechst33258 (10 µg/ml, Molecular Probes), the cells were mounted in HistoGel (Linaris) and analyzed directly by fluorescence microscopy using a Zeiss microscope (Axioskop 20) with a 40 x objective lens (Plan Neufluar). Pictures were obtained by digital imaging using the software Axio Vision 4.5.
For Western blotting analysis the transfected HeLa P4 cells were harvested 24 hours after the treatment, washed and boiled in SDS-PAGE loading buffer.

#### 2.2.3.3 Immunofluorescence and Immunodetection

The subcellular localization of endogenous proteins or overexpressed proteins harbouring a non-fluorescent tag was analyzed by indirect immunofluorescence. For that, untransfected HeLa P4 cells or HeLa P4 cells 24 hours post-transfection were fixed with 3 % paraformaldehyde in PBS for 15 minutes, washed three times, permeabilized with 0.5 % Triton X-100 in PBS for 10 minutes and blocked with 3 % bovine serum albumin in PBS (blocking buffer). After an incubation of 30 minutes, the primary antibody diluted in blocking buffer was incubated for 1 hour at 37°C. Cells were washed with PBS and subsequently incubated with the appropriate secondary antibody for 1 hour at 37°C. The nuclei were visualized with 4',6-diamidino-2'-phenylindole (DAPI) as part of the mounting medium (Vectashield®, Vector Laboratories).

#### 2.2.3.4 Cell cycle arrest experiments

To analyze cell cycle phase specific distribution of different proteins complexes, transfected HeLa P4 cells were treated with sodium butyrate, Fluorouracil or Aphidicolin. All three different agents inhibit cell growth leading to a cell cycle arrest of the HeLa cells at different stages. While sodium butyrate inhibits the cyclin D1 expression, leading to an arrest of HeLa P4 in early G1 period (Archer et al., 1998; Kress et al., 1986; Vaziri et al., 1998), Aphidicolin inhibits eukaryotic nuclear DNA replication by blocking DNA polymerase α and δ (Pedrali-Noy et al., 1980). Thus, HeLa P4 cells treated with Aphidicolin were blocked specifically in early S-phase. As a pyrimidine analogue, Fluorouracil is transformed into cytotoxic metabolites which are incorporated into DNA, specifically inducing a cell cycle arrest in S-phase (Takeda et al., 1999).
HeLa P4 cells were treated with 40 µM Fluorouracil (Sigma) 24 hours post transfection for 12 hours or with 10 µM Aphidicolin (Alexis) and 5 mM sodium butyrate (Sigma) for another 24 hours. The effect of the inhibitors was determined by fluorescence microscopy.

#### 2.2.3.5 Inactivation of exportin 1

Inactivation of exportin 1 mediated nuclear export in transfected HeLa P4 cells was performed 24 hours post transfection with 10 ng/µl Leptomycin B (LMB, Sigma). LMB, a secondary metabolite produced by *Streptomyces spp*. specifically inhibits the transport receptor exportin 1/CRM1 by alkylation (Nishi et al., 1994).

#### 2.2.3.6 *In vitro* nuclear import assay

The *in vitro* import assay is based on the method established by Adam et al. (1990). This assay allows the monitoring of nuclear import processes by using recombinant transport receptors or reticulocyte lysate in combination with recombinant substrates. Thus, interference of endogenous proteins is excluded. Import reactions were performed as described previously (Jäkel and Görlich, 1998).
HeLa P4 cells were grown on 10 mm coverslips to a confluence of 40-80 %. Permeabilization of the plasma membrane was performed with 40 µg/ml digitonin (Calbiochem). Treatment with digitonin as a non-ionic detergent selectively leads to a permeabilization of the cholesterol-rich plasma membrane while the nuclear envelope stays intact. Digitonin treatment was performed on ice for 5-10 minutes. In the following three washing steps (1 minute, 5 minutes and, 10 minutes) with ice-cold transport buffer (20 mM HEPES-KOH (pH 7.4), 110 mM KAc, 5 mM $MgAc_2$, 1 mM, EGTA, 250 mM Sucrose, 2 mM DTT) the endogenous soluble transport receptors were

now washed out of the cells. The amount of remaining soluble proteins in the cells was between 20-30 %. Afterwards, the permeabilized cells were incubated with 20 µl of transport reaction mix at 37°C for 30 minutes. This reaction mix contained 0.4 µM substrate, either 10 µl of reticulocyte lysate (Promega) as general source of import receptors or 0.4 µM recombinant, purified importin, and an energy-regenerating system consisting of 0.5 mM ATP, 0.5 mM GTP, 10 mM creatine phosphate, 50 µg/ml creatine kinase. When performing reconstitution experiments with recombinant transport factors 3 µM RanGDP and 0.5 µM NTF2 were added to generate an artificial RanGTP gradient across the nuclear membrane. For negative controls, the assay was done in the absence of import receptors. HeLa P4 cells were fixed with 3 % paraformaldehyde in PBS for 15 minutes and analyzed by indirect immunofluorescence microscopy with a Zeiss microscope (Axioskop 20). For that purpose, fixed cells were permeabilized with 0.5 % Triton X-100 in PBS for 10 minutes and blocked for 30 minutes with 3 % bovine serum albumin in PBS. Afterwards, anti-GST polyclonal rabbit antibody (Santa Cruz) and anti-rabbit Alexa 488 antibody (Molecular Probes) was used to detect the applied substrate. The cell nuclei were visualized with 4',6-diamidino-2'-phenylindole (DAPI, Vectashield®; Vector Laboratories)

#### 2.2.3.7 Preparation of HeLa low salt extract (LSE)

$5x10^9$ HeLa cells (15 ml) were added to 20 ml buffer K (20 mM Tris (pH 7.5), 1 mM MgAc, 0.5 mM EGTA, 2 mM, β-mercaptoethanol, and 1 % Complete® protease inhibitor solution) and homogenized with a tightly fitting Dounce homogenizer 10 times. The lysate was pelleted by centrifugation at 4000 rpm for 5 minutes at 4°C. The supernatant (S1) was stored at 4°C, and the pellet was resuspended in 25 ml buffer K and 'dounced' again 5 times. After that, the lysate was again centrifuged at 4000 rpm for 5 minutes. The resulting supernatant (S2) was pooled with S1 and centrifuged at 15000 rpm for 15 minutes. In order to receive the low salt extract (LSE), the supernatant of the previous centrifugation (S3) was again centrifuged for 2 hours at 40000 rpm and this supernatant was subsequently mixed with 250 mM Sucrose. 2 ml aliquots of the LSE were stored at -80°C.

#### 2.2.3.8 RNA interference (RNAi) experiments

RNAi knockdown experiments were done to inhibit translation of specific mRNAs by transfection of short interfering RNAs (siRNA) into HeLa P4 cells. In this study three different Stealth™ RNAi (Invitrogen, Karlsruhe) duplex oligoribonucleotides were generated, using the

BLOCK-IT™ RNAi Express design and ordering tool (https://rnaidesigner.invitrogen.com/rnaiexpress/rnaiExpress.jsp) from Invitrogen. The following oligoribonucleotides turned out to be the most efficient and were used for the experiments: Primer 1: IPO5HSS105868 (RNAi) - UCG GAG ACU GCA GCU GCU AUG UUA A and Primer 2: IPO5HSS105868 (RNAi) - UUA ACA UAG CAG CUG CAG UCU CAG A. As stated by the manufacturer, Stealth™ RNAi provides higher specificity and increased stability in serum and cell culture.

#### 2.2.3.8.1 RNAi transfection procedure

Transfection of HeLa P4 cells with siRNA was performed using Oligofectamine™ Reagent. One day before transfection, cells with a density of $10^5$ cells/well were plated in a 6 well plate with 3 ml DMEM containing 10 % FBS and 2mM glutamine but without further addition of antibiotics. Transfection was carried out according to the manufacturer's instructions. For that, 10 µl of the 20 µM oligonucleotide stock were diluted in 175 µl Opti-MEM® I Reduced Serum Medium (Invitrogen, Karlsruhe) and mixed gently. Subsequently, 4 µl of Oligofectamine™ were diluted in Opti-MEM® I Reduced Serum Medium to a final volume of 15 µl, mixed gently and incubated at room temperature for 5-10 minutes. The diluted Oligonucleotides were combined with diluted Oligofectamine™ to a final volume of 200 µl and incubated for another 15-20 minutes at room temperature. While forming transfection complexes, HeLa P4 cells were washed once with FCS deficient DMEM medium. Prior to the Oligonucleotides/Oligofectamine mix, 800 µl of this medium was added to each well. Cells were incubated for 4 hours at 37°C. Afterwards, 500 µl of DMEM with 3 x FCS were added, without removing the transfection mixture. HeLa P4 cells were assayed for gene knockdown by Western blotting analysis 3 and 7 days post transfection.

# 3 Results

As integral parts of the human chromatin accessibility complex (CHRAC) (Poot et al., 2000), the histone fold subunits CHRAC-15 and CHRAC-17 are proteins that fulfill their function in the nuclear compartment. The dimerized subunits bring the other two CHRAC subunits ACF1 and SNF2H in close proximity to the DNA, thus enhancing the nucleosomal sliding processes (Kukimoto et al., 2004). So far, the mechanism how CHRAC-15 and CHRAC-17 are imported into the nucleus has not been characterized. Indeed, several possibilities exist: (i) autonomous import of CHRAC-15 and CHRAC-17 via independent NLSs, (ii) import as a preassembled complex, (iii) 'piggyback' transport into the nucleus via binding partners such as ACF1 or, (iv) import into the nucleus via passive diffusion, since both proteins are smaller than the exclusion size of the nuclear pore complex (NPC). In the latter case, passive diffusion would theoretically be possible even for the heterodimer. The aim of this study was to systematically analyze the nuclear import of the CHRAC-15 and CHRAC-17 subunits. In parallel, the nuclear transport of a related histone fold pair, p12/CHRAC-17 was studied.

## 3.1 Individual overexpression of CHRAC-15 and CHRAC-17 leads to their localization in the cytoplasm

First we analyzed the subcellular localization of endogenous CHRAC-15 and CHRAC-17 in HeLa P4 cells. The endogenous proteins were detected using specific antibodies against the histone fold motif containing CHRAC subunits. Both endogenous proteins, CHRAC-15 and CHRAC-17 were dominantly localized in the nucleus (Fig. 5*A*, upper panel), as expected by their nuclear function. To analyze the nuclear transport of the individual subunits CHRAC-15 and CHRAC-17 were fused to enhanced green fluorescent protein (EGFP), red fluorescent protein (RFP), and EGFP-EGFP-GST (EEG). Subsequently, HeLa P4 cells were transiently transfected with plasmid DNA encoding the different fusion proteins. The subcellular localization of the gene products, all under the control of a strong CMV promoter, was determined 24 hours after the transfection by direct fluorescence microscopy.

Overexpression of each of the fusion proteins led to a cytoplasmic localization, except for EGFP-CHRAC-15 which was homogenously distributed in transfected cells. Despite their similar molecular weight (~ 30 kDa), the fusion of EGFP and RFP to CHRAC-15 resulted in a different subcellular localization. Since EGFP and RFP diffuse passively into the nucleus when expressed in HeLa P4 cells (Fig. 5*B*), the nuclear transport of EGFP-CHRAC-15 might

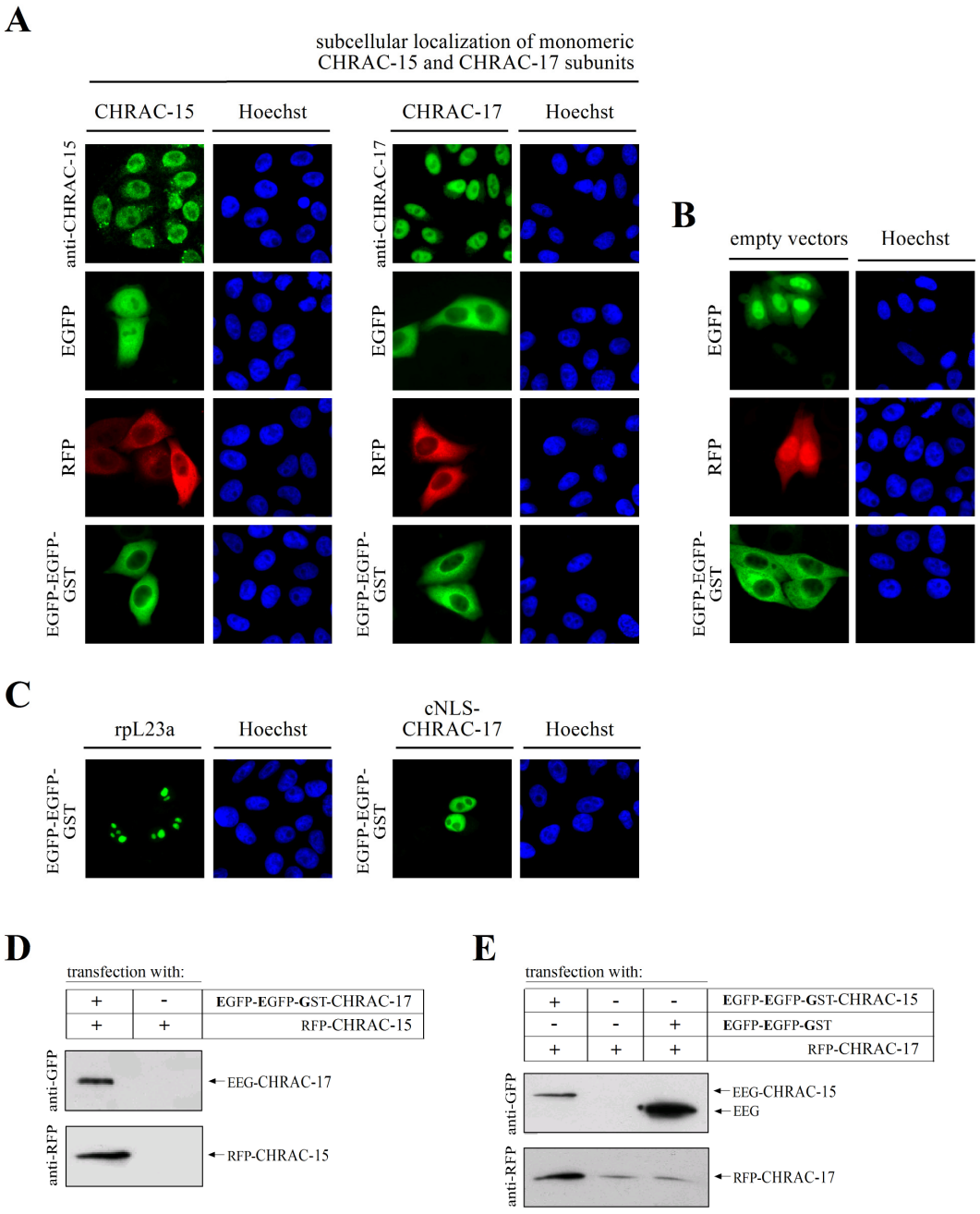


**FIG. 5: Subcellular localization of CHRAC-15 and CHRAC-17.** *(A)* The subcellular localization of different CHRAC-15 and CHRAC-17 subunits in HeLa P4 cells is shown. Endogenous proteins were detected using specific antibodies against CHRAC-15 and CHRAC-17. HeLa P4 cells were also transiently transfected with plasmids coding for EGFP-, RFP- and EGFP-EGFP-GST (EEG)-tagged CHRAC-15 and CHRAC-17. The subcellular localization was determined after 24 hours by direct fluorescence. The nuclei were stained using Hoechst. The endogenous proteins localized mainly in the nucleus, whereas the CHRAC-15 and CHRAC-17 subunits fused to a reporter protein localized exclusively in the cytoplasm, with the exception of EGFP-CHRAC-15. *(B)* EGFP, RFP, and EEG alone were overexpressed in HeLa P4 cells and the subcellular localization determined 24 hours post transfection by direct fluorescence. Hoechst was used to visualize the nuclei. EGFP and RFP diffused into the nucleus, whereas EEG localized exclusively in the cytoplasm. *(continued on page 65)*

be accomplished by passive diffusion. Interestingly, Seibel and coworkers (2007) recently described the problem of transport receptor independent diffusion of EGFP into the nucleus, and the authors concluded that the use of EGFP as reporter protein fused to small proteins is not adequate to investigate nuclear import processes.

However, contrary to EGFP-CHRAC-15, EGFP-CHRAC-17 was strictly detected in the cytoplasm and obviously no diffusion of the fluorescent protein occurred. The cytoplasmic retention of CHRAC-15 and CHRAC-17 fusion proteins, when overexpressed in HeLa P4 cells could have sterical reasons in contrast to the endogenous proteins. The fusion of EGFP or RFP to the CHRAC subunits doubles the size of both proteins and might result in partial masking of a given NLS. Fusion to EGFP-EGFP-GST even increases the size of the protein nearly 5 fold. However, by fusing the ncNLS (BIB domain) containing ribosomal protein L23a (rpL23a) to EEG the reporter protein showed a strict localization in the nucleoli (Fig. 5*C*, left panel), which shows the general functionality of EEG as a reporter protein. Therefore, the cytoplasmic localization of the CHRAC-fusion proteins could be due to the lack of nuclear localization signal (NLS) in the monomeric proteins. Evidence that both proteins, CHRAC-15 and CHRAC-17, might indeed lack a NLS was provided when the bipartite cNLS (KRPAATKKAGQAKKKKL) of the nuclear chaperone nucleoplasmin (for review, see Frehlick et al., 2007) was inserted between the amino-terminal EEG and CHRAC-17 resulting in a nuclear localization of the fusion protein (Fig. 5*C*, right panel).

To additionally analyze whether fusion of the EEG tag still allows dimerization of the two CHRAC subunits, *in vivo* GST-Pulldown assays were done next. HeLa P4 cells were transiently transfected with CHRAC-15 fused to RFP and CHRAC-17 fused to EEG (Fig. 5*D*) or *vice versa*

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**FIG. 5: continued**

*(C)* To test the functionality of the EEG vector, transfection experiments with modified EEG and EEG-CHRAC-17 fused to a ncNLS were performed. Fusion of the ribosomal protein L23a (rpL23a) to EEG reporter led to a nuclear localization strictly restricted to the nucleoli (left panel). The fusion of the bipartite cNLS from the nuclear chaperone nucleoplasmin results in a nuclear localization of EEG-cNLS-CHRAC-17 (right panel). *(D)* and (*E)* The dimerization between CHRAC-15 with CHRAC-17 in HeLa P4 cells was verified by *in vivo* GST-Pulldown assays. For that purpose, cells were transiently transfected as indicated in the scheme. Twenty-four hours post transfection cells were harvested and lysed. Glutathione-Sepharose was used to precipitate the respective EGFP-EGFP-GST-fused subunit (CHRAC-17 in *(D)* CHRAC-15 in *(E)*) from the cell lysates. Bound proteins were analyzed by SDS-PAGE followed by immunoblotting. *(D)* RFP-fused CHRAC-15 coprecipitated with the EEG-tagged CHRAC-17 subunit, whereas RFP-CHRAC-15 alone was not precipitated. *(E)* RFP-tagged CHRAC-17 bound specifically to EEG-CHRAC-15 but not to EEG alone. Coprecipitation of CHRAC-17 is subunit-specific, since the precipitation of RFP-CHRAC-17 (middle lane), equals the amount of precipitated protein that is based on unspecific binding to glutathione-Sepharose (right lane).

(Fig. 5*E*). Twenty-four hours post transfection, the cells were lysed and the EEG-fused proteins were precipitated with glutathione-Sepharose. The coprecipitated proteins were analyzed by SDS-PAGE and subsequently detected by Western blotting. Using anti-RFP and anti-GFP antibodies, specific binding of the histone fold subunits to each other was demonstrated (Fig, 5*D* and *E*). Whereas RFP-CHRAC-15 (Fig. 5*D*) had no affinity to either the EEG-tag at CHRAC-17 or the glutathione-Sepharose, RFP-CHRAC-17 bound weakly to glutathione-Sepharose (Fig. 5*E*, middle panel). Moreover, RFP-CHRAC-17 does not bind to the EEG-tag alone (Fig. 5*E*, right panel). Hence, dimerization between CHRAC-15 and CHRAC-17 occurs despite the large EGFP-EGFP-GST-tag fused to either of the subunits. The cytoplasmic retention of the overexpressed CHRAC subunits suggests that nuclear import of CHRAC-15 and CHRAC-17 depends either on heterodimerization of the two subunits or on their formation of a complex with other proteins. Previous work on NF-YC/NF-YB and NC2α/NC2β (Kahle et al., 2005; Kahle et al., 2009), two H2A/H2B-like histone fold pairs that are related to the CHRAC-15/CHRAC-17 complex (in the following referred to as CHRAC-15/17), showed that in these cases the histone fold subunits are imported into the nucleus as a complex.

## 3.2 The CHRAC-15/17 heterodimer is imported as a complex

To analyze whether the heterodimeric CHRAC-15/17 complex is imported in a receptor-mediated fashion, we performed *in vitro* nuclear import assays. This method was developed by Adam and Gerace (1990) and allows the study of receptor-mediated transport processes through the nuclear pore complex. In this assay, HeLa P4 cells were grown on glass coverslips, the plasma membrane was selectively permeabilized with digitonin leaving the nuclear envelope structurally intact, and the cytosol was removed by washing the permeabilized cells. After this treatment, the nuclear pore complex maintain its ability to facilitate import processes of NLS-containing recombinant cargo, which allows the reconstitution of import processes without the interference of endogenous import factors. The semi-permeabilized cells were incubated with recombinant purified GST-CHRAC-15/His-CHRAC-17 complex, which had before been coexpressed in *E. coli* and affinity purified using the 6xHis-tag on the CHRAC-17 subunit (Fig. 6*A*). Nuclear import of the CHRAC-15/17 heterodimer was analyzed in the presence of rabbit reticulocyte lysate as a general source of exogenous import receptors or recombinant purified importins in combination with RanGDP/NTF2 and an energy-regenerating system. The subcellular distribution of the CHRAC-15/17 heterodimer was detected by indirect immunofluorescence using an anti-GST antibody against the recombinant GST-CHRAC-15. To

discriminate between the nucleus and cytoplasm and thus allow controlling nuclear entry of the recombinant proteins, the nuclei were visualized with DAPI.

Incubation with reticulocyte lysate led to a strong accumulation of the CHRAC-15/17 complex in the nucleus. In contrast, in the absence of transport factors (buffer) a strict cytoplasmic retention was observed, suggesting that nuclear import of the CHRAC-15/17 heterodimer is energy-dependent and receptor-mediated (Fig. 6*A*). To further specify this import process,

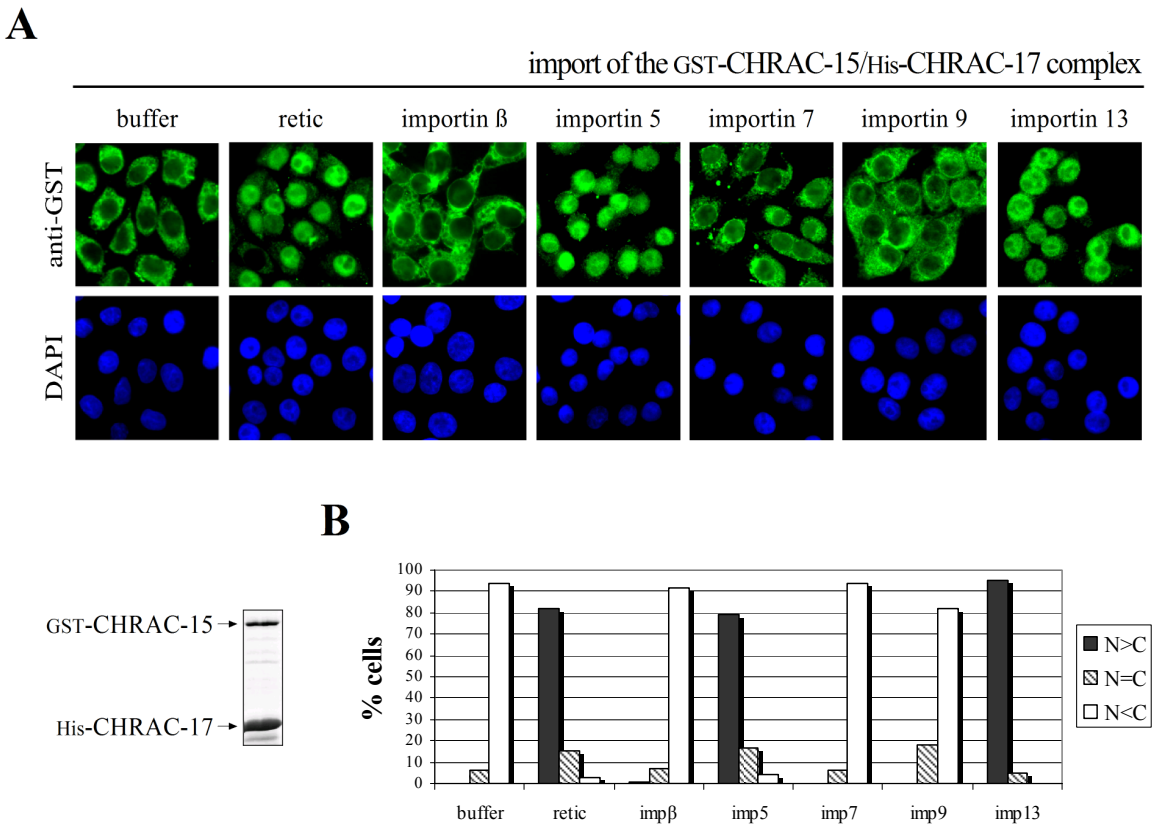


**FIG. 6: Accumulation of the CHRAC-15/17 heterodimer in the nucleus of permeabilized cells is mediated by importin 5 and importin 13.** *(A)* Digitonin-permeabilized HeLa P4 cells were incubated with 0.4 µM purified GST-CHRAC-15/His-CHRAC-17 heterodimer, rabbit reticulocyte lysate (retic) or ~ 0.4 µM of the indicated nuclear transport receptors in combination with a RanGDP/NTF2-mix and an energy regenerating system for 30 minutes at 37°C. As a negative control, reticulocyte lysate was replaced by transport buffer (buffer). Cells were fixed and the subcellular localization of the CHRAC-15/17 heterodimer was visualized by indirect immunofluorescence using anti-GST antibodies. The DNA was counterstained using 4',6-diamidino-2'-phenylindole (DAPI). Nuclear import of the CHRAC-15/17 heterodimer is energy dependent and requires importin 5 or importin 13. A Coomassie-stained SDS-PAGE gel of purified GST-CHRAC-15/His-CHRAC-17 complex used for the *in vitro* import assay is shown. *(B)* The mean distribution of the CHRAC-15/CHRAC-17 heterodimer was quantified. For that, 100 fluorescent cells per condition were scored in three categories: N > C (more reporter protein in the cytoplasm), N = C (equal distribution of reporter protein between nucleus and cytoplasm), and N < C (more reporter protein in the cytoplasm). imp, importin.

reconstitutions experiments with importin β, importin 5, importin 7, importin 9, and importin 13 were performed. Nuclear accumulation of the heterodimeric complex was strictly dependent on the presence of either recombinant importin 5 and importin 13. Other importins had no significant effect. In addition, since purification was mediated using the 6xHis-tag on the CHRAC-17 subunit, but detection of the complex was accomplished using an anti-GST antibody directed against GST-CHRAC-15, we excluded the possibility of detecting monomeric subunits. For description of the mean distribution of the CHRAC-15/17 complex in this import assay, fluorescent cells were classified in three different categories: N > C (more reporter protein in the nucleus), N = C (equal distribution of reporter protein between nucleus and cytoplasm), and N < C (more reporter protein in the cytoplasm). For the quantification 100 fluorescent cells were scored per condition (Fig. 6*B*).

The results demonstrate that the heterodimeric histone fold complex is actively imported into the nucleus by at least importin 5 and importin 13.

## 3.3 Dimerization of CHRAC-15 and CHRAC-17 is the prerequisite for transport receptor binding

To further characterize binding of importin 5 and importin 13 to the CHRAC-15/17 complex as well as to the individual subunits, we next performed *in vitro* binding studies with recombinant import receptors (Fig. 7). GST-CHRAC-15 and His-CHRAC-17 were coexpressed in *E. coli* and purified on nickel NTA-agarose. After elution, a second purification step on glutathione-Sepharose followed to immobilize the heterodimeric complex. The CHRAC-15/17 dimer was then incubated with equal concentrations of recombinant, purified nuclear transport receptors, namely preincubated importin α/importin β, importin β alone, importin 5, importin 7, importin 9, and importin 13. The concentration of the recombinant transport receptors was analyzed after the expression and purification by determining the total protein concentration in the fractions using the Bradford Protein Assay. The percentage of full length import receptor in these fractions was calculated using the program GelEval, comparing the band intensity of the full length protein, verified by Western blotting, with the remaining bands on a Coomassie gel. Based on these percentages the protein concentration of the representative import receptor was calculated and appropriate amounts were used for subsequent analysis (Fig. 7*A*). Import cargoes bind to their transport receptor only in the absence of RanGTP but dissociate upon RanGTP binding (Görlich et al., 1996b; Izaurralde et al., 1997; Rexach and Blobel, 1995). Thus, to ensure binding specificity between the import receptor and the CHRAC-15/17 complex and to mimic the

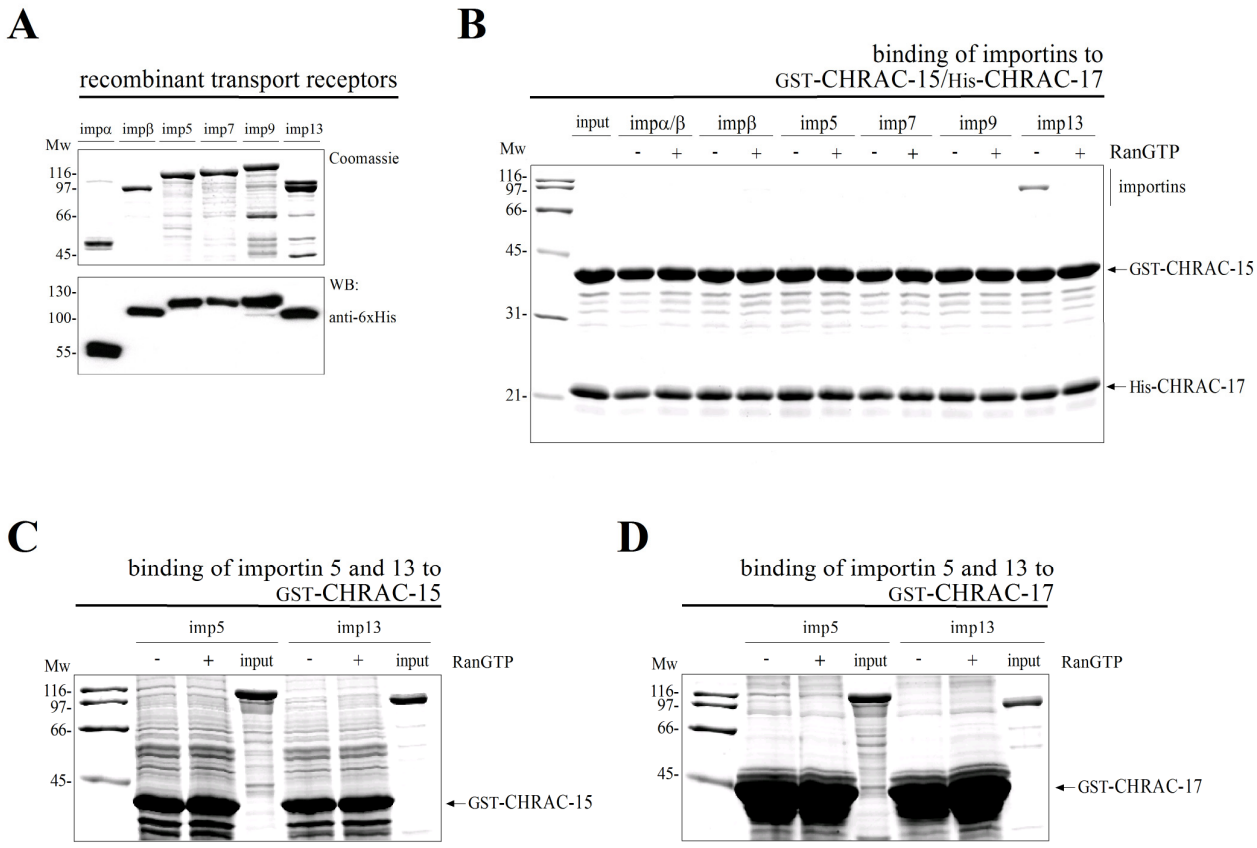


**FIG. 7: The heterodimeric CHRAC-15/17 complex is recognized by importin 13.** *(A)* 6xHis-tagged nuclear transport receptors used for *in vitro* studies, were expressed in *E. coli*, purified and analyzed by SDS-PAGE followed by Coomassie staining. In addition, the band representing full length importins was verified by Western blotting using anti-His antibody. The amount of the recombinant transport receptors was calculated by comparing the percentages of the full length import receptor with remaining impurities in the fraction, calculated with the program GelEval. Based on these percentages and the total protein concentration of the fraction, prior determined before using the Bradford Protein Assay, the protein concentration of the representative import receptor was calculated. *(B)* GST-CHRAC-15 and His-CHRAC-17 were coexpressed in *E. coli* and immobilized on glutathione-Sepharose. The heterodimer was incubated with equal concentrations (~ 0.2 µM) of recombinant purified importin α/importin β dimers, importin β, importin 5, importin 7, importin 9, and importin 13. Binding was tested in the absence (-) or presence (+) of RanGTP (Q69L mutant) to simulate cytoplasmic and nuclear conditions, respectively. Bound fractions were analyzed by SDS-PAGE followed by Coomassie staining. The CHRAC-15/17 heterodimer binds predominantly to importin 13 in a RanGTP-sensitive manner. Binding of other nuclear transport receptors was not detected. *(C* and *D)* GST-CHRAC-15 and GST-CHRAC-17 were expressed and immobilized on glutathione-Sepharose. After incubation with either recombinant importin 5 or importin 13, bound proteins were analyzed by SDS-PAGE and stained with Coomassie. Binding was performed in the absence (-) or presence (+) of RanGTP. 10 % of the recombinant transport receptor used in this study is indicated as input. Neither GST-CHRAC-15 nor GST-CHRAC-17 showed significant binding to importin 5 or importin 13. imp, importin; Mw, molecular weight; WB, Western blot.

cytoplasmic or the nuclear environment, the binding studies were performed in the absence or presence of RanGTP, respectively. In this study, we used the RanGTP mutant Q69L which prevents RanGAP-mediated hydrolysis of GTP to GDP, keeping Ran constantly in its GTP-bound state. The transport receptors interacting with the CHRAC-15/17 heterodimer were analyzed and displayed by SDS-PAGE followed by Coomassie staining (Fig. 7*B*). Consistent with the results in Figure 6, immobilized CHRAC-15/17 showed strong binding to recombinant importin 13. In addition, because binding was abolished in the presence of RanGTP, as expected for an import cargo, it can be concluded that the interaction between importin 13 and the CHRAC-15/17 complex is specific. Binding of other import receptors to the heterodimer and surprisingly even binding of importin 5 was not detected.

Taken together, our data from binding studies and *in vitro* import assays suggest that importin 13 represents a primary nuclear import receptor for the CHRAC-15/17 heterodimer. The reason for the discrepancy concerning importin 5 between the import assay (Fig. 6*A*) and the binding studies (Fig. 7*B*) remains unclear.

Studies in the lab of Dirk Görlich concerning the transport factor importin 13 showed that importin 13 acts as an import and export factor. Binding studies with HeLa extract and subsequent identification by mass spectrometry have identified CHRAC-17, referred to as NF-YB-like protein, as putative import substrate of importin 13 (Mingot et al., 2001). The histone fold partner CHRAC-15 however was not detected in this study. Hence it remained unsolved whether CHRAC-17 was bound to importin 13 as a monomer or in a complex. To address this question, we analyzed the binding of importin 13 and importin 5 to the individual subunits, although we had seen before their cytoplasmic retention *in vivo* (Fig. 5*A*).

For that purpose, GST-CHRAC-15 (Fig. 7*C*) and GST-CHRAC-17 (Fig. 7*D*) were independently expressed in *E. coli* and immobilized on glutathione-Sepharose. As described above, both subunits were incubated in the absence or presence of RanGTP with equal concentrations of importin 5 and importin 13. Bound proteins were analyzed by SDS-PAGE followed by Coomassie staining. In both assays, neither importin 13 nor importin 5 bound significantly to CHRAC-15 or CHRAC-17. Together with the data from the *in vivo* transfection experiments with the monomeric subunits (Fig. 5), these results demonstrate that the individual CHRAC subunits do not contain a NLS and nuclear import via importin 13 depends on heterodimerization of CHRAC-15 and CHRAC-17, recognized by either importin 5 or importin 13. Once again, these results are consisted with recent data obtained for the heterodimeric histone fold pairs NF-YB/NF-YC (Kahle et al., 2005) and NC2α/NC2β (Kahle et al., 2009). These two complexes are also imported into the nucleus via importin 13. Thus, it is likely that

the CHRAC-15/17 complex represents the third histone fold motif containing complex that is imported into the nucleus as a heterodimer via an importin 13-dependent pathway. This underlines the role of importin 13 for nuclear import of histone fold complexes that belong to the H2A/H2B family.

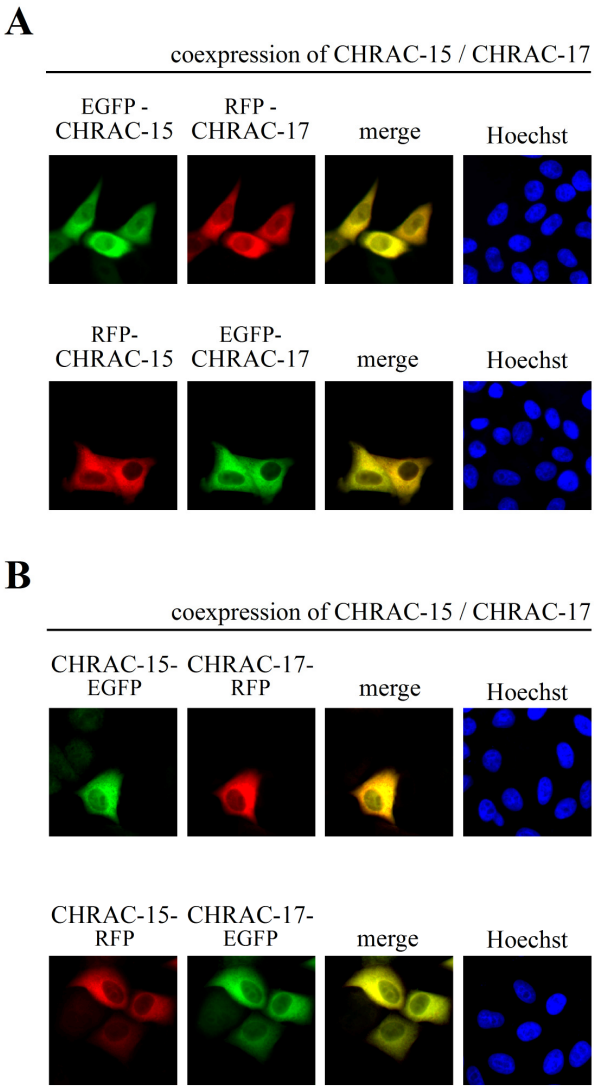


**FIG. 8: *In vivo* coexpression of CHRAC-15 and CHRAC-17 results in a cytoplasmatic localization.** *(A)* HeLa P4 cells were transiently transfected with plasmid DNA encoding EGFP-CHRAC-15 and RFP-CHRAC-17 (upper row) or RFP-CHRAC-15 and EGFP-CHRAC-17 (lower row). The subcellular localization of the green EGFP and the red RFP fusion proteins was determined by direct fluorescence 24 hours post transfection. The overlap is shown in yellow (merge). The DNA was counterstained with Hoechst. Coexpression of CHRAC-15 and CHRAC-17 amino terminally fused either to EGFP or RFP, respectively, results in a colocalization in the cytoplasm of transfected cells. *(B)* As in *A*, HeLa P4 cells were transiently transfected with plasmid DNA coding for CHRAC-15 and CHRAC-17 fused at the carboxy terminus to either EGFP or RFP. The merged pictures represent the overlap of the green EGFP and the red RFP fusion proteins. The carboxy-terminally labeled subunits remained also exclusively in the cytoplasm of transfected cells. The position of the fluorescent tags has no effect on the cytoplasmic retention of the CHRAC subunits.

## 3.4 Importin 13 facilitates nuclear uptake of the CHRAC-15/17 complex *in vivo*

The *in vitro* data suggested that the CHRAC-15/17 heterodimer is actively imported into the nucleus via the nuclear transport receptor importin 13. To investigate nuclear import of the heterodimeric CHRAC-15/17 complex *in vivo* transfection experiments were performed. Therefore, HeLa P4 cells were transiently cotransfected with plasmid DNA coding for CHRAC-15 fused amino-terminally to EGFP and CHRAC-17 fused amino-terminally to RFP and *vice versa* (Fig. 8*A*). The subcellular localization of the gene products was analyzed 24 hours post transfection by fluorescence microscopy. Surprisingly, both fluorescently-labelled complexes, EGFP-CHRAC-15/RFP-CHRAC-17 and RFP-CHRAC-15/EGFP-CHRAC-17, remained exclusively in the cytoplasm of transfected cells (Fig. 8*A*). To exclude the possibility of a partial masking of the importin 13 recognition motif by the fused fluorescent tag, as recently shown by Wagstaff and Jans (2006), we changed the position of the fluorescent tags from the amino- to the carboxy-termini (Fig. 8*B*). However, the localization of the resulting overexpressed CHRAC-15-EGFP/CHRAC-17-RFP and CHRAC-15-RFP/CHRAC-17-EGFP complexes remained unchanged. Hence, the position of the fluorescent tags within the CHRAC-15/17 complex had presumably no effect on its cytoplasmic retention.

Since the cytoplasmic localization of proteins does not only result from the absence of NLSs or the masking of specific sequence elements within the proteins but can also be based on nuclear export, we examined a possible role of exportin 1 in mediating nuclear export of the CHRAC-15/17 complex. HeLa P4 cells were cotransfected with plasmid DNA encoding EGFP-CHRAC-15 and RFP-CHRAC-17 (Fig. 9*A*, upper row) or RFP-CHRAC-15 and EGFP-CHRAC-17 (Fig. 9*A*, lower row). Twenty-four hours after transfection the cells were treated with the exportin 1 inhibitor Leptomycin B (Nishi et al., 1994) for 6 hours. Leptomycin B (LMB) is an antifungal cytotoxin isolated from *Streptomyces spp*. (Hamamoto et al., 1983). It specifically inhibits exportin 1 by covalent binding and alkylation of exportin 1, which interferes with the formation of export complexes (Fornerod et al., 1997). As shown in Figure 9*A*, the subcellular cytoplasmic distribution of both fluorescently labeled CHRAC-15/17 complexes was not affected by the addition of 10 ng/µl LMB. These results indicate that the CHRAC-15/17 complex did not localize in the cytoplasm due to exportin 1 mediated export.

Next we wanted to examine, if inaccurate folding of the histone fold complex as a result of the strong overexpression was responsible for the cytoplasmic retention. For that purpose, HeLa P4 cells were transfected as described above, and in addition the heat shock protein 70 (HSP70) chaperone, a member of the functionally related heat shock protein family and essential

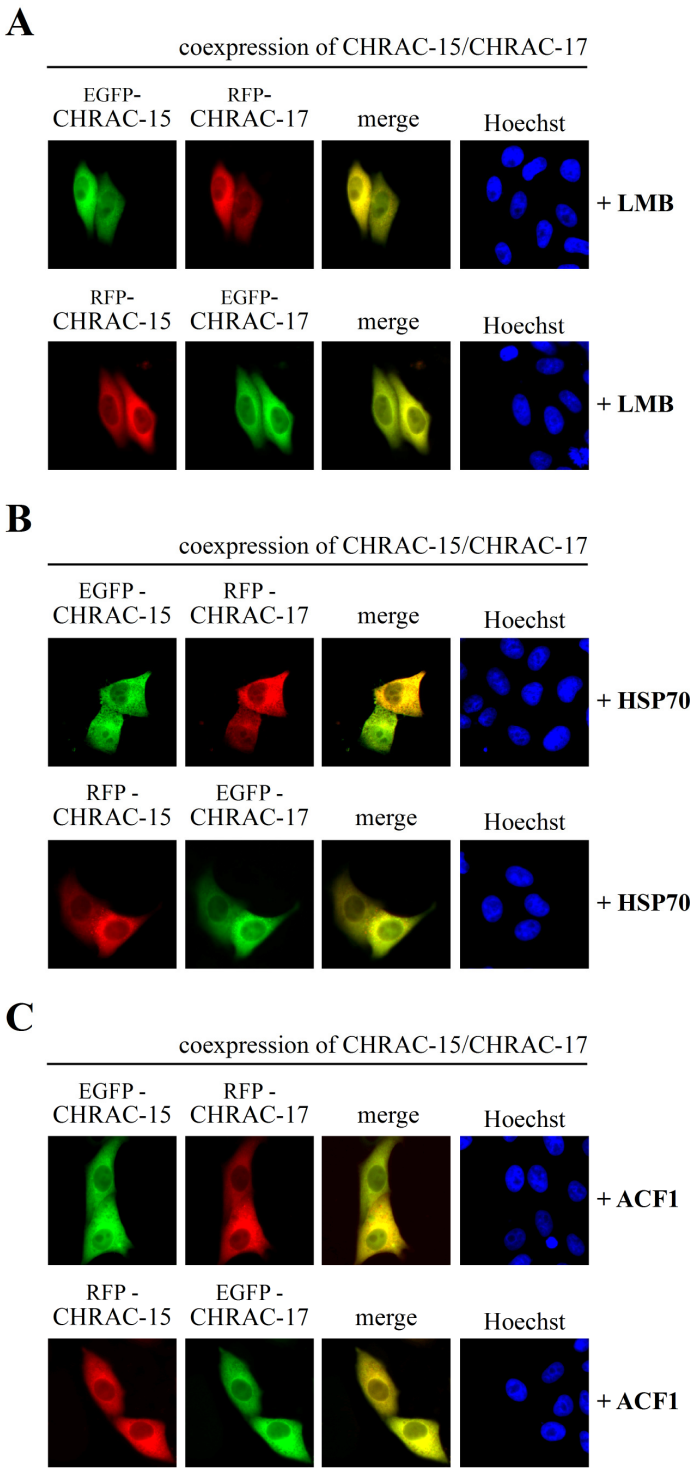


**FIG. 9: The CHRAC-15/17 heterodimer is not actively exported from the nucleus by exportin 1 and the localization is not affected by either HSP70 or ACF1.** HeLa P4 cells were transiently transfected with plasmid DNA encoding EGFP-CHRAC-15 and RFP-CHRAC-17 or RFP-CHRAC-15 and EGFP-CHRAC-17 under the influence of Leptomycin B (LMB) (*A*), or the additional cotransfection of HSP70 *(B)* and ACF1 *(C)*. The subcellular localization of the overexpressed CHRAC-15/17 heterodimer was determined by direct fluorescence of the individual subunits 24 hours post transfection, and colocalization is shown in yellow (merge). The DNA was counterstained using Hoechst. *(continued on page 74)*

machinery for protein folding and the establishment of proper protein conformation (for review, see Morano, 2007), was coexpressed. The coexpression of the chaperone as shown in Figure 9*B* had no effect on the cytoplasmic localization of the CHRAC-15/17 complex. This may allow the conclusion that the deficiency in nuclear uptake of the heterodimeric complex is not based on misfolding as a result of the strong CMV promoter-driven overexpression of the fusion proteins.

Since inaccurate folding and export from the nucleus were not responsible for the cytoplasmic retention of CHRAC-15/17 we wanted to investigate whether the CHRAC-15/17 dimer is imported in the nucleus via a piggyback mechanism. Piggyback transport depends on the additional binding of an NLS-containing protein. Thus, since flag-tagged ACF1 was detected in the nucleus of HeLa cells (Patrick Varga-Weisz, personal communication), the possible role of the regulatory subunit ACF1, the binding partner of the CHRAC-15/17 heterodimer in CHRAC (Eberharter et al., 2001; Ito et al., 1999; Poot et al., 2000; Varga-Weisz et al., 1997), for the nuclear uptake of the histone fold pair was analyzed. HeLa P4 cells were transiently cotransfected with CHRAC-15 and CHRAC-17, fused N-terminally either to EGFP or RFP, and plasmid DNA coding for flag-tagged ACF1 (Fig. 9*C*). The overexpression of ACF1 had no influence on the subcellular localization of the heterodimeric CHRAC-15/17 complex compared to transfection experiments with the CHRAC subunits alone (see again Fig. 8). Therefore, ACF1 has presumably no function in the nuclear uptake of the heterodimeric CHRAC complex.

Finally, we tested the influence of additionally coexpressed transport receptors. EGFP-CHRAC-15 and RFP-CHRAC-17 were transiently cotransfected with either flag-tagged importin 13, importin β, importin 5, importin 7 or importin 9, which are all under the control of a strong CMV promoter. The subcellular distribution of the fluorescent CHRAC-15 and CHRAC-17 fusion proteins was determined 24 hours post transfection (Fig. 10*A*). The additional coexpression of exogenous importin 13 led to an exclusively nuclear distribution of the CHRAC-15/17 complex.

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**FIG. 9: continued**

*(A)* The subcellular localization of the histone fold heterodimer was not affected by blocking the Leptomycin B sensitive exportin 1 pathway. Thus, the cytoplasmic distribution of CHRAC-15/17 does not result from an active export from the nucleus mediated by exportin 1. *(B)* To enhance the proper folding of the overexpressed gene products, HeLa cells were additionally cotransfected with HSP70. Despite the overexpression of the chaperone, the distribution of the histone fold heterodimer did not change and the subunits remained in the cytoplasm. *(C)* CHRAC-15/17 were cotransfected with ACF1 to analyze the potential influence of ACF1 in the nuclear import via a piggyback mechanism. The overexpression of ACF1 did not affect the subcellular distribution of the heterodimer compared to transfection experiments of the CHRAC subunits alone (see Fig. 8*A*). The results are valid for both tag combinations.

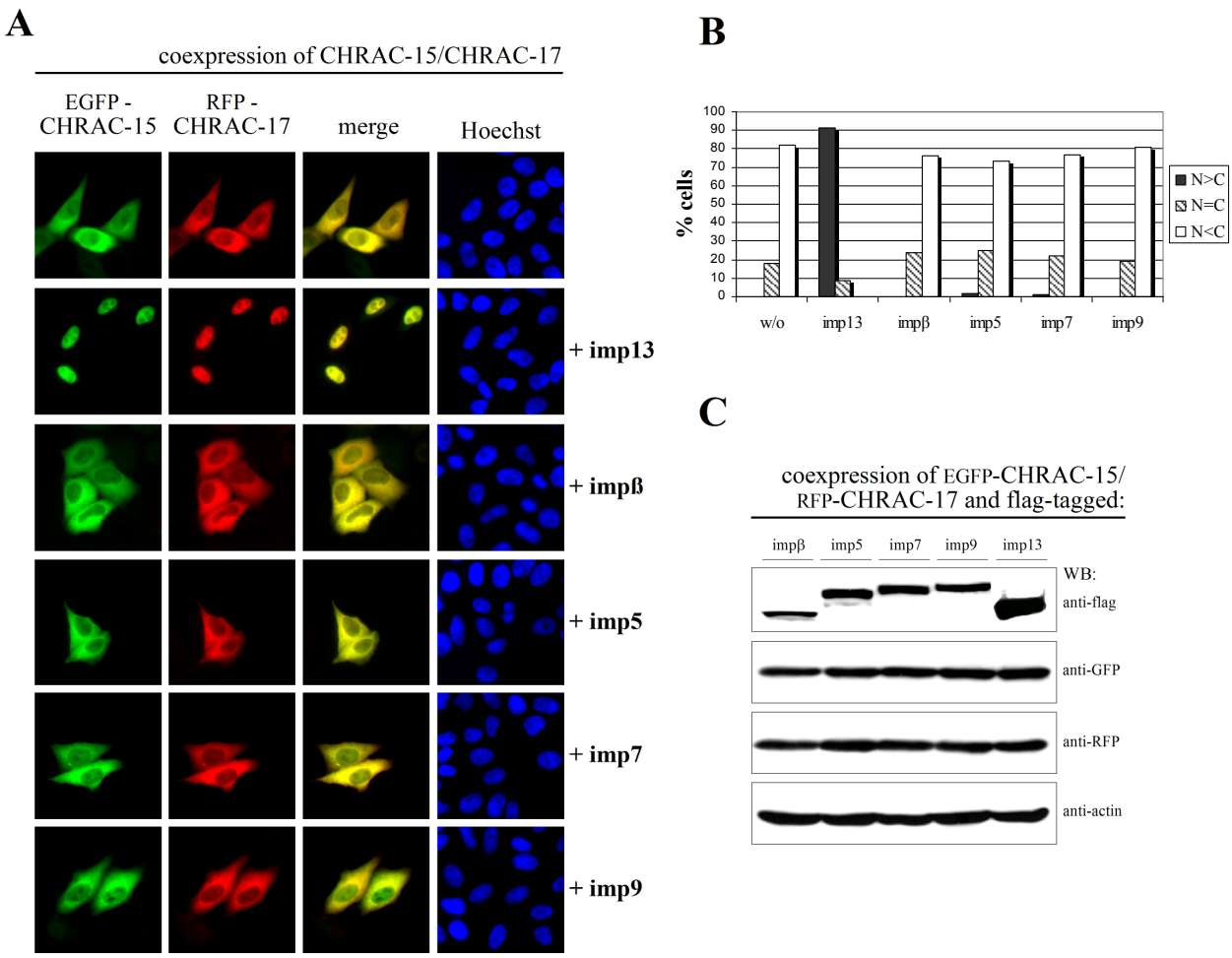


**FIG. 10: Importin 13 mediates the nuclear import of the CHRAC-15/17 heterodimer *in vivo*.** *(A)* HeLa P4 cells were transiently transfected with plasmid DNA encoding EGFP-CHRAC-15, RFP-CHRAC-17 and flag-tagged importins as indicated. The subcellular localization of the green EGFP and the red RFP fusion proteins was determined by direct fluorescence 24 hours post transfection. The overlap is shown in yellow (merge). The DNA was counterstained with Hoechst. Coexpression of EGFP-CHRAC-15 and RFP-CHRAC-17 results in a colocalization in the cytoplasm of transfected cells (upper row). The additional coexpression of flag-importin 13 leads to a nuclear accumulation of the CHRAC-15/17 complex. Additional coexpression of importin β, importin 5, importin 7, and importin 9 in contrast did not change the subcellular distribution of the EGFP-CHRAC-15/ RFP-CHRAC-17 complex. *(B)* The mean distribution of the EGFP-CHRAC-15/RFP-CHRAC-17 heterodimer with different coexpressed import factors was scored into the following three categories: N > C, N = C, and N < C. *(C)* Plasmid DNA coding for EGFP-CHRAC-15, RFP-CHRAC-17 and flag-tagged importin β, importin 5, importin 7, importin 9, or importin 13, respectively, were cotransfected in HeLa P4 cells. Twenty-four hours post transfection the cells were lysed, and the import factors, CHRAC-17 and CHRAC-15 were analyzed by immunoblotting using anti-flag, anti-RFP and anti-GFP antibody. Anti-actin antibody was used to control equal loading. imp, importin; WB, Western blot.

In contrast, coexpression of importin β, importin 5, importin 7, and importin 9 did not result in any nuclear accumulation of the heterodimer. A quantitative analysis of the localization pattern is shown in Figure 10*B*, where the mean distribution of the heterodimeric complex was scored into three different categories. This result underlines the important role of importin 13 in facilitating nuclear import of the CHRAC-15/17 heterodimer and also supports the data from our *in vitro* experiments. The expression of EGFP-CHRAC-15, RFP-CHRAC-17 and the flag-tagged nuclear transport receptors was additionally analyzed by immunoblotting to check for similar protein expression (Fig. 10*C*). Although importin 13 was coexpressed to a slightly higher degree than importin β, importin 5, importin 7, and importin 9, no functional significance of these import factors was found for nuclear import of the CHRAC-15/17 complex *in vivo*. Similar results were obtained when the fluorescent tags were exchanged among the histone fold subunits (Fig. 11*A*). Hence, coexpression of importin 13 is necessary to facilitate nuclear uptake of the CHRAC-15/17 complex *in vivo*. In addition, the exchange of EGFP and RFP from the amino-terminus to the carboxy-terminus did not alter the nuclear localization of the CHRAC-15/17 complex upon coexpression of importin 13 (Fig. 11*B*).

A possible explanation for the necessity of importin 13 coexpression is that high amounts of CHRAC-15 and CHRAC-17 require also high amounts of importin 13 to accomplish proper nuclear uptake. In other words, the endogenous importin 13 levels may just not suffice to support the transport of the strongly overexpressed histone fold complexes. Since the cotransfected nuclear transport receptors localized mainly in the nucleus or at the surface of the nuclear envelope ('rim staining'), flag-tagged importin β, importin 5, importin 7, importin 9, and importin 13 seem to be properly expressed and functional (Fig. 11*C*). Differences in the subcellular localization of importin 13 and importin β, importin 5, and importin 9 may be due to the fact that importin 13 facilitates import as well as export processes (Mingot et al., 2001).

## 3.5 Importin 5 mediates the nuclear import of PGC7/Stella but is dispensable for nuclear import of CHRAC-15/17

We next wanted to elucidate a possible role of importin 5 in the nuclear import of CHRAC-15/17 and to illuminate the discrepancy between the data from the *in vitro* import assay (Fig. 6) and the *in vivo* transfection experiments (Fig. 10*A*). Therefore, RNAi experiments with siRNA against importin 5 were performed in order to control a potential significance of importin 5 in the nuclear transport of CHRAC-15/17. For that purpose, RNAi duplex oligoribonucleotides against importin 5 were transfected in HeLa P4 cells. After three and seven days the depletion of

importin 5 was controlled by Western blotting (Fig. 12*A*) using a specific antibody against importin 5. Cells transfected with control siRNA and mock treated cells served as expression

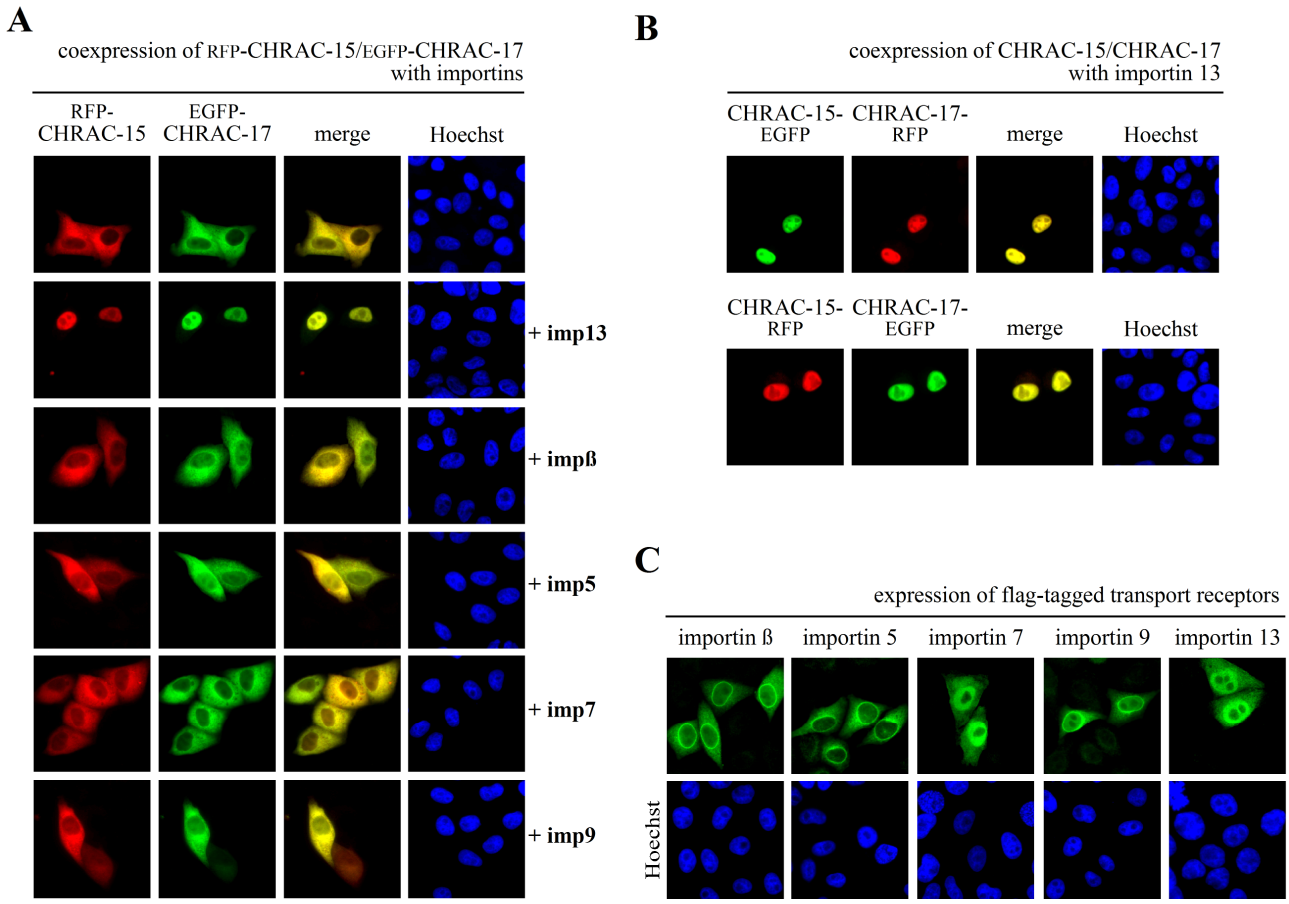


**FIG. 11:** ***In vivo*** **coexpression of importin 13 is necessary for the nuclear accumulation of differently tagged CHRAC-15/17 complexes.** *(A)* HeLa P4 cells were transiently cotransfected with DNA coding for RFP-CHRAC-15 and EGFP-CHRAC-17. In addition, plasmid DNA coding for different flag-tagged transport receptors was added as indicated. The subcellular localization of the gene products was examined 24 hours post transfection by direct fluorescence. Using Hoechst the DNA was counterstained. The merged picture results from the overlap between the green EGFP and red RFP fluorescence. Coexpression of importin 13 leads to an exclusive nuclear accumulation of the CHRAC-15/17 complex. Neither importin β, importin 5, importin 7 nor importin 9 mediates the nuclear accumulation of the CHRAC-15/17 heterodimer when cotransfected. *(B)* CHRAC-15 carboxy-terminally fused to EGFP (upper panel) or RFP (lower panel) and CHRAC-17 likewise fused to EGFP or RFP were cotransfected with flag-importin 13 in HeLa P4 cells. The subcellular distribution of the heterodimeric complex was determined 24 hours post transfection. The DNA was counterstained with Hoechst. The exchange of EGFP and RFP in CHRAC-15 and CHRAC-17 from the amino-terminus to the carboxy-terminus did not influence the nuclear localization upon importin 13 coexpression. *(C)* Flag-tagged importins used in the study were overexpressed in HeLa P4 cells. The localization of each nuclear transport receptor was determined using an anti-flag antibody. The DNA was stained with Hoechst. Whereas flag-tagged importin 7 and importin 13 localized predominantly in the nucleus, flag-tagged importin β, importin 5, and importin 9 showed a strong rim staining. imp, importin.

controls. Comparing the band intensities of importin 5 in mock treated cells, set 100 %, with the cells transfected with siRNA a decrease in the importin level of 74 % after three days and 90 % after 7 days became evident. Importin 5 levels, however, was even slightly increased (9 %) in cells treated with control siRNA. To analyze the effect of importin 5 depletion on the nuclear import of CHRAC-15/17, antibodies against CHRAC-17 were applied to detect its subcellular localization (Fig. 12*B*). Despite the depletion of importin 5 in the cells, the localization of endogenous CHRAC-17 was not affected. This indicates that importin 5 has no significant role in the import of the heterodimeric CHRAC-15/17 complex *in vivo*. To further investigate the primary role of importin 13, we performed RNAi experiments with Stealth siRNA to deplete endogenous importin 13. Transfection of Stealth siRNA against importin 13 in HeLa P4 cells were performed using Oligofectamine and in addition, the electroporation system Nucleofector® II Device. Unfortunately, the protein levels of importin 13, verified by Western blotting remained unchanged (data not shown).

However, since the flag-tagged importin 13 expression construct was able to translocate the CHRAC heterodimer from the cytoplasm into the nucleus, but flag-importin 5 was not (Fig. 10*A*), the functionality of the overexpressed importin 5 construct was controlled. Therefore, PGC7/Stella, a known importin 5 cargo protein (Nakamura et al., 2007) was transfected in HeLa P4 cells. PGC7/Stella, a maternal factor essential for early development, protects the DNA methylation state and is preferentially expressed in primordial germ cells (PGCs), early preimplantation embryos and oocytes (Bortvin et al., 2004; Sato et al., 2002). Because the primary structure of PGC7/Stella, consisting of 150 amino acids, contains a putative bipartite NLS (amino acid residues 42-59) and in addition a NES (amino acid residues 32-46), it is localized in the nucleus and the cytoplasm when expressed alone (Nakamura et al., 2007). In analogy to the results from Nakamura and colleagues, flag-tagged PGC7/Stella was localized homogenously within transfected cells, showing an even distribution between the nucleus and the cytoplasm (Fig. 12*C*). In contrast, EGFP-PGC7/Stella was mainly found in the cytoplasm (Fig. 12*D*), forming cytoplasmic aggregates, pointing towards an unfolded state of the EGFP-fusion proteins. Additional cotransfection of importin 5 consequently led to a predominantly nuclear localization of PGC7/Stella (Fig. 12*C* and *D*, middle panel), analogous to the increased nuclear uptake of CHRAC-15/17 upon importin 13 coexpression. Interestingly, importin 5 not only led to an increased nuclear import of PGC7/Stella, but also acted as a molecular chaperone for the EGFP-tagged protein by preventing its aggregation upon overexpression. This ‘anti-aggregation activity’ is in line with previous reports concerning the dual role of importins by mediating nuclear transport processes and possessing a general chaperone-like role in protein

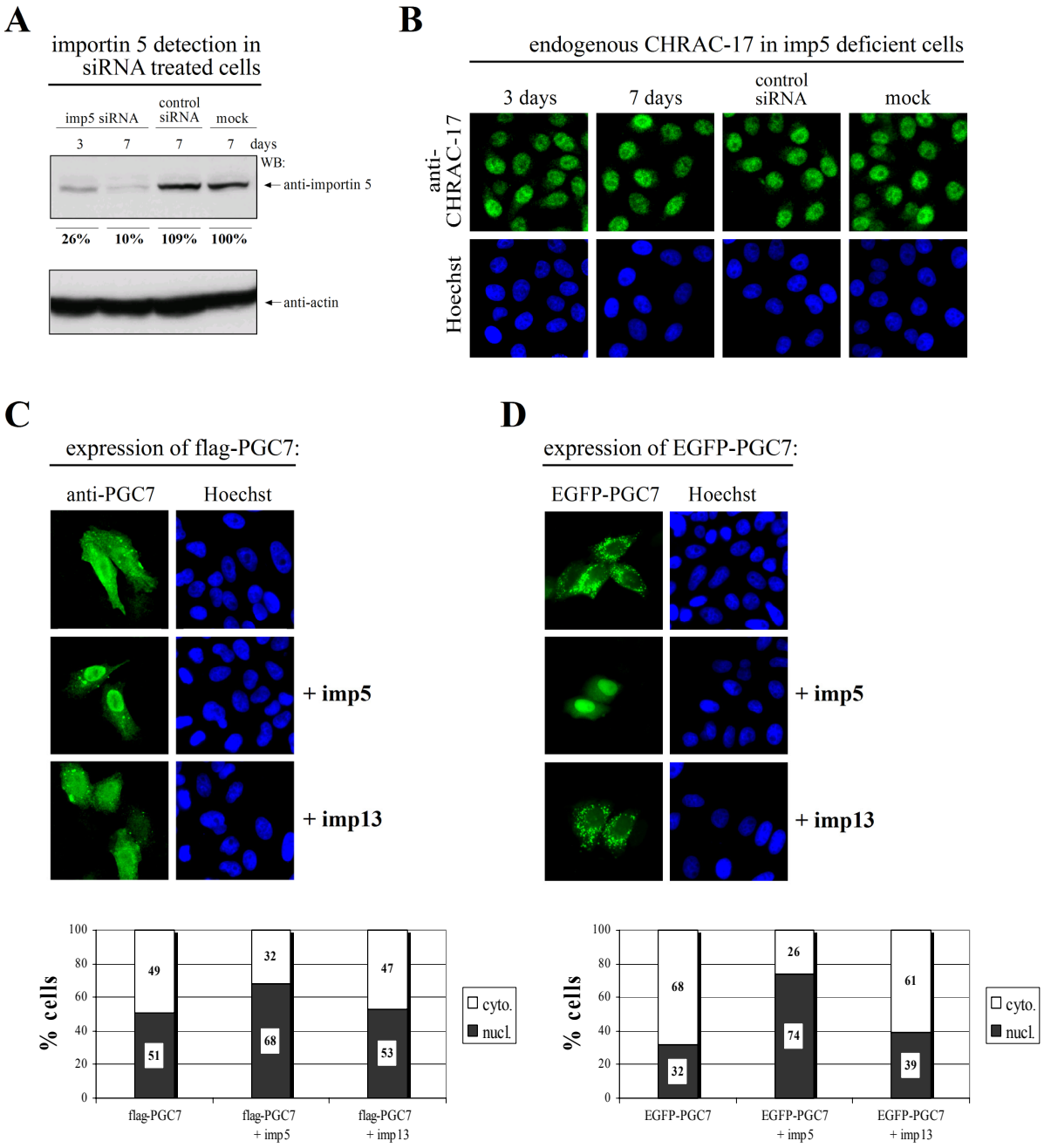


**FIG. 12: Influence of importin 5 on the endogenous distribution of CHRAC-17 and the known cargo protein PGC7/Stella.** *(A)* RNAi experiments with siRNA against importin 5 were accomplished using Stealth™ RNAi duplex oligoribonucleotides. HeLa P4 cells were transfected and cells were assayed for gene knockdown by Western blotting analysis 3 and 7 days post transfection using a specific antibody against importin 5. Mock treated HeLa P4 cells served as expression control. The software 1Dscan Ex (Scanalytics, Inc) was used to analyze band intensities. Mock treated cells were set 100 % and an importin 5 depletion of 74 % after 3 days and 90 % after 7 days was measured. An antibody against actin was used to control equal loading. *(B)* Importin 5 depleted HeLa P4 cells (3 and 7 days after siRNA treatment), cells treated with control siRNA (after 7 days) and mock treated cells were used to detect the subcellular distribution of endogenous CHRAC-17 using an anti-CHRAC-17 antibody. Hoechst was used to counterstain the DNA. Depletion of importin 5 did not affect the localization of endogenous CHRAC-17 was. *(C)* HeLa P4 cells were transiently transfected with plasmid DNA encoding *(continued on page 80)*

folding (Jäkel et al., 2002). The cotransfection of importin 13 had neither an influence on the subcellular localization of flag-tagged PGC7/Stella nor of the EGFP-PGC7/Stella (Fig. 12*C* and *D*, lower panel).

From these data, it can be concluded that, importin 5 has no functional significance in facilitating nuclear import of the CHRAC-15/17 heterodimer *in vivo*. Nuclear import of the CHRAC-15/17 complex overexpressed in HeLa P4 cells depends on the additional coexpression of importin 13.

## 3.6 Full length importin 13 is required to mediate efficient nuclear import of the CHRAC-15/17 complex

After verifying that importin 13 represents a functional import receptor for the CHRAC-15/17 complex *in vivo*, we next wanted to characterize the binding sites of the heterodimer in importin 13. For that purpose, different deletion constructs of importin 13 were generated and tested for their import capacity for CHRAC-15/17. Independent of the HEAT repeat prediction for importin 13 in *Swiss-Prot* (http://www.uniprot.org/uniprot/O94829), putative α-helices within the sequence of importin 13 were identified using the secondary structure prediction program PSIPRED (Jones, 1999; McGuffin et al., 2000). According to PSIPRED, importin 13 contains 38 α-helices. This is in line with the assumption that all transport receptors of the importin β-like family are comprised of 19 HEAT repeats (Petosa et al., 2004). Based on this prediction, we selectively deleted α-helices in importin 13 by using specific oligonucleotides (Fig. 13*A*). The resulting truncated flag-tagged importin 13 constructs (Fig. 13*C*) were coexpressed with EGFP-CHRAC-15 and RFP-CHRAC-17 in HeLa P4 cells. Among them, amino acids 1-784 and

**FIG. 12: continued**

flag-tagged PGC7/Stella. In addition, importin 5 and importin 13 were cotransfected as indicated 24 hours post transfection. The subcellular localization of flag-PGC7/Stella was determined using specific anti-PGC7/Stella antibody. The DNA was counterstained with Hoechst. Flag-PGC7/Stella localizes homogenously within transfected cells and accumulates predominantly in the nucleus upon importin 5 coexpression. Coexpression of flag-tagged importin 13 had no effect. The localization of the PGC7/Stella fusion protein was additionally quantified by measuring the fluorescence intensity in 20 cells, followed by a calculation of the ratio between nuclear (nucl.) and cytoplasmic (cyto.) distribution. *(D)* HeLa P4 cells were additionally transfected with EGFP-PGC7/Stella. The localization of the green fluorescent fusion protein was determined 24 hours post transfection. Strong overexpression of EGFP-tagged PGC7/Stella led to a cytoplasmic localization, which changed to a mainly nuclear localization when importin 5 was coexpressed. Coexpression of importin 13 had no influence on the subcellular distribution of PGC7/Stella. The percentage of cytoplasmic (cyto.) and nuclear (nucl.) localization of EGFP-PGC7/Stella was quantitatively analyzed using the ImageJ software. WB, Western blot; imp, importin.

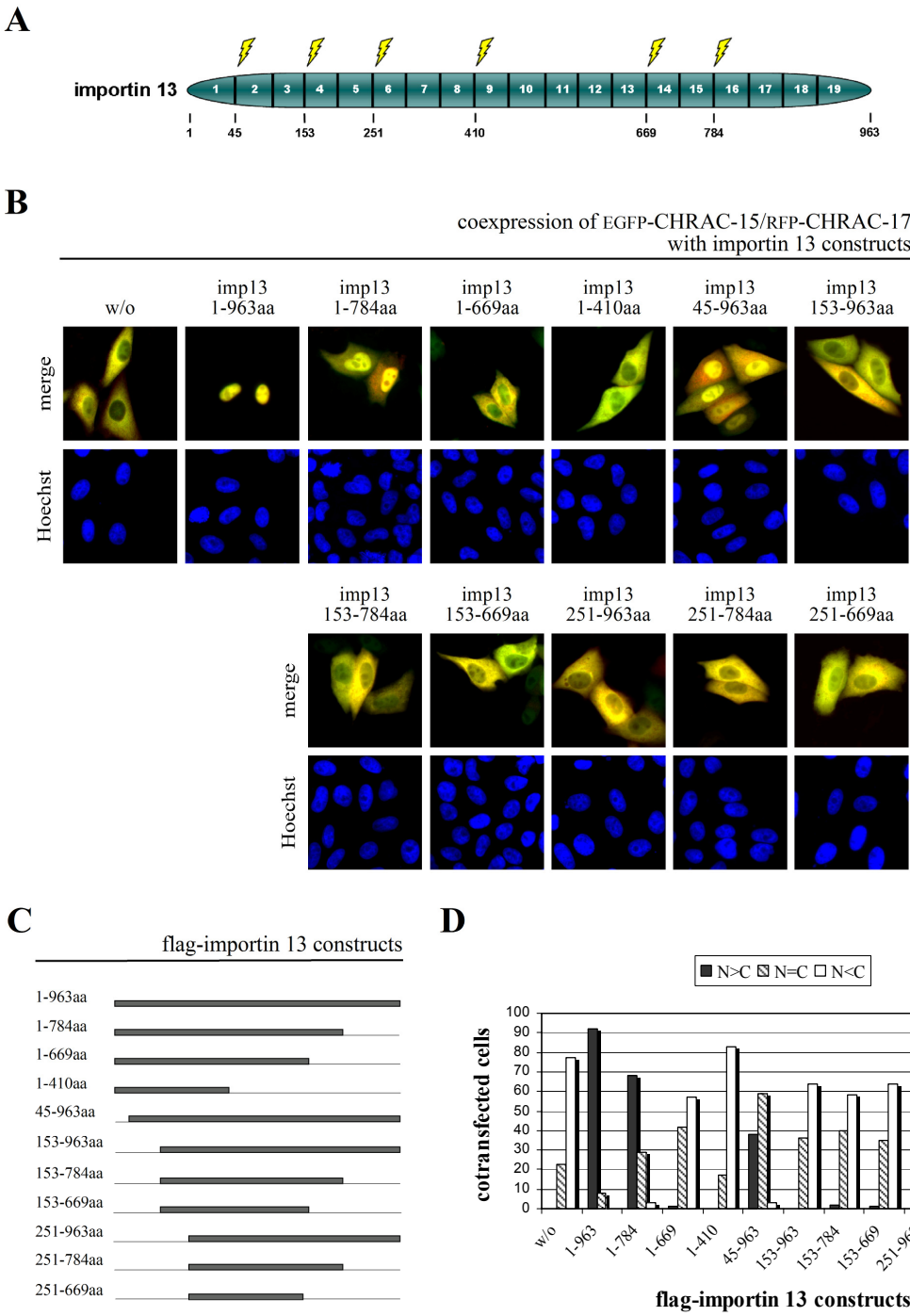


**FIG. 13: Full length importin 13 is required for efficient nuclear accumulation of the CHRAC-15/17 heterodimer.** *(A)* Importin 13 presumably consists of 19 HEAT repeats. According to the prediction of α-helices by PSIPRED, HEAT repeats were selectively deleted. *(B)* Plasmid DNA encoding EGFP-CHRAC-15 and RFP-CHRAC-17 was cotransfected in HeLa P4 cells. Different flag-tagged importin 13 fragments were additionally coexpressed as indicated. The subcellular distribution of the EGFP-CHRAC-15/RFP-CHRAC-17 complex was determined 24 hours post transfection and is shown in yellow (merge). The addition of full length importin 13 (1-963 amino acids) results in a strict nuclear accumulation (see also Fig. 10*A*). In the absence of exogenous importin 13 (w/o) the CHRAC-15/17 heterodimer localizes mainly in the cytoplasm. *(continued on page 82)*

45-963 were still capable in facilitating import of the CHRAC-15/17 complex in the nucleus of transfected cells although to a lower degree than wild type importin 13 (Fig. 13*B*). All other importin 13 fragments were not able to facilitate nuclear import of the CHRAC-15/17 complex. A quantitative analysis of the import is shown in Figure 13*D*. The same results were obtained when RFP-CHRAC-15 and EGFP-CHRAC-17 were coexpressed in HeLa P4 cells along with the different importin 13 fragments (Fig. 14*A*).

Indirect fluorescence microscopy of the importin 13 fragments revealed a dominant nuclear localization pattern. Hence, the subcellular localization of the importin 13 fragments is comparable to the full length importin 13 (see again Fig. 11*C*) and all importin 13 fragments retained their capacity to enter the nucleus (Fig. 14*B*). Based on these data, it can be concluded that the loss of nuclear uptake of the CHRAC-15/17 dimer is caused by the absence of importin 13 amino acid residues essential for the interaction with the heterodimeric CHRAC complex and not through the cytoplasmic retention of the importin 13 fragments. The deletion of one HEAT repeat at the amino-terminus and four HEAT repeats at the carboxy-terminus already negatively influenced the nuclear transport of the CHRAC-15/17 heterodimer. This indicates that, full length importin 13 is required for binding and subsequent nuclear uptake of the histone fold pair. In addition, these results point towards a broad interaction surface between the nuclear transport factor importin 13 and the CHRAC-15/17 complex.

Recently, Tao and colleagues (2004) demonstrated the same localization pattern for full length (amino acid 1-963) and a carboxy-terminally deleted fragment of importin 13 (amino acid 1-488), referred to as lgl2 (late gestation lung 2 protein). However, upon deletion of the amino acid residues 1-488 of importin 13, Tao and coworkers observed a cytoplasmic retention. In contrast, in our study, the loss of the first 251 amino acids did not result in a cytoplasmic localization. This might indicate that the remaining 238 amino acid residues of importin 13 harbor binding pockets, which are essential for the interaction between importin 13 with

**FIG. 13: continued**

Relative to wild type importin 13 (1-963), the C-terminally truncated importin 13 fragment 1-784 and the N-terminally truncated importin 13 fragment 45-963 show a reduced potential to translocate the heterodimer into the nucleus of transfected cells. All other importin 13 fragments were not able to import the CHRAC-15/17 complex in the nucleus. *(C)* Importin 13 constructs used in this assay are listed. The amino acids or the importin 13 fragments are indicated and are represented by grey bars. Deleted regions are depicted by thin lines. Construct 1-963 amino acid represents wild type importin 13. *(D)* Quantification of the nuclear import of EGFP-CHRAC-15/ RFP-CHRAC-17 mediated by full length and truncated importin 13. 100 transfected cells were quantitatively analyzed and the nucleocytoplasmic colocalization of the CHRAC-15/17 heterodimer scored into the following three categories: N > C, N = C, and N < C. imp13, importin 13; aa, amino acid.

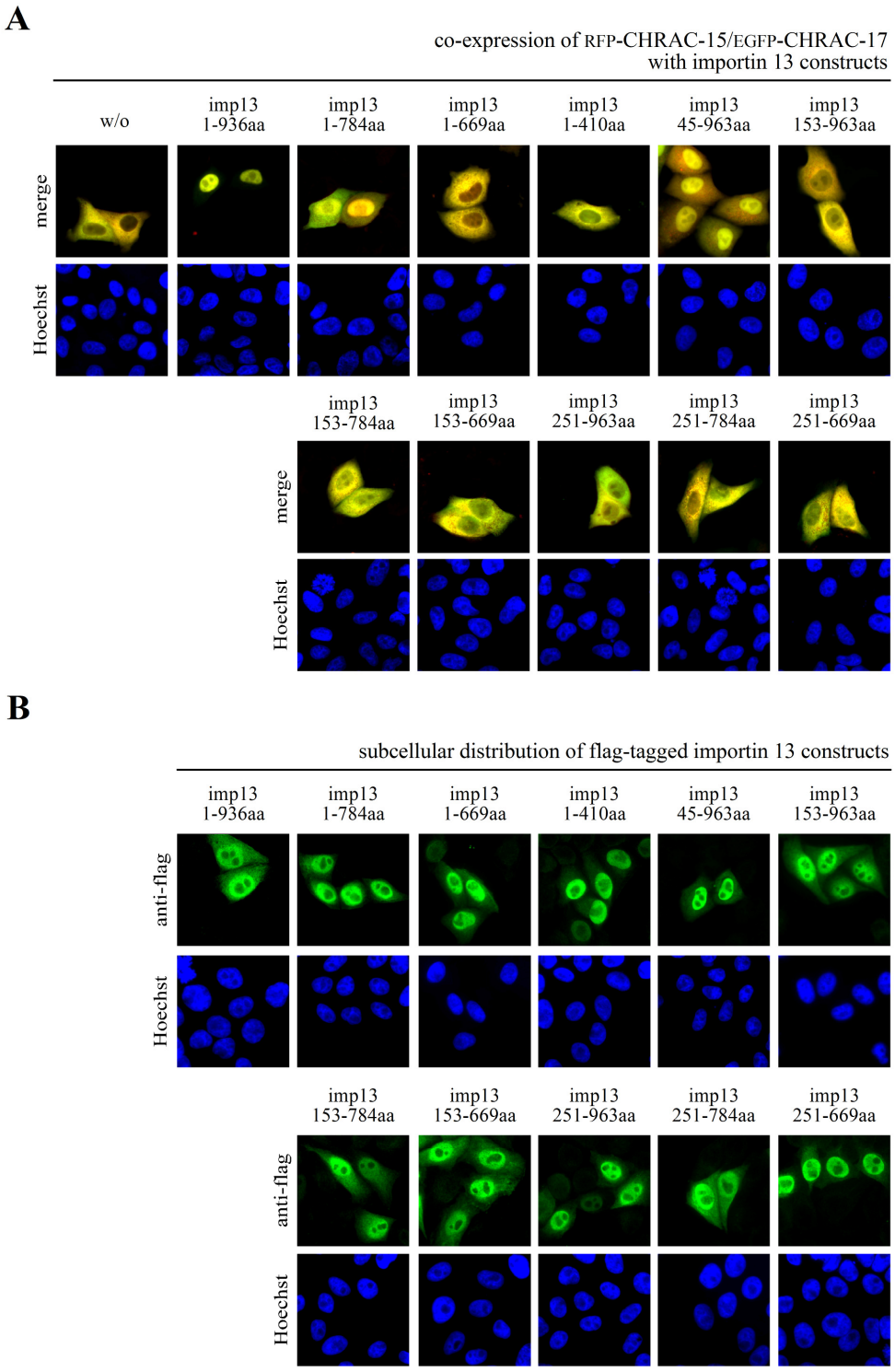


**FIG. 14: Transport characteristics of different importin 13 constructs for the CHRAC-15/17 complex do not differ upon exchange of EGFP and RFP among the subunits.** *(A)* Plasmid DNA coding for RFP-CHRAC-15 and EGFP-CHRAC-17 were cotransfected in HeLa P4 cells. As in Figure 13, importin 13 fragments were cotransfected as indicated. The subcellular distribution of the CHRAC-15/17 complex in transfected cells is shown in yellow (merge). Full length importin 13 (1-963aa) mediates nuclear uptake of the heterodimers, whereas the carboxy-terminally truncated 1-784 fragment and the *(continued on page 84)*

FG-containing nucleoporins. In fact, for importin β several binding pockets in the corresponding HEAT repeats 6, 7, and 8 have been identified (Bayliss et al., 2000; Liu and Stewart, 2005). For the importin 13 fragment 45-963 amino acid we still observed import capacity for the CHRAC-15/17 heterodimer. However, since this importin 13 fragment lacks at least parts of the putative RanGTP binding site, which is essential for the dissociation of receptor-cargo-complex, the nuclear release of the CHRAC-15/17 complex from this importin 13 fragment remains unclear. Conceivable for this particular last step of nuclear transport is however a direct competition for either the cargo or the nuclear transport receptor.

## 3.7 Importin 13 does not influence the nuclear localization of monomeric CHRAC-15 and CHRAC-17

Concerning the subcellular localization of the overexpressed monomeric CHRAC-15 and CHRAC-17 subunits *in vivo*, we have demonstrated a cytoplasmic retention (see again Fig. 5*A*). In addition, neither binding to importin 13 nor to importin 5 could be detected *in vitro*. However, since overexpression of importin 13 is a prerequisite for nuclear accumulation of the CHRAC-15/17 complex in HeLa P4 cells we also wanted to analyze the influence of these importins, in particular the role of importin 13, on the subcellular localization of the individual CHRAC-15 and CHRAC-17 subunits. For that purpose, cotransfection experiments with green and red fluorescently labeled CHRAC-15 (Fig. 15*A*) or CHRAC-17 (Fig. 15*B*) were performed. To exclude the possibility that another nuclear transport receptor is able to mediate the import of either one of the two subunits, either importin β, importin 5, importin 7, or importin 9 was coexpressed. While EGFP-CHRAC-15 shows a homogenous distribution in transfected cells at steady state, RFP-CHRAC-15 and fluorescently labeled CHRAC-17 are exclusively localized in the cytoplasm. In contrast to the heterodimeric CHRAC-15/17 complex the subcellular distribution of the monomeric histone fold subunits did not change upon coexpression of either importin 13 or any other nuclear import receptor. These data confirm our results of the previous *in vitro* binding studies where neither significant binding of importin 13 nor of importin 5 had

**FIG. 14: continued**

amino-terminally truncated 45-963 importin 13fragment show a reduced potential for nuclear import of CHRAC-15/17. All other fragments were not able to import the complex into the nucleus. *(B)* The flag-tagged importin 13 constructs were transfected in HeLa P4 cells and their subcellular distribution was determined using an anti-flag antibody. As the full length importin 13, all other truncated importin 13 fragments localized dominantly in the nucleus. imp, importin.

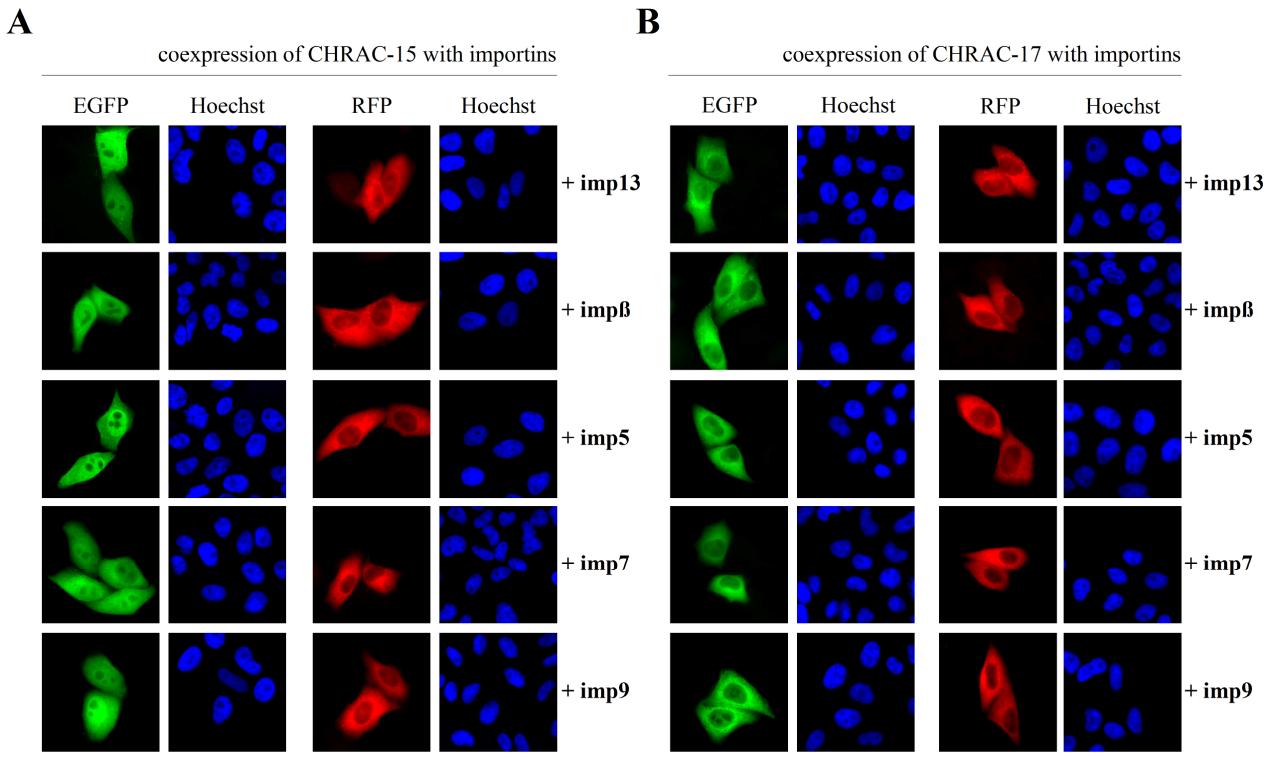


**FIG. 15: Monomeric CHRAC-15 and CHRAC-17 are not imported into the nucleus of HeLa P4 cells.** HeLa P4 cells were transiently transfected with plasmid DNA encoding CHRAC-15 *(A)* and CHRAC-17 *(B)* amino-terminally fused to either EGFP or RFP. As indicated, flag-tagged nuclear transport receptors were coexpressed. The subcellular distribution of the transfected fusion proteins was determined by direct fluorescence 24 hours post transfection. The DNA was counterstained with Hoechst. While EGFP-CHRAC-15 shows a homogenous distribution in transfected cells at steady state, RFP-CHRAC-15 and fluorescently labeled CHRAC-17 are exclusively localized in the cytoplasm. imp, importin.

been detected (see again Fig. 7*C* and 7*D*). In addition, these results demonstrate that the individual CHRAC subunits lack a NLS. In conclusion, nuclear import of CHRAC-15 and CHRAC-17 does strictly depend on the heterodimerization of the histone fold subunits in the cytoplasm.

## 3.8 Potential influence of phosphorylation on the subcellular localization of CHRAC-15 and CHRAC-17

The reversible phosphorylation of eukaryotic proteins on serine, threonine and tyrosine residues is an important and ubiquitous post-transcriptional regulatory mechanism, responsible for protein stability, function, and interaction but also for the localization of proteins (for review, see Poon and Jans, 2005). Recently, Gnad and colleagues (2007) established a phosphorylation site

database (PHOSIDA) where the time courses of phosphorylation of about 6600 phosphorylation sites in response to growth factors stimuli were analyzed by high resolution mass spectrometry-based proteomics (http://www.phosida.com). The analysis of the PHOSIDA database revealed three potential phosphorylation sites in the carboxy-terminal tails of CHRAC-15 and CHRAC-17. Whereas one phosphorylation site is located within CHRAC-17 at serine 122 (S122), the others are located at serine 124 and 131 in CHRAC-15 (S124/S131). Since phosphorylation can increase as well as decrease the affinity to import receptors (Jans et al., 2000) due to the introduction of a conformational change in the structure, we elucidated the

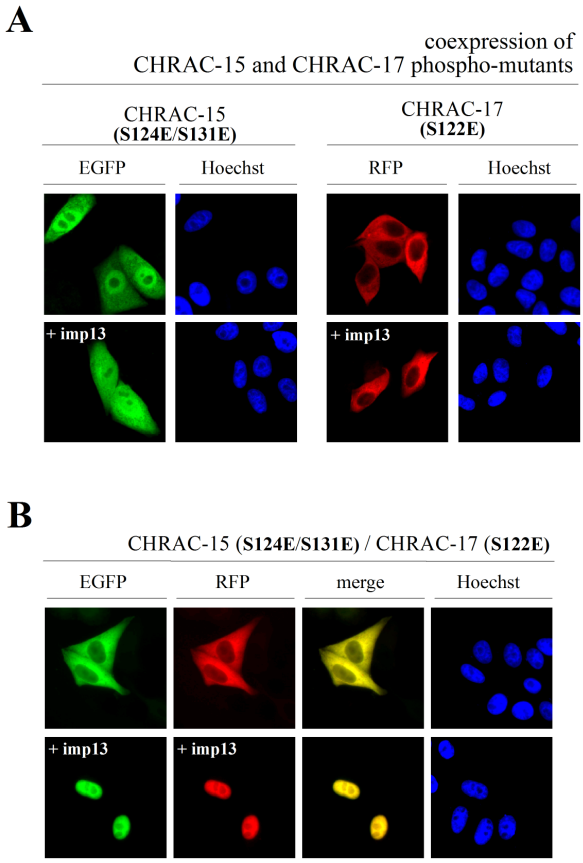


**FIG. 16: Nuclear import of monomeric CHRAC-15 and CHRAC-17 and the dimeric CHRAC-15/17 complex was not altered upon substitution of serine residues.** The indicated serine residues in CHRAC-15 (S124 and S131) and CHRAC-17 (S122) were substituted for glutamate to mimic phosphorylation. HeLa P4 cells were transiently transfected with DNA coding for substituted EGFP-CHRAC-15 or RFP-CHRAC-17 monomers *(A)* or EGFP-CHRAC-15 and RFP-CHRAC-17 *(B)* in the absence or presence of flag-tagged importin 13. The subcellular localization of the monomers and the dimeric complex was identified by direct fluorescence 24 hours after the transfection. DNA staining was performed using Hoechst. Compared to the wild type fusion proteins (see Fig. 5 and 8), the subcellular distribution of the mutated subunits as well as the mutated CHRAC-15/17 complex did not change. Coexpression of importin 13 results in the same subcellular localization as shown for the wild-type monomers (see Fig. 15) or the CHRAC-15/17 complex (see Fig. 10). S, serine; E, glutamate; imp13, importin 13.

potential role of phosphorylation on the nuclear transport of the monomeric subunits as well as for the CHRAC-15/17 complex. For that purpose, we substituted the three serine residues in the subunits for glutamate to mimic the effect of phosphorylation. The resulting constructs were transfected and the localization of the fluorescently tagged proteins was determined. The results are shown in Figure 16. Substitution of the serines did neither alter the subcellular distribution of the individual CHRAC-15 and CHRAC-17 (Fig. 16*A*) subunits nor of the CHRAC-15/17 heterodimer (Fig. 16*B*). Compared to the data of the wild type proteins, similar results were observed when importin 13 was additionally coexpressed. Importin 13 led to a strong nuclear accumulation of the mutated CHRAC-15 (S124E/S131E)/CHRAC-17 (S122E) complex (Fig. 16*B*, lower panel), whereas the mutated monomeric subunits remained largely in the cytoplasm (Fig. 16*A*, lower panel).

Since PHOSIDA also matches topics like accessibility, subcellular detection and kinase motifs to the different phosphorylation sites, casein kinase 2 (CK2; with the loose recognition motif **S**/**T**-X-X-E) was identified to perform phosphorylation on serine 122 in CHRAC-17. It has been demonstrated that CK2 facilitates different cellular functions, such as control of cell cycle control progression, maintenance of cell survival, response to stress and to DNA damage. Thus, CK2 seems to have a dual role by the involvement in proliferation and cell growth as well as in suppression of apoptosis (for review, see Ahmad et al., 2008; Unger et al., 2004). However, because CK2 localizes in both compartments, cytoplasm and nucleus, it remains unclear where CHRAC-17 phosphorylation takes place. Surprisingly and in contrast to your results, Gnad and colleagues (2007) found both phosphorylated CHRAC subunits in the cytoplasmic fraction.

## 3.9 Importin 13 also mediates nuclear import of the histone fold motif containing p12/CHRAC-17 complex

The importin 13-dependent nuclear import of the CHRAC-15/CHRAC-17 complex shows analogies between the CHRAC-15/17 heterodimer and its histone fold relatives NF-YB/NF-YC (Kahle et al., 2005) and NC2α/NC2β (Kahle et al., 2009). We therefore asked, whether even more histone fold heterodimers of the H2A/H2B type are also transported into the nucleus via importin 13. To address this question, we analyzed the nuclear import of the p12/CHRAC-17 heterodimer of the DNA polymerase ε (Li et al., 2000). First we tested the interaction of the p12/CHRAC-17 complex with different nuclear transport receptors using *in vitro* binding experiments. GST-p12 and His-CHRAC-17 were coexpressed in *E. coli*, affinity purified and

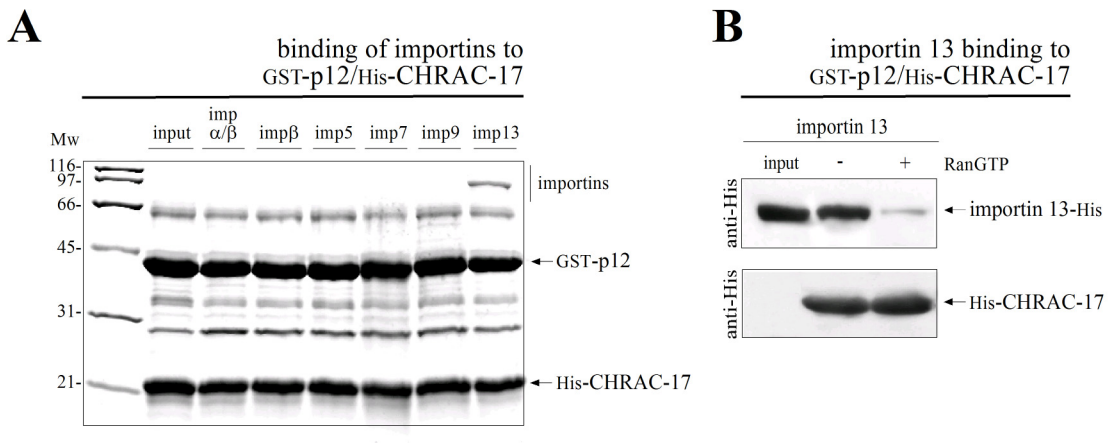


**FIG. 17: Importin 13 binds RanGTP-sensitive to the p12/CHRAC-17 complex.** *(A)* GST-p12 and His-CHRAC-17 were coexpressed in *E. coli*, immobilized on glutathione-Sepharose and incubated with equal concentrations (0.2 µM) of recombinant purified importin α/ß heterodimer, importin β, importin 5, importin 7, importin 9 or importin 13. Bound fractions were analyzed by SDS-PAGE followed by Coomassie staining. The histone fold heterodimer p12/CHRAC-17 binds exclusively to importin 13. *(B)* The binding assay with His-tagged importin 13 was repeated in the absence (-) or presence (+) of RanGTP. Bound importin 13 was detected by Western blotting using an anti-His antibody. As a control, 20 % of the recombinant importin 13 was assayed as input. To control equal loading His-tagged CHRAC-17 was also detected with the anti-His antibody. p12/CHRAC-17 binds to importin 13 in a RanGTP-dependent manner, although weak interactions were also detected in the presence of RanGTP. Mw, molecular weight; imp, importin.

immobilized on glutathione-Sepharose. The immobilized p12/CHRAC-17 complex was incubated with recombinant nuclear transport receptors and the concentration of the purified transport factors was determined as described earlier (see chapter 3.3). Analogous to the previous binding assays, 0.2 µM of preincubated importin α/importin β, importin β, importin 5, importin 7, importin 9, and importin 13 were used. After washing, the bound nuclear transport receptors were analyzed and displayed by SDS-PAGE followed by Coomassie staining. As shown in Figure 17*A*, the p12/CHRAC-17 heterodimer revealed strong binding to importin 13. Binding to other import factors was not detected in this experiment. To assure binding specificity between the p12/CHRAC-17 complex and recombinant His-tagged importin 13 binding experiments were repeated in the absence or presence of RanGTP to mimic cytoplasmic or nuclear environment. This time, importin 13 binding to the histone fold pair was analyzed by Western blotting using an anti-His antibody (Fig. 17*B*). As before, in the absence of RanGTP immobilized p12/CHRAC-17 showed efficient binding to importin 13, which was strongly reduced in the presence of RanGTP. Hence, importin 13 does not represent an export factor but

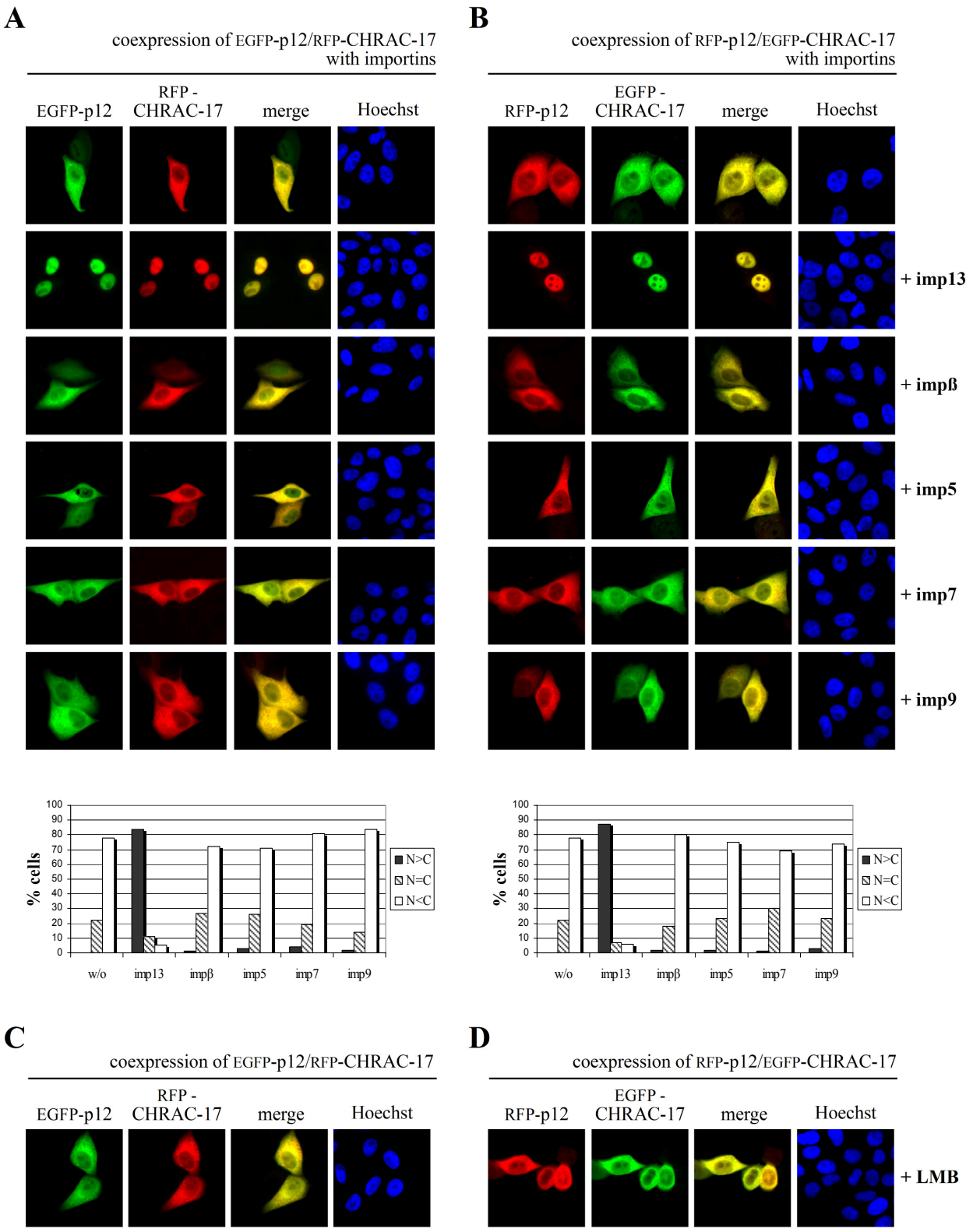


**FIG. 18: Importin 13 mediates nuclear uptake of the heterodimeric p12/CHRAC-17 complex.** HeLa P4 cells were cotransfected with DNA coding for the histone fold subunits p12 fused to RFP and CHRAC-17 fused to EGFP *(A)* and *vice versa (B)*. In addition, plasmid DNA coding for different flag-tagged nuclear transport receptors was added as indicated. The subcellular localization of the gene products was examined 24 hours post transfection by direct fluorescence. Using Hoechst, the DNA was counterstained. *(continued on page 90)*

import factor for the p12/CHRAC-17 complex. To examine the subcellular distribution of the p12/CHRAC-17 complex *in vivo*, EGFP-p12 and RFP-CHRAC-17 were coexpressed in HeLa P4 cells (Fig. 18*A*, top row). To rule out an influence of the fluorescent tag on the localization of the complex, RFP-p12 and EGFP-CHRAC-17 were additionally coexpressed (Fig. 18*B*, top row). As detected for CHRAC-15/CHRAC-17 before, overexpression of the p12/CHRAC-17 complex also led to a cytoplasmic localization. Since the cytoplasmic retention was not changed upon LMB treatment, the cytoplasmic localization of p12/CHRAC-17 does not result from exportin 1 mediated nuclear export (Fig. 18*C* and *D*). Coexpression of importin 13, however, changed the subcellular localization of p12/CHRAC-17, the complex now becoming predominantly nuclear. Analogous to previous data from CHRAC-15/CHRAC-17, the coexpression of exogenous importin 13 is required for nuclear accumulation of the related histone fold pair p12/CHRAC-17. In contrast, coexpression of other flag-tagged nuclear transport receptors did not influence the cytoplasmic distribution of the p12/CHRAC-17 complex (see again Fig.18*A* and *B*). A quantitative analysis reflects the results of the performed experiment.

Interestingly, a similar effect was observed for another H2A/H2B related histone fold complex. In the case of NC2α/NC2β, exogenous importin 13 also led to an efficient nuclear accumulation of a cNLS deficient RFP-NC2α/EGFP-NC2β dimer (Kahle et al., 2009). Taken together, these results demonstrate that importin 13 is responsible for the nuclear uptake of at least four different histone fold motif containing heterodimers, namely CHRAC-15/17, p12/CHRAC-17, NC2α/NC2β, and NF-YB/NF-YC. Thus, besides the known monomeric importin 13 substrates such as human UBC9, RBM8 (Mingot et al., 2001), paired-type homeodomain transcriptions factors (Ploski et al., 2004), the glucocorticoid receptor (Tao et al., 2004), and the actin-binding protein myopodin (Liang et al., 2008), importin 13 is likely to have a general role in mediating the nuclear import of histone fold pairs of the H2A/H2B family.

**FIG. 18: continued**

The merge picture results form the overlap of the green EGFP and the red RFP fluorescence. For a semi-quantitative analysis, the mean distribution of the p12/CHRAC-17 heterodimers with or without the additional coexpression of flag-tagged import receptors was classified in the categories: N > C, N = C, and N < C. Coexpression of importin 13 leads to an exclusive nuclear accumulation of the EGFP-p12/RFP-CHRAC-17 *(A)* and RFP-p12/EGFP-CHRAC-17 *(B)*. Neither importin β, nor importin 5, importin 7 or importin 9 mediates the nuclear accumulation of the heterodimer when cotransfected. *(C)* and *(D)* Analogous to *(A)* and *(B)*, HeLa P4 cells were cotransfected. Twenty-four hours post transfection the cells were treated with 10 ng/µl Leptomycin B (LMB) for 6 hours to block exportin 1-mediated nuclear export. LMB treatment did not affect the cytoplasmic localization of the p12/CHRAC-17 complex. imp, importin.

## 3.10 Basic amino acid residues are conserved among the histone fold pairs

Unlike the positively charged consensus sequence of monopartite or bipartite cNLSs (Boulikas, 1993; Conti et al., 1998), ncNLS are usually composed of long heterogeneous stretches of amino acids. This versatility and the non-existing consensus sequence within ncNLSs makes a prediction very difficult (Christophe et al., 2000; Ström and Weis, 2001). To identify sequence elements necessary for the recognition of heterodimeric histone fold complexes by importin 13 we searched for sequence similarities among the four different histone fold pairs that were bound by importin 13. Hence, we aligned the related histone fold subunits NF-YB, NC2β, and

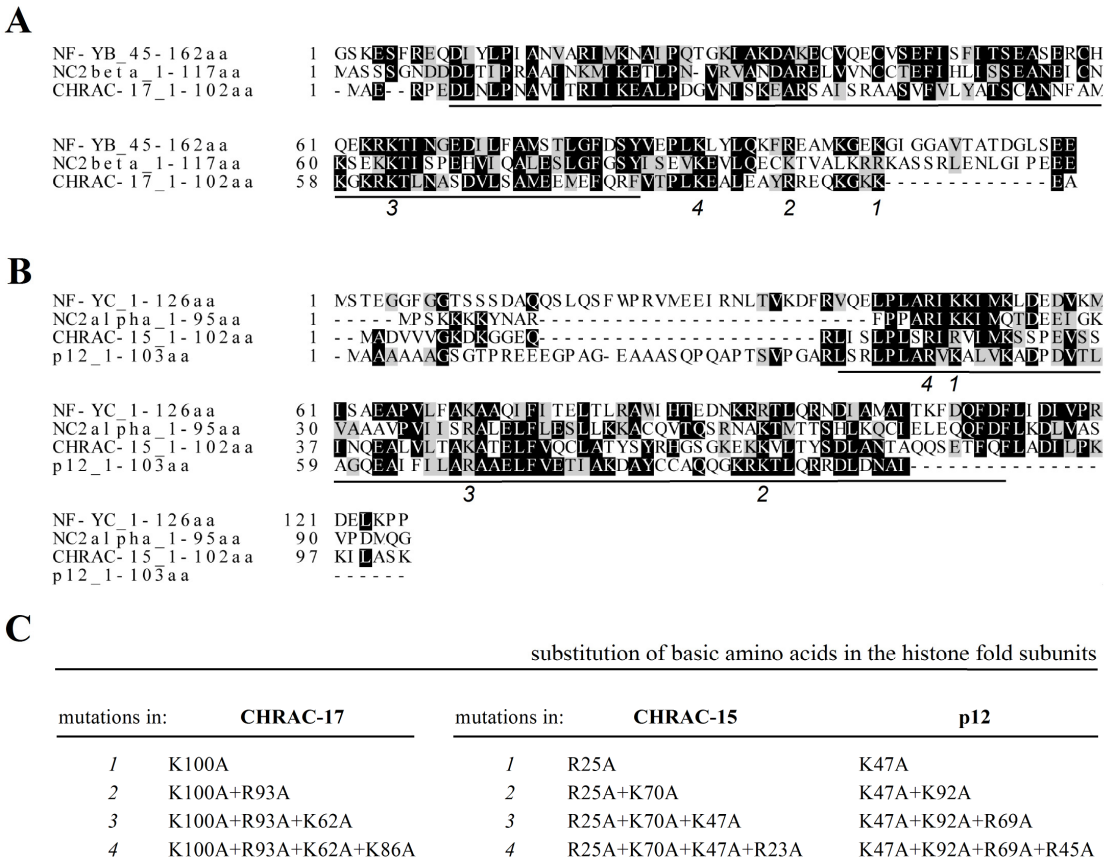


**FIG. 19: Basic amino acids are conserved between related histone fold subunits.** *(A)* and *(B)* Partial sequence alignments of histone fold proteins of the H2B *(A)* and H2A *(B)* family. Identical amino acids are presented by a black background whereas related residues are shaded in grey. The histone fold motifs of CHRAC-17 and CHRAC-15 (taken from Poot et al., 2000) are indicated by a black bar underneath the sequences. *(C)* Conserved basic amino acids responsible for DNA binding but presumably not involved in subunit heterodimerization were selected for progressive substitution with up to four basic amino acids against alanine. The substituted lysine or arginine residues in the aligned sequences are indicated by a number (*1-4*) and this also represents the order of consecutive mutations.

CHRAC-17 of the H2B protein family (Fig. 19*A*) and NF-YC, NC2α, CHRAC-15, and p12 of the H2A protein family (Fig. 19*B*). Since DNA binding regions frequently overlap with NLSs (Cokol et al., 2000; LaCasse and Lefebvre, 1995) we searched, based on data of the NC2α/NC2β and NF-YB/NF-YC heterodimers (Kamada et al., 2001; Romier et al., 2003), for basic, conserved basic amino acids with DNA binding properties. To elucidate the potential role of these residues for importin 13 binding, point mutations were gradually introduced stepwise by site-directed mutagenesis substituting up to four conserved basic amino acids against alanine. In Figure 19*C*, the different mutations in CHRAC-17, CHRAC-15, and p12 are listed. In addition, the number of substitutions is indicated, which also represents the order of consecutive mutation (also shown in Fig. 19*A* and 19*B*). To exclude an influence on the heterodimerization of the subunits, we tried to select residues not involved in subunit heterodimerization. Nevertheless, according to structural informations by Kamada and colleagues (2001) R16 and R40 of NC2α, corresponding to R23 and K47 in CHRAC-15 and to R45 and R69 in p12, respectively, seem to be involved in intermolecular interactions. The effects of the introduced mutations on the binding of CHRAC-15/17, p12/CHRAC-17, and also NC2α/NC2β were analyzed next *in vivo* and *in vitro*.

## 3.11 CHRAC-15/17 binding to importin 13 depends on basic amino acids

To study the effect of the mutations on the subcellular localization of the CHRAC-15/17 complex, we coexpressed wild type and mutated EGFP-CHRAC-15 and RFP-CHRAC-17 in HeLa P4 cells. Additionally, flag-tagged importin 13 was coexpressed as described earlier. The progressive substitution of lysine (K) or arginine (R) residues led to an increased cytoplasmic distribution of the CHRAC-15/17 complex as compared to the wild type complex (Fig. 20*A*). While single (Mut1) and double (Mut2) mutations in the CHRAC subunits still allowed nuclear uptake by importin 13, nuclear import of CHRAC-15/17 was nearly abrogated when four basic amino acids (Mut4), were substituted in each subunit. The influence of the substituted basic amino acids on nuclear uptake of the CHRAC-15/17 complex was quantitatively analyzed using the software ImageJ. The percentages of nuclearly and cytoplasmically localized CHRAC-15/17 complex were calculated and are shown in Figure 20*B*. The nuclear localization of the CHRAC-15/17 complex is reduced from 87 % to 42 %, when four basic amino acids were mutated on each subunit.

To further analyze the role of the substituted basic amino acids on the binding of importin 13 to CHRAC-15/17, we performed *in vitro* binding assays with the mutated CHRAC-15/CHRAC-17

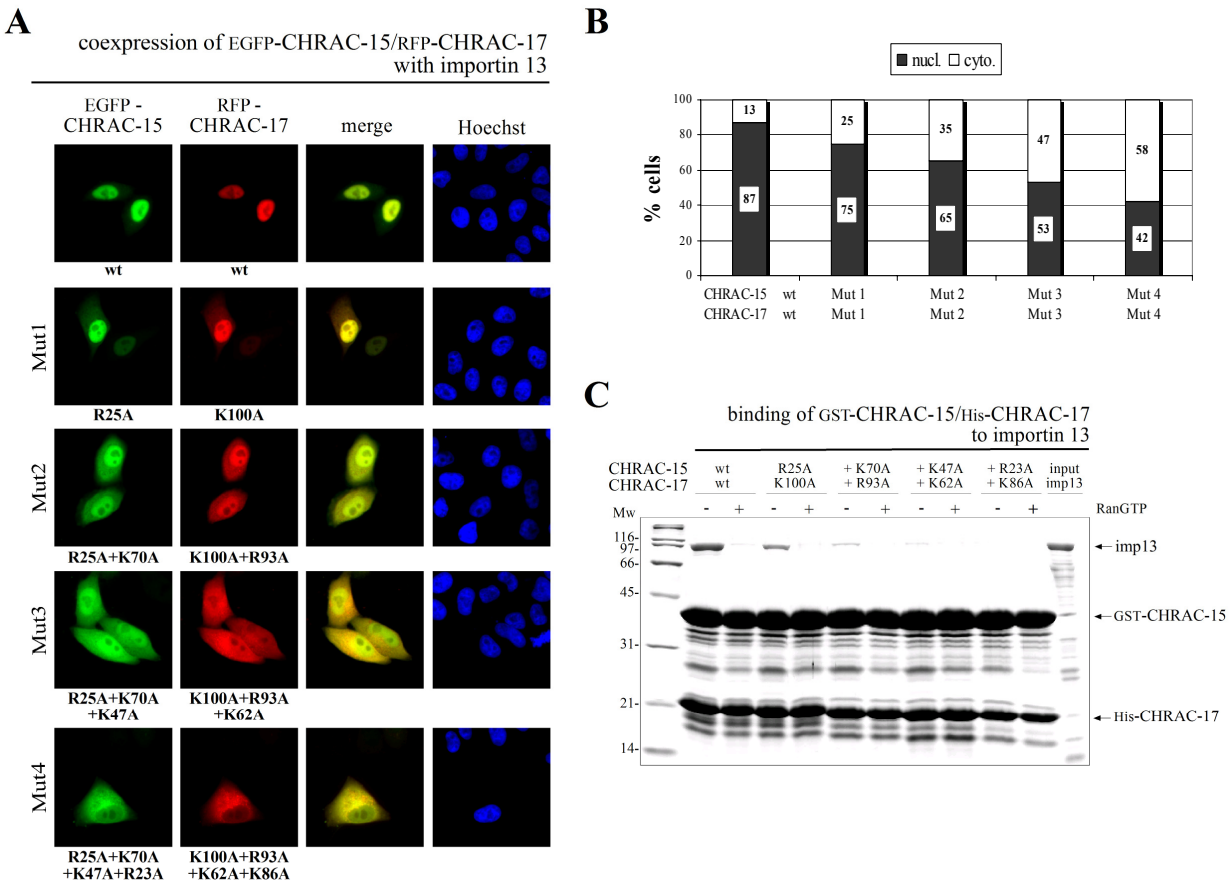


**FIG. 20: Basic amino acids in the CHRAC-15/17 subunits are necessary for importin 13 binding.** *(A)* HeLa P4 cells were transiently cotransfected with plasmid DNA coding for wild type (wt) EGFP-CHRAC-15 and RFP-CHRAC-17 (upper row) and fusion proteins carrying an increasing number of mutated basic amino acid residues as indicated (see also Fig. 19). Flag-importin 13 was coexpressed and the subcellular localization of wild type and mutated CHRAC-15/17 complexes was determined by direct fluorescence 24 hours post transfection. The colocalization of RFP- and EGFP-fusion proteins is shown in yellow (merge). DNA was counterstained with Hoechst. The stepwise substitution of basic amino acids in CHRAC-15 and CHRAC-17 interferes with the importin 13 mediated nuclear transport and results in an increased cytoplasmic accumulation of the CHRAC-15/17 complex in transfected cells. *(B)* For quantitative analysis, the fluorescence intensity of colocalized wild-type and mutated CHRAC-15/17 complexes was measured in 20 cells using the ImageJ software (NIH). The percentage of nuclear (nucl.) and cytoplasmic (cyto.) localization was calculated (sites of mutations are indicated with Mut1, Mut2, Mut3, and Mut4 as in *(A)*). *(C)* GST-CHRAC-15/His-CHRAC-17 with alanine substitutions of conserved basic amino acids were coexpressed in *E. coli* and immobilized on glutathione-Sepharose. Binding studies with the wild type (wt) and the mutated heterodimers were performed using recombinant purified importin 13. 20 % of importin 13 used in this assay are displayed as input. Each indicated amino acid substitution is additional compared to the preceding column. Binding was performed in the absence (-) or presence (+) of RanGTP and bound fractions were displayed by SDS-PAGE followed by Coomassie staining. The loss of positively charged amino acids in the CHRAC-15/17 complex results in a reduced binding of importin 13. wt, wild type; Mw, molecular weight; imp13, importin 13.

complexes and recombinant importin 13. Due to the substitution of R23A and K47A, CHRAC-15 lacked two amino acid residues that are presumably involved in the heterodimerization with CHRAC-17. However, the coexpression and purification of the mutated GST-CHRAC-15/His-CHRAC-17 heterodimer (Mut4) was not negatively influenced. Despite the loss of up to four positively charged amino acids all mutated GST-CHRAC-15 and His-CHRAC-17 subunits did interact with each other and therefore could be immobilized on glutathione-Sepharose as one complex (Fig. 20*C*). As before, the binding assay was performed in the absence or presence of RanGTP. As shown in Figure 20*C*, the strong RanGTP-sensitive binding of importin 13 to the wild type CHRAC-15/17 heterodimer, also shown above in Figure 7*B*, was gradually reduced with increasing numbers of mutations and disappeared completely when four basic amino acids on each CHRAC-subunit were mutated.

The need for basic amino acids in both subunits of the CHRAC-15/17 complex for efficient binding to importin 13 provides an explanation for the results of the *in vivo* transfection experiments presented in Figure 20*A*. Transport into the nucleus of course does only occur when the cargo (CHRAC-15/17) is properly bound and recognized by the corresponding import factor (importin 13). Thus, it can be concluded that positively charged amino acids distributed among the two histone fold subunits, CHRAC-15 and CHRAC-17, are essential for importin 13 binding. Further, these results suggest that binding of importin 13 to the CHRAC-15/17 complex is based on the existence of a *binding platform* within the dimerized histone fold complex. This binding interface is generated upon the heterodimerization of CHRAC-15 and CHRAC-17, whereas the individual histone fold subunits do not contain this binding interface. Importin 13 specifically binds to the complexed histone fold subunits exposing the appropriate binding motif, which leads to the nuclear import of the CHRAC-15/17 complex.

## 3.12 The importin 13 binding platform is conserved between different histone fold heterodimers

In addition to the CHRAC-15/17 complex, we also wanted to verify the role of the conserved positively charged amino acid residues for the nuclear import of the related histone fold pairs, p12/CHRAC-17 and NC2α/NC2β. The NC2α/NC2β (NC2) complex binds to the promoter-bound TATA-binding protein (TBP) and the formation of the NC2-TBP complex results in a sterical hindrance for the recruitment of the general transcription factors IIB and IIA (Goppelt et al., 1996; Kamada et al., 2001; Mermelstein et al., 1996). Hence, the NC2 complex functions as a transcriptional repressor (Inostroza et al., 1992; Meisterernst and Roeder, 1991). As recently

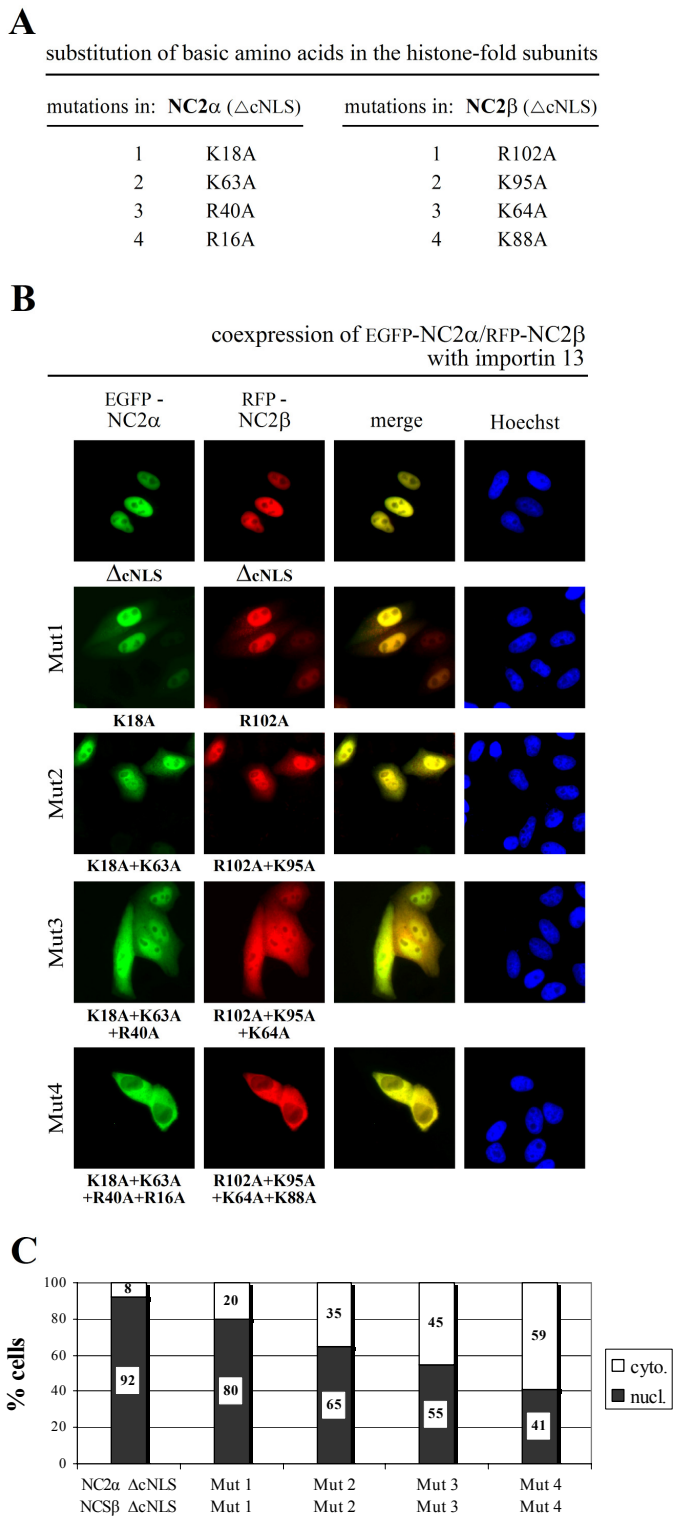


**FIG. 21: Nuclear import of NC2α/NC2β is mediated by importin 13 and depends on basic amino acids.** *(A)* Conserved basic amino acids in cNLS deficient (ΔcNLS) NC2α and NC2β were progressively substituted. The numbers (1-4) represents the order of consecutive mutations against alanine (see also Fig. 19*A* and 19*B*). *(B)* HeLa P4 cells were transiently transfected with plasmid DNA encoding flag-importin 13. Additionally, EGFP-NC2α and RFP-NC2β, both mutated in their cNLS by site-directed mutagenesis and then consecutively mutated in their basic amino acids as indicated, were cotransfected. The subcellular localization of the NC2α/NC2β complex was examined 24 hours post transfection by direct fluorescence. *(continued on page 96)*

shown in our group (Kahle et al., 2009) both NC2 subunits, NC2α and NC2β, contain a cNLS. The NC2 complex is therefore recognized and imported by the importin α/β heterodimer. However, when the cNLSs in NC2α ($^{4}$KKKK$^{7}$) and NC2β ($^{100}$KRRRK$^{103}$) are mutated via a substitution of K5 in NC2α and R101 in NC2β against alanine (in Fig. 21 referred to as ΔcNLS), the NC2 complex is still imported into the nucleus in an importin 13-dependent pathway (Fig. 21*B*, upper row). To analyze the influence of the conserved basic amino acids in the NC2 complex (Fig. 21*A*), EGFP-NC2α and RFP-NC2β, both deficient of its cNLS and progressively mutated in their basic amino acids were coexpressed with importin 13 in HeLa P4 cells. The subcellular distribution was detected by direct fluorescence. For a quantitative analysis, the fluorescence intensity in cotransfected cells was measured and the ratio between cytoplasmic and nuclear localization was calculated (Fig. 21*C*). Similar to the changes observed in the subcellular localization of CHRAC-15/17 complex, the transfection of the NC2α/NC2β complex also led to a decreased nuclear uptake when progressively mutated at the respective sites (Fig. 21*B*). The strong nuclear accumulation of the cNLS deficient NC2 complex (92 %), was lost when three basic amino acids on each subunit were mutated leading to a homogenous localization (55 %). When eight basic amino acids in the cNLS deficient NC2 complex were mutated, 59 % of the complex accumulated in the cytoplasm.

Finally we analyzed the nuclear transport of the p12/CHRAC-17 complex deficient in conserved basic amino acids. Analogous to previous transfection experiments with CHRAC-15/17 HeLa P4 cells were cotransfected with plasmid DNA coding for importin 13 and mutated p12 and CHRAC-17 (Fig. 22). Although the overall effect of progressive substitution of basic residues was less dramatic compared to mutated CHRAC-15/17 and NC2α/NC2β, nuclear transport of p12/CHRAC-17 was reduced strongly if four basic amino acids on each subunit were mutated (Fig. 22*A* and *B*). Additionally, we performed *in vitro* GST-Pulldown assays with mutated p12/CHRAC-17 complexes (Fig. 22*C*). Again, we found no evidence for reduced heterodimerization, caused by the substitution of R45 and R69 for alanine in p12. The loss of basic amino acids in the p12/CHRAC-17 complex resulted in decreased importin 13 binding,

**FIG. 21: continued**

DNA was counterstained with Hoechst. Colocalization of the red RFP-fusion protein and the green EGFP-fusion protein is shown in yellow (merge). The loss of conserved basic amino acids in cNLS deficient NC2α and NC2β results in an increased cytoplasmic accumulation of the NC2complex in transfected cells. *(C)* The subcellular distribution of colocalized cNLS-deficient (ΔcNLS) and mutated NC2α/NC2β complexes was quantitatively analyzed using the program ImageJ (NIH). The fluorescence intensity of 20 cotransfected cells was measured and the ratio between nuclear (nucl.) and cytoplasmic (cyto.) localization was calculated. R, arginine; K, lysine; A, alanine.

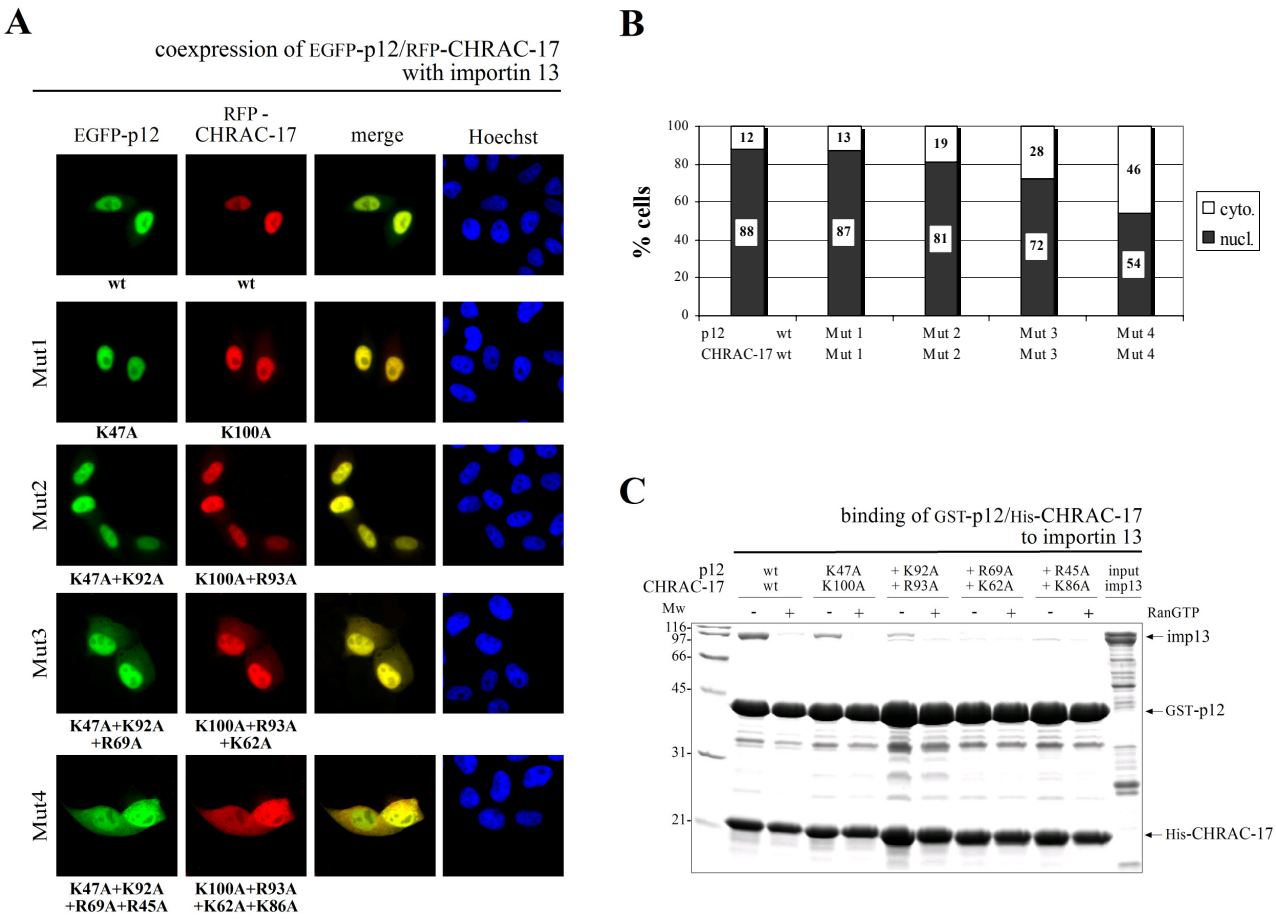


**FIG. 22: Basic amino acids conserved among the H2A/H2B histone fold family are necessary for the interaction between importin 13 and the p12/CHRAC-17 heterodimer.** *(A)* HeLa P4 cells were transiently cotransfected with plasmid DNA encoding wild type (wt) or stepwise mutated EGFP-p12 and RFP-CHRAC-17 as indicated. Flag-importin 13 was additionally coexpressed. Subcellular localization of the p12/CHRAC-17 heterodimer was detected 24 hours post transfection by direct fluorescence. Colocalization of the fluorescently-labelled subunits is shown in yellow (merge). DNA was stained with Hoechst. The loss of basic amino acid residues in p12 and CHRAC-17 does change the subcellular distribution of the heterodimer towards a homogeneous localization. *(B)* The subcellular distribution of colocalized wild type (wt) and mutated p12/CHRAC-17 complex was quantitatively analyzed using the program ImageJ (NIH). The fluorescence intensity of 20 cotransfected cells was measured and the ratio between nuclear (nucl.) and cytoplasmic (cyto.) localization was calculated. *(C)* Recombinant GST-p12/His-CHRAC-17 wild type heterodimer (wt) and heterodimers with alanine substitutions of conserved basic amino acids were incubated with recombinant, purified importin 13. Binding was determined in the absence (-) or presence (+) of RanGTP (Q69L-mutant). 20 % of the applied importin 13 is shown as input. Bound fractions were analyzed by SDS-PAGE followed by Coomassie staining. Substitution of basic residues in p12 and CHRAC-17 led to a decreased binding of importin 13 to the p12/CHRAC-17 complex. wt, wild type; R, arginine; K, lysine; A, alanine; Mw, molecular weight; imp13, importin 13.

similar to the observations made for the mutated CHRAC-15/17 complex (Fig. 22*C* and see again Fig. 20*C*). In more detail, upon the substitution of three conserved basic amino acids against alanine in each histone fold subunit, p12 and CHRAC-17, binding of importin 13 was lost completely.

## 3.13 The importin 13 binding platform in histone fold pairs is created by conserved basic residues on both subunits

The results of *in vivo* transfection experiments clearly showed that mutations of selected basic amino acids in the subunits of CHRAC-15/17 and p12/CHRAC-17 significantly reduced nuclear accumulation of the histone fold pairs. However, since CHRAC-17 is part of both heterodimers, CHRAC-15/CHRAC-17 and p12/CHRAC-17, one could argue that the reduced nuclear uptake is caused solely by CHRAC-17. To test this possibility, we additionally performed transfection experiments with wild type RFP-CHRAC-17 in combination with either gradually mutated EGFP-CHRAC-15 (Fig. 23*A*) or EGFP-p12 (Fig. 23*B*). The loss of positively charged amino acids in CHRAC-15 alone also led to a constant decrease in nuclear accumulation of the corresponding heterodimers. The mutation of all four basic residues in CHRAC-15 resulted in a more or less homogenous localization of the CHRAC-15-17 heterodimer (see quantification in Fig. 23*A*). Progressive mutation of the conserved residues in p12 also led to a decrease of nuclear localization of the p12/CHRAC-17 complex. However, as previously noticed, the effect of the p12-containing histone fold heterodimer was less dramatic, but nevertheless visible compared with the CHRAC-15/17 complex (Fig. 23*B*). Therefore, a decrease of just 13 % was measured for the nuclear uptake of the histone fold complex in which p12 is mutated relative to the wild type complex.

In summary, the loss of positively charged amino acids in CHRAC-15 and p12 alone reduces the nuclear accumulation of the corresponding heterodimers. Hence, the reduced nuclear import does not result exclusively from mutations in CHRAC-17, but results from the substitution of basic amino acid residues distributed among both histone fold subunits. In conclusion, basic amino acids on both histone fold motif containing subunits are essential for binding of importin 13 to histone fold complexes. Since the importin 13 binding site in the histone fold complexes contains positively charged amino acid residues, the interaction between the histone fold complexes and importin 13 is presumably based on electrostatic interactions with negatively charged residues of importin 13.

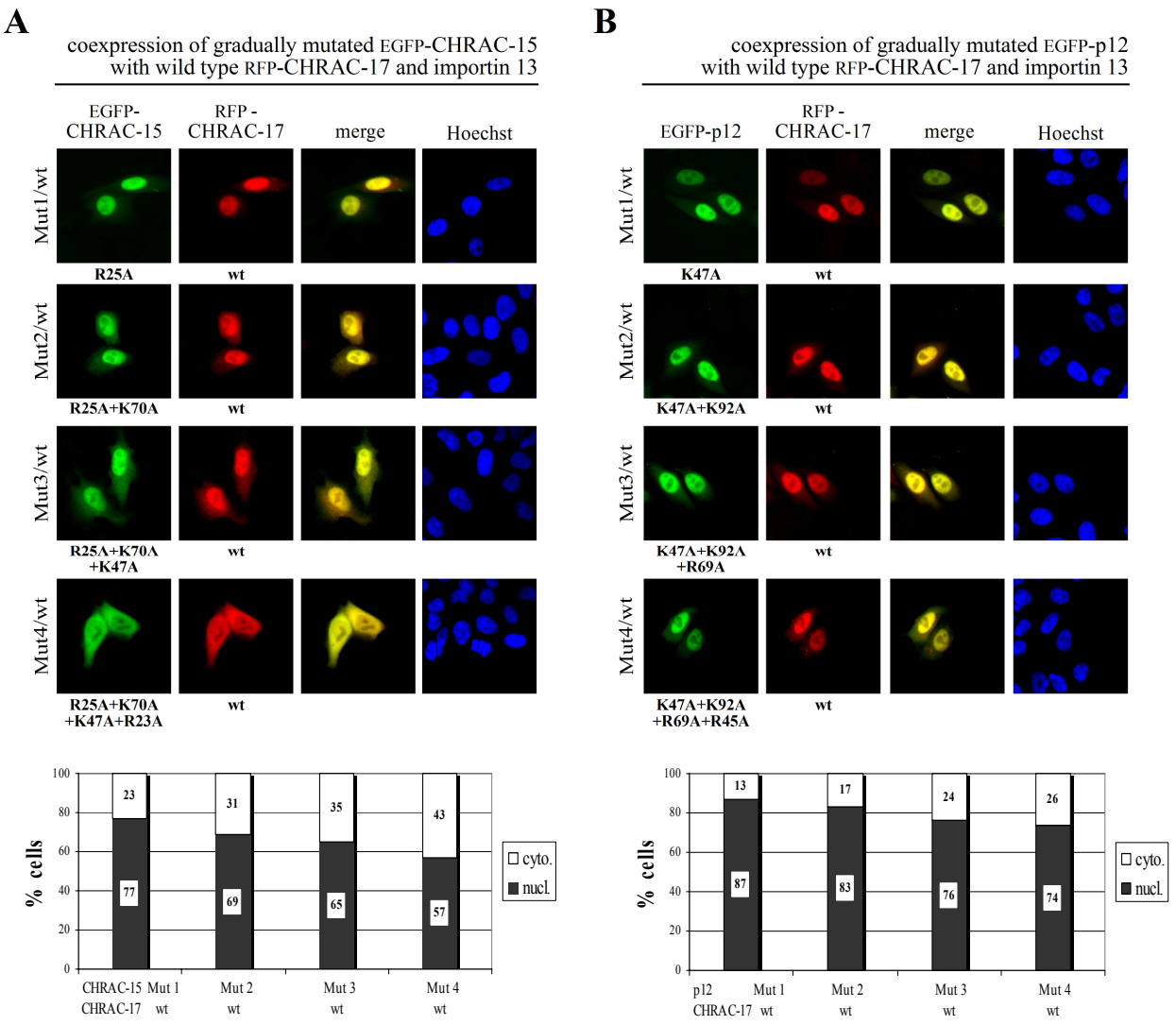


**FIG. 23: Mutation of positively charged amino acids in CHRAC-15 and p12 reduces the nuclear accumulation of the corresponding histone fold complexes.** *(A)* HeLa P4 cells were transiently transfected with plasmid DNA coding for mutated EGFP-CHRAC-15 and wild type (wt) RFP-CHRAC-17 as indicated. Flag-tagged importin 13 was additionally coexpressed. The subcellular localization of the resulting CHRAC-15/17 complexes was determined 24 hours post transfection by direct fluorescence. The DNA was stained with Hoechst and colocalization of the histone fold subunits is shown in yellow (merge). The subcellular distribution of the heterodimerized CHRAC-15/17 complex was quantitatively analyzed with the ImageJ software (NIH). The percentage between nuclear (nucl.) and cytoplasmic (cyto.) localization of the heterodimers was calculated by measuring the fluorescence intensity of 20 cotransfected cells. Stepwise mutation of basic amino acids in the CHRAC-15 subunit increases the cytoplasmic localization of the CHRAC-15/17 heterodimer. *(B)* HeLa P4 cells were transiently cotransfected with plasmid DNA encoding flag-tagged importin 13, wild type (wt) RFP-CHRAC-17 and EGFP-p12 with an increasing number of mutated basic amino acids as indicated. The subcellular localization was determined by direct fluorescence 24 hours post transfection. The DNA was counterstained with Hoechst. (*continued on page 100*)

## 3.14 The subcellular localization of the heterodimeric p12/CHRAC-17 complex is cell cycle regulated

The loss of nuclear uptake upon transfection of the gradually mutated p12/CHRAC-17 heterodimers was not as strongly affected as the transport of the CHRAC-15/17 and NC2α/NC2β heterodimers. In addition, the results of the *in vitro* binding studies did not always reflect the data generated upon transfection *in vivo*. Whereas binding of importin 13 to the heterodimeric p12/CHRAC-17 complex could not be detected *in vitro* when three basic residues were mutated on each subunit (Fig. 22*C*), nuclear import was still accomplished under these conditions *in vivo*. This discrepancy between *in vitro* and *in vivo* data might therefore point towards an additional regulation mechanism within the cell or an alternative import pathway via an unidentified transport receptor that does not depend on the basic amino acid residues distributed among the two subunits.

However, as described before, post-transcriptional modifications like phosphorylation may influence the function and structure of proteins *in vivo*. In fact, using mass spectrometry-based *in silico* analysis (Gnad et al., 2007) a putative phosphorylation site at threonine 11 ($^{9}$SG**T**PREE$^{15}$) was identified in the p12 subunit. Therefore, to analyze if the p12/CHRAC-17 complex is transported in a cell cycle-dependent manner, we performed drug induced cell cycle arrest experiments. For that purpose, HeLa P4 cells were transfected with flag-tagged importin 13, EGFP-p12 and RFP-CHRAC-17. Twenty-four hours after transfection the cells were treated with Fluorouracil, Aphidicolin or sodium butyrate. Fluorouracil induces a cell cycle arrest in S-phase (Takeda et al., 1999), Aphidicolin blocks the transition between G1- and S-phase (Pedrali-Noy et al., 1980) both by inhibiting DNA synthesis, and sodium butyrate specifically synchronized HeLa P4 cells in early G1-phase by inhibiting the expression of cyclin D1 and the up regulation of p21 (Archer et al., 1998; Kress et al., 1986; Vaziri et al., 1998). The effect of the corresponding cell cycle arrests on the subcellular localization of the p12/CHRAC-17 complex is presented in Figure 24. Whereas the histone fold complex was primarily found in the nucleus when the cells are blocked in late G1-phase or S-phase (middle und lower panel), the sodium butyrate-induced block in G1-phase led to a dominant cytoplasmic localization of the

**FIG. 23: continued**

The overlap of the green EGFP and red RFP fusion proteins is shown in yellow (merge). For quantitative analysis, the fluorescence intensity of colocalized wild-type CHRAC-17 and mutated p12 was measured in 20 cells using the ImageJ software (NIH). The percentage of nuclear (nucl.) and cytoplasmic (cyto.) localization of the heterodimer was calculated. The stepwise mutation of basic amino acids in p12 increases the cytoplasmic localization of the p12/CHRAC-17 complex. wt, wild type; R, arginine; K, lysine; A, alanine.

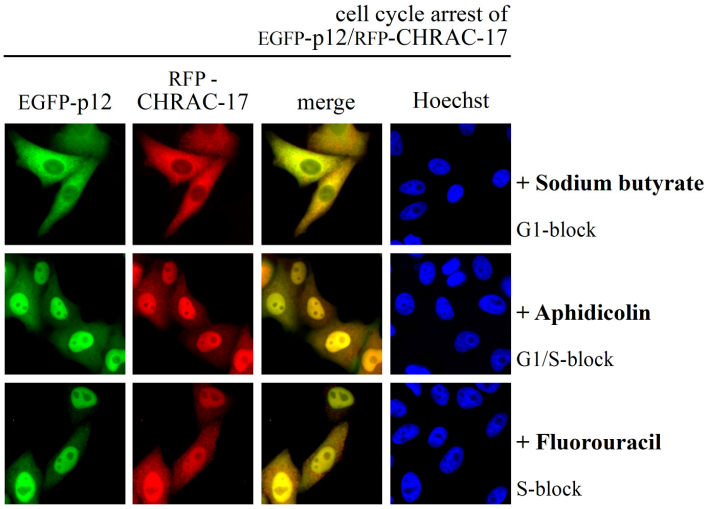


**FIG. 24: Subcellular localization of the p12/CHRAC-17 complex is cell cycle dependent.** HeLa P4 cells were transiently transfected with plasmid DNA encoding EGFP-p12, RFP-CHRAC-17, and flag-importin 13. Twenty-four hours post transfection the cells were treated with sodium butyrate or Aphidicolin for 24 hours or with Fluorouracil for 12 hours. The subcellular localization of the p12/CHRAC-17 heterodimer was displayed by direct fluorescence. The overlap of green EGFP-p12 and red RFP-CHRAC-17 is shown in yellow (merge). The DNA was counterstained with Hoechst. The drug induced cell cycle arrest is indicated. While p12/CHRAC-17 is localized in the cytoplasm in G1-phase the heterodimer accumulated in the nucleus in late G1/S-phase.

p12/CHRAC-17 complex (upper panel). The same results were obtained when HeLa cells instead of HeLa P4 cells were taken (data not shown). These preliminary data might in fact indicate that at least subunits of the polymerase ε, in this case the heterodimeric p12/CHRAC-17 complex, not only fail to bind to histones (Tackett et al., 2005), but perhaps even are exported from the nucleus in a cell cycle-dependent manner. Following this idea, we used a NES Finder software, provided by the Fornerod laboratory (http://research.nki.nl/fornerodlab/index.html), to search for a potential export signal. In doing so, a leucine-rich NES at the carboxy terminus of p12 ($^{103}$**I**EA**V**DE**F**A**F**$^{111}$) with the loose consensus (F/I/L/M/V)$X_2$(F/I/L/M/V)$X_2$(F/I/L) X [but not: F/I/L/M/V/W] (F/I/L/M/V) was identified.

Because of its carboxy-terminal position, the putative NES seems not to be involved in the dimerization with CHRAC-17. Hence, the p12/CHRAC-17 heterodimer could theoretically be exported from the nucleus via the putative NES in the p12 subunit. However, treatment with LMB had no effect on the subcellular distribution of the complex in transfected cells (see again Fig. 18 *C* and *D*). The putative nuclear export pathway therefore must be independent of exportin 1 and may occur via a different export receptor.

## 3.15 Is importin 13 the only nuclear transport receptor for the CHRAC-15/17 complex?

In the previous *in vitro* and *in vivo* experiments, we have clearly demonstrated that importin 13 mediates nuclear uptake of the two histone fold pairs, CHRAC-15/17 and p12/CHRAC-17. In addition, we excluded the importin α/β heterodimer, importin β, importin 5, importin 7, and importin 9 to play a role in the nuclear transport of these complexes via *in vitro* binding studies and *in vivo* cotransfection experiments in HeLa P4 cells. However, the question whether importin 13 represents the only nuclear transport factor, for the p12/CHRAC-17 and

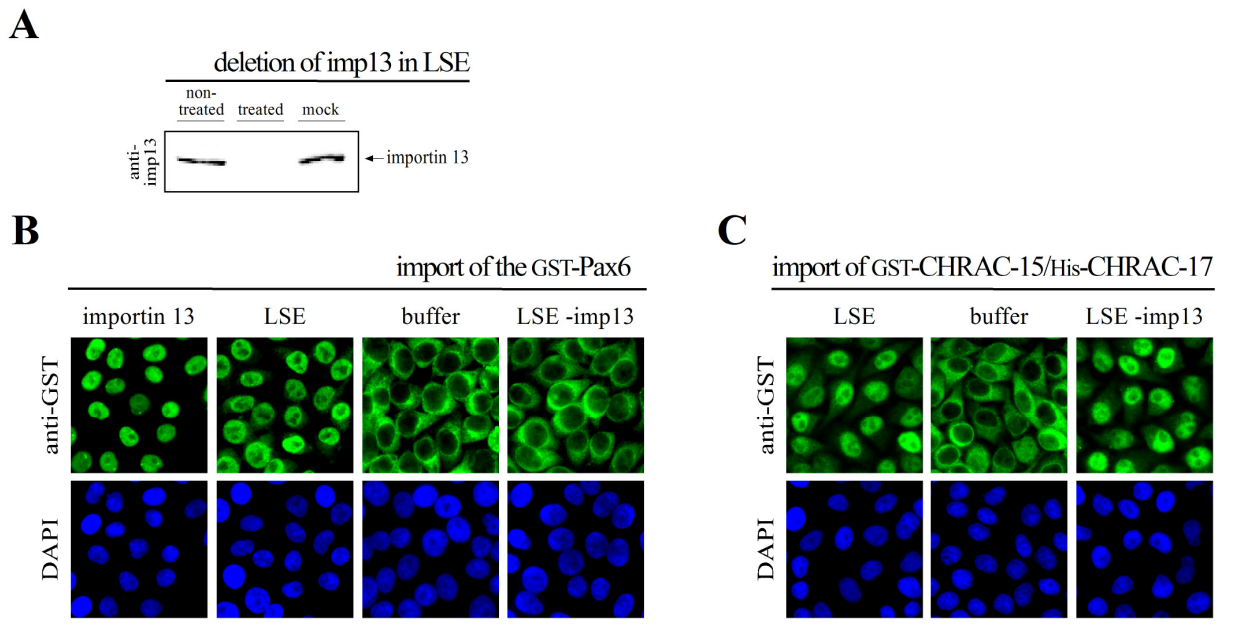


**FIG. 25: *In vitro* nuclear import of CHRAC-15/17 is not dependent on importin 13 alone.** *(A)* Low salt cell extract (LSE) from HeLa cells (left lane) were incubated with an anti-importin 13 antibody. After 4 hours at 4°C Gamma-Bind-Sepharose was added for 6 hours to precipitate the antibody-associated importin 13. To remove the nuclear transport receptor from the LSE, the matrix was spun down and the supernatant was analyzed by Western blotting, using the specific importin 13 antibody. Importin 13 was completely depleted from the LSE (middle lane in panel *A*). The importin 13 level of mock-treated LSE was not affected (right lane). *(B) In vitro* nuclear import assays with GST-Pax6 were performed. Whereas recombinant, purified importin 13 and LSE from HeLa cells facilitated nuclear uptake of GST-Pax6, no import into the nucleus of digitonin-permeabilized cells was detected in the presence of importin 13-deficient LSE (LSE -imp13). As a negative control, LSE was replaced by transport buffer (buffer). *(C)* Nuclear import assay with the GST-CHRAC-15/His-CHRAC-17 complex in the presence of LSE (left panel) and LSE -imp13 (right panel) were performed. Nuclear transport of the CHRAC-15/17 heterodimer was not affected upon importin 13 depletion. In the absence of nuclear transport receptors (buffer) the GST-CHRAC-15/His-CHRAC-17 complex remained in the cytoplasm of semi-permeabilized HeLa P4 cells and was not imported into the nucleus (middle panel). LSE, low salt extract.

CHRAC-15/17 complexes remained unanswered. Since both subunits of the NC2α/NC2β heterodimer contain a cNLS for nuclear import of the complex via the classical importin α/β import pathway, one cannot exclude the possibility that other, non-tested transport factors also mediate nuclear transport of p12/CHRAC-17 or CHRAC-15/17. To address this possibility, we performed additional *in vitro* nuclear import assays with the CHRAC-15/17 complex. In contrast to the previous experiments (see again Figure 6) in which recombinant, purified import receptors were used, we now applied low salt extract (LSE) from HeLa cells. LSE, as reticulocyte lysate, contains the complete cytoplasmic constituents, including importin 13 (Fig. 25*A*, left lane). To analyze alternative nuclear transport pathways independent of importin 13, the nuclear transport receptor was depleted form the LSE by immuno-precipitation with a specific anti-importin 13 antibody. After 4 hours incubation at 4°C, the antibody-antigen complex was precipitated with Gamma-Bind-Sepharose. To control the importin 13 depletion we performed Western blotting analysis (Fig. 25*A*). Using the same antibody as for the immuno-precipitation, importin 13 could not be detected (Fig. 25*A*, middle panel) whereas the amount of importin 13 in the mock-treated LSE remained unaffected. To test the importin 13-deleted LSE, we performed *in vitro* nuclear import assays with GST-tagged Pax6 which is a paired-type homeodomain transcription factor responsible for the control of development and differentiation (Gehring, 1987) and a known importin 13 cargo (Ploski et al., 2004). GST-tagged Pax6 was efficiently transported into the nucleus in the presence of recombinant importin 13 and LSE and remained strictly cytoplasmic in the absence of any transport receptor (Fig. 25*B*). In line with the published data, cytoplasmic retention of Pax6 was also observed when importin 13-deficient LSE (LSE -imp13) was used as source of importins.

Analogous to GST-tagged Pax6, the GST-CHRAC-15/His-CHRAC-17 complex was transported into the nucleus of semi-permeabilized HeLa P4 cells in the presence of LSE. In the absence of LSE (buffer), no import occurred resulting in a distinct cytoplasmic fluorescence, which showed again that the GST-CHRAC-15/His-CHRAC-17 complex does not passively diffuse into the nucleus (Figure 25*C*). In contrast to GST-tagged Pax6, the cytoplasmic retention of CHRAC-15/17 changed when importin 13-deficient LSE was used as source of nuclear transport receptors. Here, the heterodimeric CHRAC-15/17 complex was transported into the nucleus. This allows the conclusion that, nuclear import of the CHRAC-15/17 complex is not only facilitated by importin 13 but it is likely that an alternative nuclear transport pathway does exist.

# 4 Discussion

The compartmentalization of eukaryotic cells separates the sites of transcription and translation and necessitates the controlled nucleocytoplasmic exchange of molecules between the cytoplasm and the nucleus. The majority of nuclear transport processes is mediated by soluble nuclear transport receptors of the importin β family. Due to the variety of 20 different transport factors in human and the much larger list of putative cargoes it is a difficult task to identify the transport receptors for every given cargo. In fact, more than 17 % of all eukaryotic proteins are localized in the nucleus at some stages of their life (Cokol et al., 2000). The aim of this study was the identification of the transport factor/s, for the nuclear import of the heterodimeric CHRAC-15/17 and p12/CHRAC-17 complexes. The function of both complexes is indeed nuclear as shown by Poot et al. (2000) and Li et al. (2000). Poot and colleagues demonstrated that CHRAC-15/17 is associated with the chromatin remodelling complex CHRAC, composed of the ATPase SNF2H and the regulatory subunit ACF1 and responsible for efficient nucleosome sliding in highly condensed heterochromatin (Kukimoto et al., 2004). Li and colleagues showed that the p12/CHRAC-17 complex is an integral part of DNA polymerase ε. The CHRAC-15/17 and the p12/CHRAC-17 complex are related to each other, which is based on the presence of a H2A/H2B-like *histone fold motif* (HFM) as the main structural feature in the sequence of each subunit.

In this study, we have identified importin 13 as major import receptor for both complexes, CHRAC-15/17 and p12/CHRAC-17. These data allowed us to connect the nuclear import of CHRAC-15/17 and p12/CHRAC-17, with the import of the two related complexes NF-YB/NF-YC and NC2α/NC2β, which are also imported into the nucleus via importin 13.

## 4.1 Heterodimerization as a prerequisite for nuclear access

Importin 13 was identified by Mingot and colleagues (2001) as a member of the importin β-like transport receptor family mediating the import and surprisingly also the export of several different proteins. The human SUMO-conjugating enzyme UBC9 and the RNA-binding motif protein 8 (RBM8; also referred to as Y14) were identified among the import cargoes, but importin 13 also accounts for nuclear import of the paired-type homeodomain transcriptions factors Pax6, Pax3, and Crx (Ploski et al., 2004). As export cargo, the translation initiation factor eIF1A was identified (Mingot et al., 2001). These cargoes are all monomeric proteins. In addition to these data, work in our lab revealed that the importin 13 mediated nuclear import of

the H2A/H2B family member NF-YB/NF-YC depends on complex formation (Kahle et al., 2005). A similar behaviour has been recently shown for another histone fold pair of the H2A/H2B-type, namely NC2α/NC2β (Kahle et al, 2009). In this case, binding of the complex to importin 13 was also strictly dependent on subunit dimerization, thus showing the same binding properties as the NF-YB/NF-YC complex. With the new data from this work, we have expanded the spectrum of importin 13 recognized heterodimers of the H2A/H2B family by two novel heterodimeric cargoes, namely CHRAC-15/CHRAC-17 and p12/CHRAC-17.

Binding of both histone fold heterodimers to importin 13 was identified by performing *in vitro* GST-Pulldown assays with immobilized CHRAC-15/17 and p12/CHRAC-17, respectively. The specific loss of binding capacity between the nuclear transport factor importin 13 and the histone fold heterodimers in the presence of RanGTP pointed towards importin 13 as a specific nuclear import factor for CHRAC-15/17 and p12/CHRAC-17. Hence, within the cell importin 13 binds to CHRAC-15/17 or p12/CHRAC-17 at low RanGTP concentration in the cytoplasm. The import complex is then transferred into the nucleus and the dimeric cargo finally dissociates from importin 13 in the nucleus via RanGTP, which is present at high concentrations. The results from the GST-Pulldown assays also ruled out that importin 13 may function as an export factor as shown for eIF1A (Mingot et al., 2001). However, the fact that at least CHRAC-17 is recognized by importin 13 is not new. Mingot and colleagues (2001) already showed that CHRAC-17, referred to as NF-YB-like protein, binds to importin 13 in a RanGTP dependent manner. In the study by Mingot and colleagues, importin 13 was immobilized and a cytosolic HeLa cell extract was used to identify importin 13 interacting proteins. However, it remains unclear if CHRAC-17 was bound to importin 13 as a monomer or complexed with another protein, since only selected binding proteins were analyzed by mass spectrometry. However, our data concerning the monomeric subunits demonstrate that regardless of the absence or presence of RanGTP neither CHRAC-15 nor CHRAC-17 specifically binds to importin 13. Hence, only the CHRAC-15/17 heterodimer but not the monomeric components are recognized by importin 13.

This type of cooperativity between the individual histone fold subunits for nuclear import was also observed *in situ* for the nuclear accumulation of the p12 and CHRAC-17 homologues Dpb3 and Dpb4 in the fission yeast *Schizosaccharomyces pombe*. In that case, the repression of Dpb3 expression abrogated nuclear localization of Dpb4 (Spiga and D'Urso, 2004). These results support our data that the monomeric subunits are only imported in the nucleus when complexed with their histone fold partner.

In line with our interaction data, recombinant importin 13 was also able to mediate nuclear uptake of CHRAC-15/17 *in vitro*. In the *in vitro* import assays, described by Adam et al. (1990),

digitonin permeabilized cells were used to reconstitute nuclear import processes by substituting endogenous cytosolic components. The addition of importin 13 to the recombinant CHRAC-15/17 heterodimer led to a nearly 100 % nuclear localization of the histone fold pair. This nuclear localization was analogous to that of the endogenous CHRAC-15 and CHRAC-17 subunits. In addition, by using antibodies against the GST-tag fused to CHRAC-15 for detection, but the His-tag of CHRAC-17 for purification of the complex, we excluded the possibility of detecting monomeric subunits. We therefore conclude that the nuclear uptake of CHRAC-15/17 is energy-dependent and facilitated by importin 13.

The prerequisite of heterodimerization for nuclear uptake of the CHRAC-15/17 complex was further confirmed by the results of *in vivo* transfection experiments. The subcellular localization of monomeric CHRAC-15 and CHRAC-17 when fused to either RFP or EGFP-EGFP-GST (EEG) was strictly cytoplasmic. In contrast, EGFP-fused CHRAC-15 was localized homogeneously within the cell whereas EGFP-CHRAC-17 fusion protein was localized exclusively in the cytoplasm. The homogeneous localization of EGFP-CHRAC-15 may be explained by the tendency of EGFP to diffuse passively into the nucleus. Seibel et al. (2007) showed that even EGFP homohexamers diffuse into the nucleus of different mammalian cell lines. In addition to Seibel and coworkers, we show that RFP protein also tends to diffuse passively into the nucleus of HeLa P4 cells. However, these *in vivo* results implied that neither CHRAC-15 nor CHRAC-17 contain a NLS. Alternatively, the cytoplasmic retention of histone fold subunits could have also resulted from the masking of a NLS as shown by Wagstaff and Jans (2006). Therefore, we analyzed whether nuclear import of EEG alone or EEG fused to CHRAC-17 occurs in the presence of a non-classical or classical NLS. Thus, EEG was fused to the ribosomal protein L23a or was inserted between the bipartite cNLS of Nucleoplasmin and CHRAC-17.

The non-classical NLS in L23a, namely beta-like importin receptor binding (BIB) domain, consists of a highly basic stretch of 43 amino acids. It has been shown that L23a as well as the ribosomal proteins S7 and L5, are recognized by at least importin β, importin 5, importin 7, and transportin (Jäkel and Görlich, 1998). Fusions of the BIB alone (data not shown) or full length L23a to EEG therefore consequently result in a strict localization of the fusion proteins in the nucleoli, resembling the localization of ribosomal proteins. The NLS in Nucleoplasmin represents the prototype of a bipartite classical NLS with two basic clusters, separated by a stretch of 10-12 amino acids. Fusion of the bipartite cNLS between EEG and CHRAC-17 resulted also in a nuclear accumulation of the EEG-cNLS-CHRAC-17 fusion protein. Therefore, it was reasonable to assume that (i) neither the fluorescent EEG nor probably EGFP or RFP have

a significant influence on the interaction between a putative NLS in the monomeric CHRAC subunits and importin α/importin β or other transport receptors, (ii) both subunits, CHRAC-15 and CHRAC-17, do not possess a NLS for individual nuclear transport, and (iii) nuclear uptake therefore depends on the heterodimerization of the histone fold subunits.

## 4.2 Nuclear import of overexpressed HFM complexes

Based on the *in vitro* data one would have expected a nuclear accumulation of the CHRAC-15/17 complex *in vivo* mediated at least by importin 13. However, coexpression of CHRAC-15 and CHRAC-17 in HeLa P4 cells resulted in a strict cytoplasmic localization. Exchanging the EGFP- and the RFP-tag among the two subunits did not alter the cytoplasmic localization of the complex. As mentioned above, Wagstaff and Jans (2006) described the significant influence of flanking sequences and surrounding residues on the cargo-import factor interaction. In their study, intramolecular masking of the SV40 large-T antigen NLS by recombinant GFP was determined by a modified AlphaScreen-based assay. To assess the influence of flanking sequences, SV40 amino acids 111-135 containing the cNLS sequence $^{126}$P<u>KKKRK</u>V$^{132}$ were fused amino- and carboxy-terminally to GFP. The construct where GFP was carboxy-terminally fused to the SV40 residues ($^{111}$SV40$^{135}$-GFP) was recognized by the importin α/importin β dimer with high affinity ($K_d$ of 6.3 nM) whereas the GFP-$^{111}$SV40$^{135}$ fusion construct showed extremely weak binding with only 2 % of wild type SV40. This value was however equivalent to the binding between importin α/importin β dimer and GFP alone and proved the striking interference of fused protein moieties like fluorescent tags with binding interactions. Based on these data and on the fact that CHRAC-15 and CHRAC 17, either fused to RFP or EGFF-EGFP-GST, were still capable to bind *in vivo* to each other, the position of the EGFP- and RFP-tag was changed from the amino- to the carboxy-terminus of CHRAC-15 and CHRAC-17. However, the cytoplasmic colocalization of CHRAC-15-EGFP/CHRAC-17-RFP did not change in transfected HeLa P4 cells.

In addition, the same localization pattern was observed for the p12/CHRAC-17 complex regardless of amino- or carboxy-terminal fusion of EGFP and RFP. Considering of the nuclear import of the CHRAC-15/17 complex *in vitro* and the nuclear localization of the endogenous subunits, these *in vivo* results were quite surprising. In particular, this was unexpected, since the interaction *in vivo*, the colocalization of the HFM-containing subunits in the transfection experiments, and the purification of recombinant histone fold pairs showed that heterodimerization of the subunits was not affected by the tags. After confirming that neither nuclear export via exportin 1 nor inaccurate folding due to strong overexpression accounts for

the cytoplasmic retention, we analyzed the role of ACF1 as a potential carrier of CHRAC-15/17 via a piggyback mechanism This idea was based on the fact that CHRAC-15, when dimerized with CHRAC-17, is assembled into CHRAC via interaction with ACF1 (Collins et al., 2002). Thus, CHRAC-15 plays an important role in the maintenance of the structural integrity of the chromatin accessibility complex. We wanted to test whether this interaction already occurs in the cytoplasm by additional coexpression of ACF1. However, irrespective of the tag arrangement, overexpression of ACF1 did not change the cytoplasmic localization of the CHRAC-15/17 heterodimer and is therefore presumably not responsible for the nuclear import of the CHRAC-15/17 complex.

In contrast, the CHRAC-15/17 complex localized exclusively in the nucleus upon additional coexpression of flag-tagged importin 13. The same behavior was observed for the p12/CHRAC-17 complex and in both cases no other additionally coexpressed transport receptor (importin β, importin 5, importin 7, and importin 9) facilitated the nuclear import of the histone fold pairs in transfected cells. These results agreed with our *in vivo* results showing that importin 13 bound to both HFM complexes in a RanGTP dependent manner and facilitated nuclear uptake in permeabilized cells. The question however remained why endogenous importin 13 was not capable to translocate CHRAC-15/17 and p12/CHRAC-17 into the nucleus of transfected cells. Since the nuclear transport efficiency varies with the respective conditions, one possible answer is that the endogenous amount of importin 13 in HeLa cells does not suffice for import of the strongly overexpressed histone fold complexes. In this context, using a single-molecule fluorescence microscopy approach Yang and Musser (2006) have recently shown that the maximal import rate of a signal-dependent cargo can be increased at least 10-fold by the concentration of importin β. A NLS-2xGFP construct showed an increased import efficiency as well as an increased transit speed across the nuclear envelope by increasing importin β concentrations (Yang and Musser, 2006). Hence, the import rate seems to be rather dependent on the ‘cooperativity’ of the transport receptors, the cargo molecules and the involved cofactors than on simple Michaelis-Menten saturation kinetics (Ribbeck and Görlich, 2001; Yang et al., 2004). Based on these data, we assume that increasing the cellular concentration of importin 13 in HeLa P4 cells through additional coexpression therefore most likely results in an enhanced nuclear import of the histone fold pairs CHRAC-15/17 and p12/CHRAC-17. In addition, experiments in our group concerning the nuclear import of the related histone fold pair NC2α/NC2β confirmed the necessity of additional importin 13 expression for nuclear uptake (Kahle et al., 2009).

In a different approach, Liang and colleagues (Liang et al., 2008) showed that the overexpression of amino-terminally deleted importin 13 (amino acids 489-963) blocked the nuclear uptake of the importin 13 cargo myopodin. Obviously, the competition between endogenous wild-type and truncated overexpressed importin 13 led to the loss of nuclear localization of myopodin. This might indicate that due to a comparatively low concentration of importin 13 in the cells the additional overexpression of a putative dominant-negative mutant of importin 13 has a stronger influence on nuclear import than endogenous importin 13.

Moreover, a low concentration of endogenous importin 13 has been indeed observed in HeLa cells (Thomas Güttler, personal communication). According to these data, the concentration of importin 13 in HeLa cells is, relatively low in comparison to importin β, importin 5, and importin 9. Data for importin 7 were not available. The differences in the endogenous protein levels of the nuclear transport factors in HeLa cells might also explain the slightly higher expression of flag-tagged importin 13 compared to flag-tagged importin β, importin 5, importin 7, and importin 9. A relatively low concentration of endogenous importin 13 might favor a stronger expression of exogenous importin 13 upon transfection.

## 4.3 The role of importin 5 in the nuclear uptake of the CHRAC-15/17 complex

Besides importin 13, recombinant importin 5 was capable of mediating nuclear accumulation of the CHRAC-15/17 heterodimer in semi-permeabilized cells. In contrast, neither binding of importin 5 to the CHRAC-15/17 complex nor to the monomeric subunits was detected. In addition, the overexpression of importin 5 did also not lead to nuclear accumulation of the CHRAC-15/17 dimer *in vivo*. However, importin 5 was properly expressed in HeLa P4 cells and it also was functional as demonstrated by its facilitating the nuclear accumulation of flag- and EGFP-tagged PGC7/Stella. In respect to this nuclear import of the fluorescently tagged PGC7/Stella, importin 5 was not only responsible for an increased nuclear accumulation of the NLS- and NES-containing protein (Nakamura et al., 2007), but in addition it facilitated the proper folding of the EGFP-PGC7/Stella fusion protein. This was concluded from the loss of intracellular aggregation of EGFP-PGC7/Stella upon coexpression of the flag-tagged importin 5. Hence, importin 5 like other transport factors (Jäkel et al., 2002) functions as a chaperone. Despite its functionality, coexpressed flag-tagged importin 5 did not show any capacity to mediate nuclear import of the two histone fold pairs CHRAC-15/17 and p12/CHRAC-17 *in vivo*.

Therefore, importin 5 does not seem to represent a *bona fide* import factor for CHRAC-15/17 and p12/CHRAC-17.

In addition, RNA interference with siRNA against importin 5, resulting in a depletion of 90 % after 7 days, had also no effect on the nuclear localization of endogenous CHRAC-17. Once again, we found no evidence for an involvement or a functional significance of importin 5 in the nuclear uptake of the CHRAC-15/17 complex. The reason for the import capacity of importin 5 in permeabilized cells, however, remains unexplained. In particular, this is unclear, since the same batch of recombinant purified importin 5 was used for both experiments, the *in vitro* import assay and the *in vitro* binding studies. In the latter experiment, no interaction between importin 5 and CHRAC-15/17 was detected. Unfortunately, the available quantity of importin 5 antibody was not high enough to deplete importin 5 from HeLa cell extract (LSE) and to subsequently test the import capacity of this importin 5-depleted LSE for the CHRAC-15/17 complex in semi-permeabilized cells. This experiment would perhaps solve the question whether importin 5 is involved in the import of the CHRAC-15/17 complex.

## 4.4 Importin 13 and the binding to histone fold motifs

In this study, we have convincingly shown that importin 13 mediates nuclear import of the heterodimeric CHRAC-15/17 and p12/CHRAC-17 complexes. Additional cotransfection experiments then demonstrated that importin 13-mediated nuclear uptake of CHRAC-15/17 was reduced upon deletion of HEAT repeat 1 or HEAT 16-19 of importin 13. Since the nuclear distribution of these truncated importin 13 proteins was not changed relative to full length importin 13, we assume that the heterodimeric CHRAC-15/17 complex requires a relative broad interaction surface in importin 13. In addition to these two histone fold pairs, CHRAC-15/17 and p12/CHRAC-17, it was shown that the related H2A/H2B-like complexes NF-YB/NF-YC (Kahle et al., 2005) and NC2α/NC2β (Kahle et al., 2009) are also imported by importin 13.

The calculated electrostatic surface potentials of H2A/H2B (Luger et al., 1997a), NC2α/NC2β (Kamada et al., 2001), NF-YB/NF-YC (Romier et al., 2003), and CHRAC-16/14, the CHRAC-15/17 homolog from *Drosophila melanogaster*, (Hartlepp et al., 2005) are rather similar and the surfaces are highly basic. This basicity allows low affinity binding to the negatively charged backbone of the DNA, a feature that has also been shown for CHRAC-15/17, CHRAC-16/14 and p12/CHRAC-17 (Hartlepp et al., 2005; Kukimoto et al., 2004; Poot et al., 2000). On the other hand, it would also favor the interaction to negatively charged residues in the inner surface of importin 13. Because the HFM represents the common structural feature in the subunits of the different heterodimeric complexes (Kamada et al., 2001; Li et al., 2000; Poot et

al., 2000; Romier et al., 2003), we aligned the sequences of the related subunits and looked for conserved basic amino acid residues. As an evolutionary fact, DNA binding regions frequently overlap with NLSs (Cokol et al., 2000; LaCasse and Lefebvre, 1995). Based on the structural informations of the two complexes, NF-YB/NF-YC and NC2α/NC2β (Kamada et al., 2001; Romier et al., 2003) we identified conserved, basic amino acids that are responsible for DNA binding, but not involved in the heterodimerization of the individual subunits. These arginine and lysine residues were substituted for alanine. Purification of the recombinant complexes proved that heterodimerization of at least CHRAC-15/17 and p12/CHRAC-17 was not affected by the mutations. Hence, the observed reduced nuclear import of the histone fold complexes is based on the loss of basic amino acids residues and not attenuated by the disruption of the heterodimerization between the two subunits which would in fact preclude effective import.

In summary, increasing stepwise mutation of conserved basic amino acid residues in the subunits, concomitantly reduced binding to importin 13 *in vitro* as well as nuclear import of the heterodimeric complexes CHRAC-15/17, NC2α/NC2β, and p12/CHRAC-17 *in vivo*. We therefore propose that each histone fold motif containing heterodimer provides a conserved binding platform for the recognition of importin 13. These positively charged platforms are formed by basic amino acids distributed among the subunits and assemble upon the heterodimerization of the monomeric HFM subunits. The loss of positively charged amino acids in the histone fold pairs leads consequently to a decreased binding capacity for importin 13. This mechanism would also explain why only heterodimeric CHRAC-15/17 and p12/CHRAC-17 complexes, but not the monomeric subunits are recognized and imported by importin 13. Interestingly, for other ncNLS such as the beta-like importin receptor binding (BIB) domain of the ribosomal protein L23a, an extremely basic character can be observed as well (Jäkel and Görlich, 1998). However, basicity is not a general prerequisite for ncNLS (Rosenblum et al., 1998).

Evidence for the existence of a region with a positive electrostatic potential is also given by the three-dimensional arrangement of the basic residues in the heterodimeric complexes. Because of the general head-to-tail binding of histone fold motifs, resulting in the typical handshake arrangement (Arents et al., 1991), all essential basic amino acid residues are clustered on one side of the heterodimer in close proximity to each other (Fig. 26). The only exception, however, is the lysine residue at position 70 (K70) in CHRAC-15, corresponding to K93 in p12 and K63 in NC2α. Again, we suggest that these positively charged residues in the heterodimerized histone fold subunits form a region of positive electrostatic potential that permits favorable interactions with negatively charged amino acid residues of importin 13. A similar recognition mode between

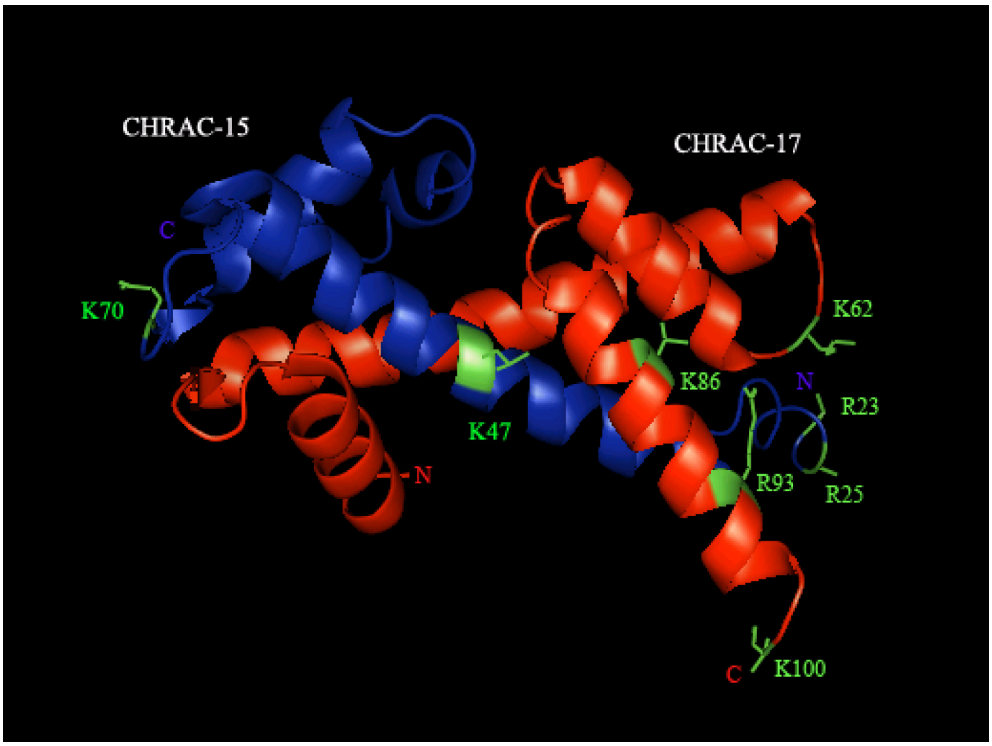


**FIG. 26: Three dimensional structure of the CHRAC-16/14 heterodimer.** Ribbon representation of the histone fold motif containing subunits CHRAC-16 and CHRAC-14 bound to each other head to tail. The crystal structure of the CHRAC-16/14 complex from *Drosophila melanogaster* solved by Hartlepp and colleagues (2005) was adapted for the human homolog CHRAC-15/17. CHRAC-15 and CHRAC-17 are depicted in blue and red, respectively. The basic amino acids selected for successive mutation (R25A, K70A, K47A, and R23A in CHRAC-15 and K100A, R93A, K62A, and K86A in CHRAC-17) are shown in green. All basic amino acids except for the lysine residue at position 70 (K70) in CHRAC-15 are clustered at one site of the heterodimeric complex in close proximity to each other forming a region of positive electrostatic potential. The figure was produced with the software PyMOL.

importin 13 and its cargo, the paired-type homeodomain transcription factor Pax6 was proposed by Ploski et al. (2004). There, the characteristic homeodomain is composed of a helix-turn-helix segment comprised of three α helices (amino acids 218-261), whereas both ends of the homeodomain are flanked by a family-specific basic cluster, which is essential for nuclear uptake. Based on the three-dimensional structure of Pax6 (Wilson et al., 1995) these basic clusters, $^{206}$LKRKLQR$^{212}$ and $^{261}$RRAKWR$^{266}$, are much closer in space than expected from the primary amino acid sequence, namely approximately 8 Å. Analogous to our suggestion for the recognition of the HFM heterodimers by importin 13, Ploski et al. (2004) proposed that the three-dimensional composition of the homeodomain has a structural role of bringing the positively charged regions together and exposing the two basic clusters in a proper position and orientation for the contact with importin 13.

## 4.5 Does a general importin 13 binding motif exist?

In modern, computer-based structural analysis, great efforts are made to analyze specific recognition motifs mediating the interaction between different proteins. This is not much different in the field of nucleocytoplasmic transport and the analysis of binding between transport factors and cargo proteins. Whereas cNLSs show loosely related consensus sequences, the prediction of ncNLS is more difficult, and the calculation of a consensus sequence is virtually impossible (Christophe et al., 2000). However, a recent study by Lee et al. (2006) has established different principles for the recognition of a NLS by the nuclear import factor transportin. Their work led to the identification of the basic 'PY-NLS' with the consensus sequence (R/H/K)$X_{2\text{-}5}$PY. According to these data, the PY-NLS is defined as a stretch of basic and hydrophobic residues φ(G/A/S)φφ; with φ representing hydrophobic and basic residues, followed by the PY consensus dipeptide. In contrast, a consensus motif for importin 13 cargoes has not been defined. In addition, the aforementioned basic clusters $^{206}$L<u>KRK</u>LQ<u>R</u>$^{212}$ and $^{261}$<u>RR</u>A<u>K</u>W<u>R</u>$^{266}$ detected in the paired-type homeodomain transcription factor family and responsible for the nuclear uptake (Ploski et al., 2004) can be found neither in the importin 13 cargoes UBC9 and RBM8 (Mingot et al., 2001) nor in the histone fold subunits analyzed in this study.

However, sequence comparison between the subunits of four different histone fold pairs showed striking similarities in positively charged residues. For three of the four histone fold complexes, we have demonstrated that these basic amino acid residues are essential for nuclear import. This raises the question, whether the conserved arrangement of positively charged residues is adopted among other species. A sequence alignment of the human homologues of CHRAC-15 and CHRAC-17 from *Mus musculus*, *Rattus norvegicus*, *Xenopus laevis*, and *Bos taurus* in fact supports this idea (Fig. 27). Hence, it is reasonable to assume that nuclear import of the homologous CHRAC-15/17 complexes in these other species is mediated by a putative importin 13 orthologue, which also depends on basic amino acids distributed among the histone fold subunits as in human. Most notably, importin 13 is occasionally highly conserved in these species, with protein identities ranging from 99 % (mouse and rat) to 83 % (*Xenopus*). In the same manner, Mingot et al. (2001) reported that putative orthologues of importin 13 are found in representative branches of eukaryotes, such as plant, insects, nematodes, and fungi and therefore suggests that importin 13 has evolved quite early in eukaryotic phylogenesis.

In contrast to the high conservation of importin 13 mentioned above, the putative importin 13 homologue in *Drosophila melanogaster* (AAF55502) shows only an identity of 49 %. As a possible result, only three of the four basic amino acids essential for nuclear uptake via

importin 13 are conserved in CHRAC-16 and CHRAC-14, the homologues of the human CHRAC-15 and CHRAC-17 subunits (Fig. 27). However, by disregarding the initially chosen requirements, like DNA-binding and non-involvement in heterodimerization, for the selection and subsequent mutation of basic amino acid residues, we could identify at least three other conserved basic amino acids in the CHRAC-15 homologue and 12 in the CHRAC-17 homologue. Whether these basic amino acids are crucial for binding of importin 13 to the CHRAC heterodimer, is unknown. However, these residues presumably increase the potential of forming an adequate basic environment, which is necessary for electrostatic interactions with acidic amino acid residues at the inner surface of importin 13.

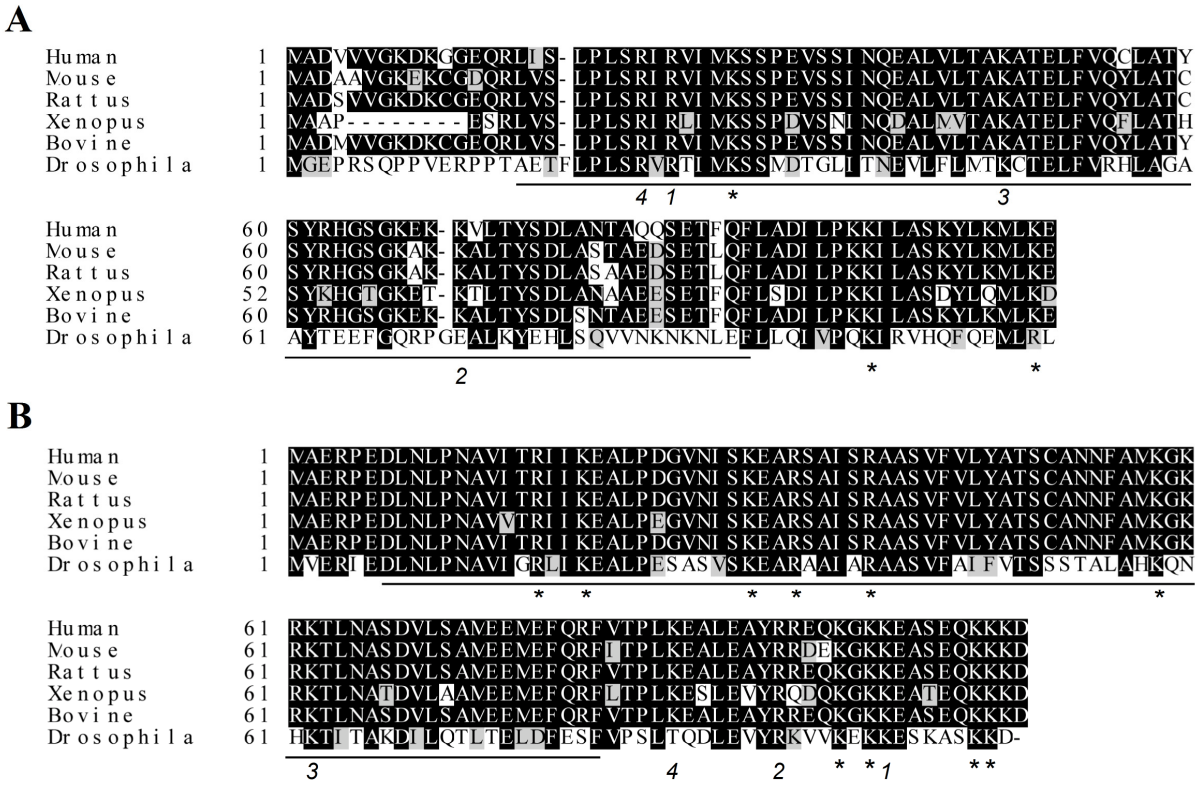


**FIG. 27: Partial sequence alignment of CHRAC-15 and CHRAC-17.** *(A)* and *(B)* Sequence alignment of CHRAC-15 *(A)* and CHRAC-17 *(B)* from human, *Mus musculus*, *Rattus norvegicus*, *Xenopus laevis*, *Bos taurus*, and *Drosophila melanogaster*. Identical amino acids are indicated by a black background, whereas identical residues in all sequences are shaded in grey. The histone fold motifs are indicated by a black bar underneath the sequences and were taken from Poot et al. (2000). The substituted lysine and arginine residues in the human subunits (compare with Fig. 19) are indicated by numbers also representing the order of consecutive mutation. Additionally conserved basic residues between the species are indicated by an asterisk (*).

## 4.6 Same protein family, but different import pathways

Regarding the fact that CHRAC-15/17, p12/CHRAC-17, NC2α/NC2β, and NF-YB/NF-YC belong to the H2A/H2B family, with sequence similarities of 31 % and 27 % for CHRAC-15 and p12 to H2A, respectively, and 25 % for CHRAC-17 to H2B (for comparison, NF-YB is 30 % identical to H2B and NF-YC is 21 % identical to H2A), the question for similarities in the nuclear transport arises. Import studies have shown that histones are transported into the nucleus in a transport receptor- and energy-dependent manner by several redundant pathways. Theoretically, their size would allow passive diffusion of monomeric histones as well as of heterodimeric H2A/H2B and H3/H4 complexes (Breeuwer and Goldfarb, 1990; Kurz et al., 1997; Schwamborn et al., 1998). However, at least five different members of the importin β superfamily, namely importin 5, importin 7, importin 9, transportin, and importin β mediate the nuclear import of the core histones (Baake et al., 2001a; Mühlhäusser et al., 2001). In addition, Mühlhäusser and colleagues reported that the binding of H2A and H2B to the import receptors as monomeric subunits was as efficient as the binding of the dimeric H2A/H2B complex (Mühlhäusser et al., 2001).

Importin 13 in contrast, has presumably no affinity to the H2A/H2B complex (Kahle, unpublished observations). Thus, the nuclear import of the H2A/H2B heterodimer is different from that of the histone fold pairs CHRAC-15/17, p12/CHRAC-17, NC2α/NC2β, and NF-YB/NF-YC. Sequence comparison of the H2A- and H2B-family members reveals that except for one amino acid residue (R23 in CHRAC-15), the positively charged amino acids essential for nuclear uptake and conserved among the histone fold pairs CHRAC-15/17, p12/CHRAC-17, NC2α/NC2β, and NF-YB/NF-YC are not conserved in the eponymous H2A and H2B subunits (Fig. 28). Lacking these positively charged amino acids, the H2A/H2B complex does not contain a *bona fide* importin 13 binding site and is consequently not imported into the nucleus by importin 13, but via alternatives. Concerning the nuclear import of the H2A and H2B, it has been shown that the HFM-containing central globular domain represents a NLS that entirely depends on the dimerization of H2A and H2B. In addition, the basic amino-terminal tail of the two core histones contains a second functional NLS, essential for binding of monomeric H2A and H2B to the nuclear import receptors (Baake et al., 2001b).

Interestingly, in the three dimensional structure of the growth factor activated protein Son of Sevenless (Sos) a intramolecular histone pseudodimer was identified (Sondermann et al., 2003). This pseudodimer comprises two tandem histone fold motifs and can be superimposed with the histone fold motif of heterodimerized H2A/H2B complex. Sos, which is analogous to the H2A/H2B complex, lacks the conserved basic amino acids residues necessary for importin 13

mediated nuclear import and any other NLS, and thus it appears logical that Sos localizes in the cytoplasm. Upon activation, Sos is, however, relocated from the cytoplasm to the plasma membrane where it stimulates the rapid conversion of the small GTPases Ras from its inactive GDP-bound form into the active GTP-bound form (Schlesinger et al., 1999). Due to the absence of a NLS, Sos is the only protein with sequence similarity to histones that is neither nuclear nor involved in DNA or RNA binding.

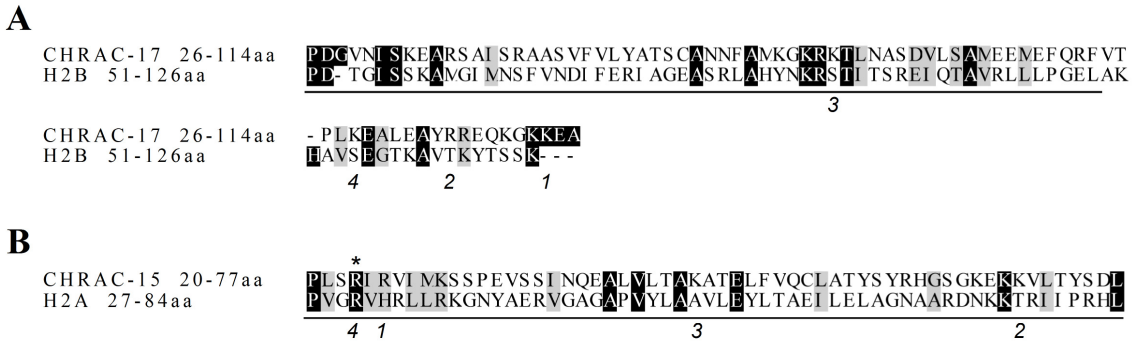


**FIG. 28: Sequence alignment of H2A and H2B with the related CHRAC subunits.** *(A)* and *(B)* Partial sequence alignment of CHRAC-15 with H2A *(A)* and CHRAC-17 with H2B *(B)*. Identical amino acids are indicated by a black background. Similar amino acids are additionally shaded in grey. The histone fold motifs are indicated by a bar underneath the alignment. Except for R23 in CHRAC-15 (*), the progressively mutated basic amino acids in CHRAC-15 and CHRAC-17, represented by numbers (*1-4*), are not conserved in H2A and H2B.

## 4.7 Importin α/β-mediated nuclear import of CHRAC-17?

Proteins containing a classical NLS (cNLS) interact with importin α and are transported into the nucleus after assembling with importin β into a trimeric import complex (Conti et al., 1998; Görlich and Mattaj, 1996; Nigg, 1997). The cNLSs resemble either the monopartite type of SV40 large T antigen or the bipartite type of nucleoplasmin (Dingwall and Laskey, 1998; Kalderon et al., 1984; Robbins et al., 1991). Initially, monopartite cNLSs were described as short sequences enriched in basic amino acids (Dingwall and Laskey, 1991; Robbins et al., 1991) following the loose consensus K(K/R)X(K/R) where X represents any amino acid (Chelsky et al., 1989). This view of the monopartite cNLS was meanwhile expanded, now also implying the amino acids that surround the cNLS consensus. According to this concept, small hydrophobic amino acids followed by an acidic residue downstream of the consensus and amino acids compatible with sharp turns like proline or glycine upstream of the consensus, are preferred (Conti, 2002).

As mentioned before, cNLSs in both histone fold motif containing subunits NC2α ($^{4}$KKKK$^{7}$) and NC2β ($^{100}$KRRK$^{103}$) were identified (Kahle et al., 2009). According to Kahle et al, the CHRAC-17 related NC2β subunit showed a striking nuclear localization upon transfection in cultured cells and a RanGTP-sensitive binding to the importin α/β heterodimer.

Since NC2β is related to CHRAC-17 we analyzed its sequence and identified a basic stretch in the unstructured, charged carboxy-terminus of CHRAC-17. This carboxy-terminal 'tail' is responsible for modulating the binding between the DNA and CHRAC and is, like the charged 'tail' of CHRAC-15, needed to enhance the nucleosome sliding process of CHRAC (Kukimoto et al., 2004). In line with the loose consensus for monopartite cNLS K(K/R)X(K/R) we identified the sequence motif $^{106}$KKDK$^{109}$ in a highly basic stretch in CHRAC-17. Furthermore, in accordance with the additional requirements for a monopartite cNLS, an arginine followed by the acidic glutamate residue was located downstream of the KKDK-motif, but in contrast to the suggestion of Conti (2002), no upstream proline or glycine was present. After all, these results might point towards an involvement of the importin α/β dimer in the nuclear uptake of the CHRAC-17 subunit. However, in our study we found no evidence for an individual import of monomeric CHRAC-17, which excludes the existence of a cNLS. As a matter of fact, Glaser et al. (1995) identified a cNLS in the sequence of the importin 13 cargo Pax6, whereas Ploski and colleagues showed that this homeodomain transcription factor is not imported in an importin α/β dependent manner and that the basic element did not represent an independent and redundant NLS (Ploski et al., 2004). It therefore seems that besides the strict definition of a consensus motif different elements act in a cooperative manner, probably in connection with other residues, to form a protein context that contributes to the recognition between a given cargo and importin α (Friedrich et al., 2006). On the other hand, some cargoes like the transcription factors STAT 1 and STAT2, influenza A virus nucleoprotein (Melen et al., 2003), and phospholipid scramblase 1 (Chen et al., 2005) even bind to importin α via sequence motifs different form the classical consensus-like type of NLSs.

Similar to CHRAC-17, we additionally screened the sequence of the homologous protein Dpb4 from *Saccharomyces cerevisiae*. In Dpb4, with a sequence identity to CHRAC-17 of 28 %, three of the four basic amino acids mutated in this study are present and five other basic residues are additionally conserved between the yeast protein and its human homologue. Based on the cNLS consensus, the sequence of Dpb4 revealed two highly basic stretches, $^{125}$RKKEK$^{129}$ and $^{177}$KKQK$^{180}$. These two potential cNLSs are both located at the carboxy-terminal end of the HFM and, additional sequence requirement for a cNLS are fulfilled via the existence of hydrophobic and acidic residues downstream of the consensus. Whether the Dpb4 subunit is

imported by the Kap60/95 heterodimer, the importin α/β homologues in yeast, or in complex with Dls1 or Dpb3, the human CHRAC-15 or p12 homologues, respectively, has to be addressed in further studies. Since *Saccharomyces cerevisiae* has no clear importin 13 orthologue and neither Dls1 nor Dpb3 exhibit a putative cNLS, the importin α/β pathway would entirely depend on Dpb4. Previous work by Liku and colleagues (2005) led to the identification of two cNLSs in Mcm2 ($^{5}$RRRRR$^{9}$) and Mcm3 ($^{766}$PKKKRQRV$^{772}$), both necessary for nuclear accumulation of the heterohexameric Mcm2-7 helicase complex in budding yeast. In addition to the Mcm2-7 complex, many other multiprotein complexes were identified, in which a single subunit provides the import signal (NLS) responsible for nuclear uptake of the entire complex (Leslie et al., 2004; Maridor et al., 1993; Pereira et al., 1998; Subramaniam and Johnson, 2004; Wendler et al., 2004). Thus, importin α/β mediated nuclear import of complexed subunits does not require import signals on every subunit and the Dpb4 subunit might be responsible for Kap60/95 mediated nuclear import of the Dls1/Dpb4 and Dbp3/Dbp4 complexes in yeast.

Concerning importin 13, the most similar protein from *Saccharomyces cerevisiae* is Kap122/Pdr6 (AAB19613) with a sequence similarity of only 34 % compared with the human importin 13. This is too low to regard it as a homologous protein. Titov and Blobel identified Pdr6, since then in agreement with the proposed nomenclature referred to as Kap122, as a member of the karyopherin β-family and responsible for nuclear import of the heterodimeric transcription factor IIA (Toa1/Toa2) (Titov and Blobel, 1999). In addition, Kap122 also mediates nuclear import of the heterodimeric R2 ββ' complex of ribonucleotide reductase (Zhang et al., 2006). Although Kap122 might not represent a clear homologue of the human importin 13, analogies in mediating nuclear import of heterodimeric complexes are nevertheless obvious. However, whether Kap122 also mediates nuclear export as shown for human importin 13 (Mingot et al., 2001) has not yet been shown.

## 4.8 Phosphorylation and nuclear export of p12/CHRAC-17

The heterodimeric complex p12/CHRAC-17 is part of the DNA polymerase ε. Polymerase ε itself serves as the leading strand synthesizing enzyme in the chromosomal DNA replication fork and thus it is part of the eukaryotic replisome (Fukui et al., 2004; Pursell et al., 2007). Based on data from Tackett et al. (2005) showing that the yeast polymerase ε was only associated with chromatin during the replication process, we analyzed the cell-cycle dependent nuclear accumulation of p12/CHRAC-17. The complex was localized in the nucleus in S-phase and during the transition of the G1-S border, whereas the p12/CHRAC-17 complex localized nearly completely in the cytoplasm when HeLa P4 cells were blocked in early G1-phase. Consistent

with this data, *in silico* analysis (http://research.nki.nl/fornerodlab/index.html) identified a putative leucine-rich NES at the carboxy-terminus of the p12 ($^{102}$AIEAVDEFAF$^{111}$) subunit, which could be responsible for the cytoplasmic accumulation of the complex. Since CHRAC-17 and p12 protein still colocalized in the cytoplasm after the treatment with sodium butyrate (inducing an arrest in G1-phase), the heterodimeric p12/CHRAC-17 complex rather than the monomeric p12 subunit seems to be exported from the nucleus. In contrast to p12 no putative leucine-rich NES was found in the CHRAC-17 subunit.

One example for a complex, whose nuclear exported is dependent on the presence of only one subunit, is the heterohexameric Mcm2-7 complex (for review, see Costa and Onesti, 2008). In this case, it has been shown that the single NES in Mcm3 is assigned for nuclear export of the whole complex (Liku et al., 2005). Hence, nuclear export of the p12/CHRAC-17 complex has to occur in a p12-dependent manner. But how could such a export mechanism be controlled? In this respect, Jans et al. (2000) have shown that transport mechanisms can directly be controlled by phosphorylation, which can either increase or decrease the affinity to a transport factor through a modification of the targeting sequences. Since Gnad and colleagues (2007) have identified a phosphorylation site in p12 at threonine 11 ($^{9}$SG**T**PREE$^{15}$), it is possible that the nuclear export of p12/CHRAC-17 is indeed regulated similar to the exportin 1-dependent nuclear export of the Mcm2-7 complex (Liku et al., 2005). There, transport out of the nucleus is regulated in a cell-cycle dependent manner by phosphorylation of Mcm3 through the yeast cyclin dependent kinase Cdc28 (Labib et al., 1999; Nguyen et al., 2000). In contrast to these results, our data excluded the involvement of exportin 1 and importin 13 in the nuclear export of the p12/CHRAC-17 complex. Nevertheless, a cell cycle regulated export mechanism that is independent of exportin 1 and importin 13 is possible in principle since (i) the serine/threonine kinases CK2 and CDK1, the human homolog of the yeast Cdc28, are responsible for phosphorylation of p12 (Gnad et al., 2007) at the respective recognition motifs **S**/**T**-X-X-E and **S**/**T**-P-K/R and (ii) these kinases have distinct roles in the progression of the cell cycle (Ahmad et al., 2008; Faust and Montenarh, 2000; Unger et al., 2004).

However, if one considers the NES in p12 as the sequence motif responsible for nuclear export, the phosphorylation at threonine 11 may modulate the transport activities towards a down-regulation of the NLS activity and the simultaneous up-regulation of the NES activity as it has been shown for the transport of the transcriptional activator Pho4 (Komeili and O'Shea, 1999). In the context of the polymerase ε complex, phosphorylation of p12 could also induce conformational changes that modify the accessibility as well as the activity of other transport

signals in the complex, even more since CHRAC-17 harbors another phosphorylation site at S122 which is also putatively phosphorylated by CK2 (Gnad et al., 2007).

## 4.9 CHRAC-17-containing complexes show equal localization in the nucleus

DNA polymerase ε and the chromatin accessibility complex CHRAC are localized in the nucleus at steady state. More specifically, it was shown that both complexes localize at late-replicating heterochromatin where CHRAC is required for efficient DNA replication (Collins et al., 2002; Fuss and Linn, 2002). In this study, we have shown that nuclear import of the CHRAC-17 containing complexes CHRAC-15/17 and p12/CHRAC-17, subunits of CHRAC and the polymerase ε, respectively, is mediated by the nuclear transport receptor importin 13. Thereby, importin 13 recognizes a specific basic binding platform formed by heterodimerization of the histone fold subunits. Moreover, we showed that this binding platform is conserved among different histone fold pairs of the H2A/H2B family but not in the heterodimeric H2A/H2B complex itself.

Recent data from *Saccharomyces cerevisiae* has revealed that the yeast homologues of the human CHRAC and DNA polymerase ε, yCHRAC and the yeast polymerase ε subunits Pol2, Dpb2, Dpb3, and Dpb4 are located at specialized boundary regions of silent chromatin (Iida and Araki, 2004; Tackett et al., 2005). These boundary regions are located between regions of transcriptionally accessible ‘active’ and inaccessible ‘silent’ chromatin and prevent the intrusion of either region into the other (for review, see Bi and Broach, 2001; Labrador and Corces, 2002). One of these isolated ‘silent’ chromatin regions is the site of the mating type loci *HML* and *HMR* (Dhillon and Kamakaka, 2002; Haber, 1998). Generally, these ‘silent’ loci represent together with the ‘active’ and boundary regions stably maintained and heritable epigenetic states of chromatin. Tackett and colleagues detected that both complexes, the DNA polymerase ε and the chromatin remodeling factor, are involved in the propagation and maintenance of the transition zone between the active and the silent states since the removal of the complexes from the boundaries results in an anomalous alteration of the epigenetic state (Tackett et al., 2005). In addition, both complexes bind similarly to modified histones. This pattern points towards the recognition of similar types of chromatin to maintain or inherit proper configuration of chromatin during cell division (Iida and Araki, 2004). For the DNA polymerase ε, this suggests participation in not only chromosomal DNA replication, but also in the duplication of chromatin structures, likely through the involvement of Dpb3 and Dpb4, which localizes the polymerase to uniquely modified chromatin. Since the human counterparts of polymerase ε and CHRAC are also localized at heterochromatin, these complexes are likely to regulate duplication of

eukaryotic heterochromatin that includes epigenetic information during or after DNA replication. That the histone fold proteins in polymerase ε are essential for cell cycle progression has been shown in the fission yeast *Schizosaccharomyces pombe*. In this organism, Spiga and D'Urso (2004) showed that upon down-regulation of the Dpb3 subunit, DNA replication and cytokinesis is inhibited, thus leading to a cell cycle delay.

It can be concluded from these data, that besides sharing the same transport pathway via importin 13, the CHRAC-15/17 and p12/CHRAC-17 complexes also colocalize at specific chromatin regions. Thus, the joint action of both histone fold pairs in DNA replication of heterochromatic regions (Collins et al., 2002; Fuss and Linn, 2002) is in accordance with their nuclear import via the same transport pathway.

## 4.10 Outlook

The histone fold pair CHRAC-15/17 clearly fulfills its function in the nucleus (Kukimoto et al., 2004; Poot et al., 2000). In this study, we have shown that nuclear import of the heterodimeric complex is mediated by importin 13 *in vitro* and *in vivo*. However, we have also shown that importin 13-deficient cytoplasmatic HeLa extract facilitates nuclear import of the CHRAC-15/17 complex in semi-permeabilized cells. Hence, additional nuclear import pathways presumably do exist for the CHRAC-15/17 complex. So far, eleven nuclear import receptors have been identified. Here, we have excluded the involvement of four of them, importin β, importin 5, importin 7, and importin 9. However, six other nuclear transport receptors, whose potential roles in nuclear import of the CHRAC-15/17 complex were not yet tested in this study, remain to be analyzed. Hence, the capacity of the remaining nuclear transport receptors in mediating nuclear import of the histone fold pairs CHRAC-15/17 and p12/CHRAC-17 should be investigated in subsequent experiments.

With regard to the general competence of importin 13 to facilitate nuclear uptake of histone fold complexes of the H2A/H2B family it also would be interesting to analyze the nuclear import of other H2A/H2B-like histone fold pairs, such as the human TAF135/20 complex (Gangloff et al., 2000). This complex would be particularly interesting since the conserved basic amino acids essential for nuclear import and distributed along the histone fold subunits are not conserved in this heterodimer.

Regarding the cell cycle-dependent nuclear export of the p12/CHRAC-17 complex, further analyses should identify the functional export factor since an exportin 1-dependent pathway was excluded. Mutational analysis of the putative NES in the p12 subunit would allow the verification of the *in silico* predicted leucine-rich nuclear export signal (NES) and would also

indicate whether other NESs are perhaps present in the heterodimer. In addition, substitution of the putatively phosphorylated threonine residue in p12 with glutamate to mimic phosphorylation would further clarify the role of kinases for the subcellular localization of p12/CHRAC-17.

Since the p12/CHRAC-17 containing polymerase ε is detached from core histones after the DNA replication (Tackett et al., 2005) the question arises whether only the p12/CHRAC-17 complex or the entire DNA polymerase ε complex is exported from the nucleus. *In vitro* interaction studies between the heterodimeric p12/CHRAC-17 complex and various export factors in the absence and presence of the other polymerase ε subunits would answer this question.

In addition, crystallographic analysis of CHRAC-15/17 or p12/CHRAC-17 cocrystallized with importin 13 would eventually reveal the exact binding mechanism of the histone fold pairs to importin 13 as well as to putative nuclear export receptors. In particular, amino acid residues essential for the binding of importin 13 to both histone fold complexes could be identified, apart from the characterized positively charged amino acid residues.

# 5 Summary

In this study, we have analyzed the nuclear import of two related histone fold pairs, CHRAC-15/17 and p12/CHRAC-17. The CHRAC-15/17 heterodimer is part of the chromatin accessibility complex (CHRAC) composed of SNF2H, the regulatory subunit ACF1 and the CHRAC-15/17 complex. CHRAC represents one of five different SNF2H-containing chromatin remodeling complexes that use ATP to shift the position of nucleosomes on DNA. The p12/CHRAC-17 heterodimer was identified as an integral component of DNA polymerase ε, composed of p12/CHRAC-17, the 261 kDa catalytic subunit (p261), and the 59 kDa subunit (p59). In human, polymerase ε is responsible for DNA repair, DNA replication and is also involved in cell cycle control.

Heterodimerization of CHRAC-15/17 and p12/CHRAC-17 is facilitated by the core region in both heterodimeric complexes, the *histone fold motif* (HFM), which has been highly conserved throughout evolution. Here, we report that both complexes, which belong to the H2A/H2B family, are imported into the nucleus by a distinct, signal-dependent, energy-dependent, importin 13-mediated pathway. Heterodimerization of the two histone fold motif containing complexes is a prerequisite for their nuclear accumulation since the monomeric subunits CHRAC-15, CHRAC-17, and p12 lack a functional NLS. Thus, importin 13 specifically binds to the dimerized histone fold complexes, while the monomeric subunits are neither bound nor imported. Mutational analysis in CHRAC-15/17, in p12/CHRAC-17, and in the related histone fold NC2α/NC2β complex reveals that basic amino acid residues conserved among the histone fold subunits are necessary for efficient nuclear import. Stepwise substitution of the basic amino acid residues therefore leads to a progressive loss of importin 13 binding and nuclear accumulation of all three histone fold heterodimers. The conservation of basic amino acid residues among the histone fold pairs that are essential for nuclear uptake suggests that heterodimerization of the subunits forms an importin 13 specific binding platform. Since this binding platform contains positively charged amino acids, the interaction between the histone fold complexes with importin 13 is presumably based on electrostatic interactions.

Moreover, deletion of one HEAT repeat at the amino-terminus and four HEAT repeats at the carboxy-terminus in importin 13 already negatively influenced the nuclear transport of the CHRAC-15/17 heterodimer *in vivo*. These data point towards a broad interaction surface between the nuclear transport factor importin 13 and the histone fold pairs.

In addition, we showed that the p12/CHRAC-17 heterodimer is exported from the nucleus in a cell cycle dependent manner. Whereas the complex was localized in the nucleus in late G1-phase

or S-phase, the p12/CHRAC-17 heterodimer was primarily found in the cytoplasm in G1-phase. Since a role for the nuclear export receptor exportin 1 in mediating the nuclear export of p12/CHRAC-17 was excluded, other export receptors have to facilitate the cell cycle dependent export of the histone fold pair.

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# 7 Appendix

**Karyopherin β family members**

| Human | *Yeast* | Cargo |
|---|---|---|
| **Import:** | | |
| Importin α/β | *Kap60/95* | cNLS-containing proteins (e.g. SV40 large T antigen, nucleoplasmin, cyclin E, c-myc, BRCA1, STAT1) |
| Importin β | *Kap95* | ribosomal proteins, core histones, HIV Rev, HIV Tat, HTLV Rex, SREBP-2, PTHrP, cyclin B1, Smad proteins, ($m_3G$ capped UsnRNPs via snurportin; Histone H1 via importin 7) |
| Transportin (Karyopherin-β2) | *Kap104* | hnRNPs (A1, A2, F), TAP, Nab2, Hrp1, core histones, ribosomal proteins; *Nab2, Hrp1* |
| Transportin SR1 | *Kap111/Mtr10* | SR proteins; *Npl3, Hrb1* |
| Transportin SR2 | | HuR |
| Importin 4 | *Kap123/Yrb4* | core histones, ribosomal proteins |
| Importin 5 | *Kap121/Pse1* | core histones, ribosomal proteins; *Pho4, Ste12, Spo12, Pdr1, Yap1, Aft1, SRP proteins, core histones, ribosomal proteins* |
| Importin 7 | *Kap119/Nmd5* | HIV RTC, core histones, ribosomal proteins; *TFIIS, Hog 1, MAPK, Crz1, Ssa4* |
| | *Kap108/SxmI* | *Lhp1, ribosomal proteins* |
| Importin 8 | | SRP19 |
| Importin 9 | *Kap114* | core histones, ribosomal proteins; *TBP, Nap1p, histones H2A and H2B* |
| Importin 11 | | UbcM2, rpL12 |
| | *Kap120* | *Rpf1, MA via Mtr10p* |
| | *Kap122* | *TFIIA (Toa1/Toa2), R2 ββ'* |

| | | |
|---|---|---|
| **Export:** | | |
| Exportin 1/Crm1 | *Kap124/Crm1/Xpo1* | Leucine-rich NES cargoes (e.g. HIV-1 Rev, PKI, MAPKK, HTLV-1, p53, HDM2, Yap1, Pap1), snurportin1, $m_7G$ |
| Exportin-t | *Los1* | tRNA |
| Exportin 4 | | eIF-5A |
| Exportin 5 | | microRNA precursors, tRNA, ILF, eEF-1A |
| Exportin 6 | | Profilin, actin |
| Exportin 7 | | p50Rho-GAP, 14-3-3σ, eIF1 |
| CAS | *Kap109/Cse1* | Importin α |
| **Import/Export:** | | |
| Importin 13 | | import: RBM8 (MGN), Ubc9, Pax6, Pax3, Crx, myopodin, GR<br>export: eIF-1A |
| | *Kap142/Msn5* | *export: Pho4, Far1, Mig1, Cdh1, Rtg1, Rtg3, Crz1, Msn2, Msn4, Swi6, Ste5p, Ssa4*<br>*import: Rpa complex* |

**Table III: Karyopherin β family members.** Members of the karyopherin β family from human are shown. If available, the yeast orthologues (in *italics*) as well as examples of specific cargoes are grouped (yeast cargoes are shown in *italics*). The data are derived from Chook and Blobel (2001), Görlich and Kutay (1999), Harel and Forbes (2004), Jans et al. (2000), Kim (2004), Liang et al. (2008), Lund et al. (2004), Mingot et al. (2004), Mosammaparast and Pemberton (2004), Pemberoton and Paschal (2005), Ploski et al. (2004), Ström and Weis (2001), and Tao et al. (2004).

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# Curriculum Vitae

| | |
|---|---|
| Name | Walker |
| Vorname | Patrick |
| Geburtsdatum | 09. Mai 1980 |
| Geburtsort | Frankfurt am Main |
| Staatsangehörigkeit | deutsch |

| | |
|---|---|
| **1986 – 1990** | Frankeschule; Grundschule in Frankfurt am Main |
| **1990 – 1999** | Liebig Schule; Gymnasium in Frankfurt am Main |
| **Juni 1999** | Abschluss: Allgemeine Hochschulreife; Note 2,3 (gut) |
| **Sept. 1999 – Okt. 2000** | Zivildienst am Institut für archäologische Denkmalpflege; Mainz |
| **Okt. 2000 – Sept. 2002** | Grundstudium Biologie an der Johann Wolfgang Goethe Universität Frankfurt am Main |
| **Okt. 2002 – Sept. 2005** | Hauptstudium Biologie mit den Schwerpunktfächern:<br>• Pflanzenphysiologie<br>• Zell- und Entwicklungsbiologie<br>• Genetik |
| **Jan. 2005 – Sept. 2005** | Diplomarbeit am Botanischen Institut für Pflanzliche Molekularbiologie und Genomforschung bei PD Dr. R. Tenhaken; Thema: „Untersuchung der Proteininteraktion und Reaktion auf exogene Stressfaktoren eines Asparagin-reichen Proteins mit DCD-Domäne aus *Arabidopsis thaliana*“.<br>Note 1,3 (sehr gut) |
| **Sept. 2005** | Abschluss: Diplom-Biologe; Gesamtnote 1,6 (gut) |
| **Feb. 2006 – März 2009** | Doktorarbeit am Institut für Biochemie und Molekulare Zellbiologie der Georg-August-Universität zu Göttingen in der Abteilung Molekularbiologie unter Anleitung von Prof. Dr. D. Doenecke; Thema: „Nuclear import of histone fold motif containing heterodimers by importin 13“ |