Molecular dissection of the nuclear pore complex in relation to nuclear export pathways

Molecular dissection of the nuclear pore complex in relation to nuclear export pathways

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To Esperanza and all of my family

CONTENTS

Chapter 1 **OUTLINE OF THIS THESIS** **9**

Chapter 2 **INTRODUCTION** **13**

1. Cellular compartmentalization and the Nuclear Pore Complex
2. NPC structure
3. NPC composition
4. Nuclear Transport
4.1. Targeting signals
4.2. RanGTP independent transport. Messenger RNPs
4.3. Transport of ribosomal RNPs
5. Models of Nuclear Translocation.
6. Dissecting the NPC
6.1. The putative oncogene Nup214/CAN.
6.2. The cytoplasmic side of the NPC.
7. Molecular dissection of the nuclear pore complex in relation to nuclear export pathways

Chapter 3 **41**

Nup358/RanBP2 attaches to the NPC via association with Nup88 and Nup214/CAN, and plays a supporting role in CRM1-mediated nuclear protein export.
Rafael Bernad, Hella Van Der Velde, Maarten Fornerod and Helen Pickersgill.
Molecular and Cellular Biology 2004 Mar;24(6):2373-84.

Chapter 4 **59**

Supraphysiological nuclear export signals bind CRM1 independently of RanGTP and arrest at Nup358
Dieuwke Engelsma, Rafael Bernad, Jero Calafat and Maarten Fornerod
The EMBO Journal 2004 Sep 15;23(18):3643-52.

Chapter 5 **77**

The Nup214/Nup88 nucleoporin subcomplex is required for CRM1 mediated 60S preribosomal nuclear export
Rafael Bernad, Dieuwke Engelsma, Helen Sanderson, Helen Pickersgill and Maarten Fornerod
The Journal of Biological Chemistry. Accepted for publication.

Chapter 6 93

Characterization of the translocation product *NUP214-ABL* in acute lymphoblastic leukaemia
Rafael Bernad, Kim de Keersmaecker,Maarten Fornerod and Jan Cools
Manuscript in preparation/Work in progress

Chapter 7 DISCUSSION 107

1. How are the cytoplasmic Nups organized?
2. What is the role of Nup358 in nuclear transport?
3. Why are nuclear export signals born to be weak?
4. What is the role of the high affinity interaction between Nup214 and CRM1?
5. Is there a specific role for Nup214/Nup88 subcomplex in nuclear export?
6. Is the Nup214/Nup88 interaction at the NPC required for gating?
7. What are the roles of Nup214 and Nup88 in cancer?

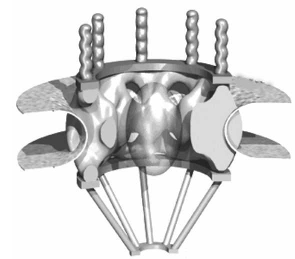
Chapter 8 Resumen en castellano para no científicos 123

List of abbreviations 129

Curriculum Vitae 131

"Nothing shocks me. I'm a scientist."

Harrison Ford (1942 -), as Indiana Jones



CHAPTER 1

OUTLINE

The nuclear pore complex (NPC) is the gateway to and from the genome. The work presented in this thesis is the result of the investigations towards understanding some of the key questions affecting NPC biology: How is the NPC built up? Can we dissect different modes of transport at the level of the NPC? Does the NPC play a role in the development of cancer? The structural localization of some of the components of the NPC, called nucleoporins (Nups), has been under intensive study. We have precisely located Nup88 using electron microscopy techniques (Chapter 3) and placed in the context of Nup214 and Nup358, the other Nups localized exclusively to the cytoplasm (Walther et al., 2002). The close localization of Nup358 in relation to the Nup88-Nup214 subcomplex suggested that they are interaction partners. In fact, we were able to show this physical relation contributing to the current knowledge of the NPC interaction map. Furthermore, the result of this study provides useful information about the behavior of the Nup88 and Nup214 as a subcomplex that shows codependence of its components on protein stability and NPC targeting, and acts as a building block required for Nup358 incorporation.

Concerning nuclear transport, several lines of evidence suggested that Nup358 plays a role in nuclear transport (Lounsbury and Macara, 1997; Singh et al., 1999; Yokoyama et al., 1995). We present data revealing that Nup358 indeed plays a supporting role in Nuclear Export Signal (NES) mediated export by facilitating the disassembly of the export complex, composed of the export receptor CRM1, RanGTP and a NES-cargo, and by facilitating a fast recycle of empty CRM1 to the nucleus (Chapter 3). In addition, we have been able to further dissect the export disassembly process by using supraphysiological (super strong) NESs which revealed export complex intermediates that arrested at Nup358 leading to a less efficient export (Chapter 4). This finding has provided the reason why NESs maintained relatively low CRM1 affinity during evolution.

Traditional transport models are unable to explain how such diversity of molecules can cross the NPC. More recent quantitative transport models discriminate transport mechanisms basing on the actual properties of the transported elements and the NPC itself (Becskei and Mattaj, 2005). Unfortunately, experimental evidence strengthening these models is missing. We have investigated in detail the role of Nup214 in CRM1 export pathways (Chapter 5). We show that CRM1-mediated export of preribosomes is dependent on the presence of Nup214, while other CRM1 cargos are not. We present clear evidence showing that the FG-domain of Nup214,

thought to play an important role in transport (Fornerod et al., 1997; Ribbeck and Görlich, 2001; Rout and Aitchison, 2001), is not relevant for this function. Instead, targeting of Nup214 to the NPC and interacting with neighbor Nups are crucial. In conclusion, we have been able to discriminate different transport modalities which are mediated by the same transport receptor and demonstrate that the characteristics of these pathways are dependent on the transport receptor, the NPC and the cargo itself.

A constitutively activated aberrant tyrosine kinase, NUP214-ABL, is overexpressed in Acute Lymphoblastic Leukemia (ALL) (Graux et al., 2004). We postulated that NPC targeting, provided by Nup214, is required for activation of Abl activity. We have analyzed the subcellular localization of this protein and found that it localizes to the nuclear envelope (Chapter 6). By using Nup88 RNAi and Nup214 overexpression on cell lines expressing NUP214-ABL, we aimed to alter the stability of this protein in an attempt to inhibit cell proliferation. These experiments may provide useful information for the development of alternative therapies for ALL.

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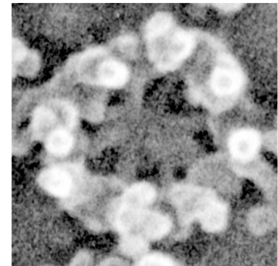
"[…]
As heads is tails
Just call me Lucifer
'cause I'm in need of some restraint"

Rolling Stones
"Sympathy for the Devil"



CHAPTER 2

INTRODUCTION

1. Cellular compartmentalization and the Nuclear Pore Complex

The eukaryotic cell has developed a membrane based system of cellular organization that led to the compartmentalization and specialization of the processes necessary to maintain vital functions. Most of the genome of the cell is located in the nucleus and separated from the cytoplasm by the nuclear envelope (NE). This involves the separation of two processes that are coupled in prokaryotes: transcription and translation (Görlich and Kutay, 1999). While genes are transcribed in the nucleus, protein synthesis occurs in the cytoplasm. In order to successfully express part of the genetic material, many different elements need to shuttle between the nucleus and the cytoplasm. Transcription factors or other chromatin remodelling proteins are required in the nucleus when activated upon signalling in the cytoplasm or in the plasma membrane. They promote transcription of genes in a process that requires the activity of complex protein machineries and leads to a messenger RNA (mRNA). Once matured, the mRNA itself is required in the cytoplasm where it provides the information necessary to assemble a protein. Protein production requires in turn, among other elements, the presence in the cytoplasm of ribosomes and transfer RNAs (tRNAs) whose synthesis occurs in the nucleus. Furthermore, more than 100 proteins and small nucleolar RNAs (snoRNAs) are involved in ribosome formation, which consists on an assembly of multiple ribosomal RNAs (rRNAs) and proteins (Warner, 2001). It is evident that compartmentalization implies the establishment of a mode of communication between the nucleus and cytoplasm. The Nuclear Pore Complex (NPC) is the structure that permits this communication while keeping the integrity of DNA and blocking access to the genome of undesired elements.

NPCs are multiproteinic assemblies that create channels interrupting the double bilayer barrier of the NE. These assemblies are linked to accessory components creating a nuclear transport machinery that establishes and regulates nucleocytoplasmic communication. Regulation of transit between the nuclear and cytoplasmic compartments is critical for the outcome of the signalling cascades that govern survival or proliferation (Vinkemeier, 2004; Xu and Massague, 2004). Furthermore, it has been proposed that nucleocytoplasmic transport itself forms part of

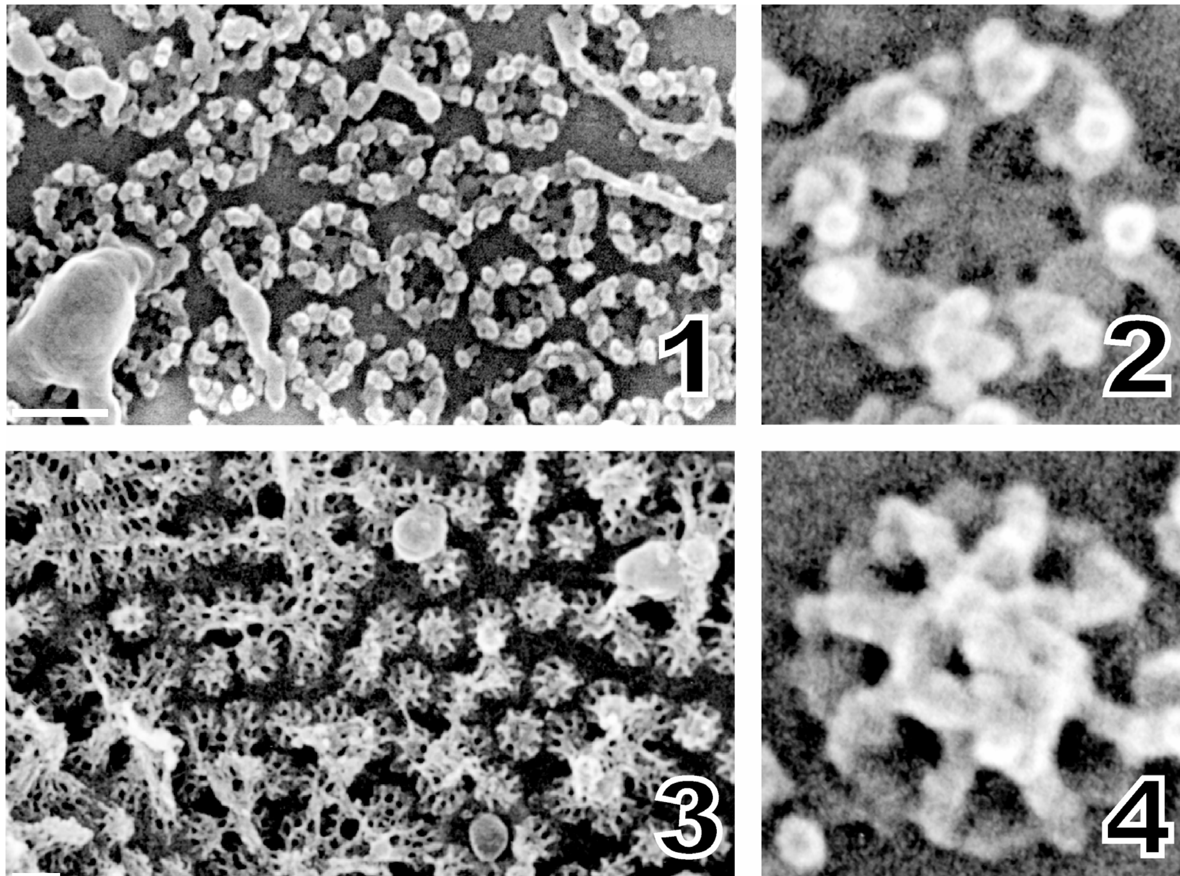


Figure 1. Scanning Electron Microscopy images of the cytoplasmic (1,2) and nuclear (3,4) sides of nuclear envelope preparations containing nuclear pore complexes. Detailed magnifications are shown (2,4). Bars represent 100 nm. Images courtesy of Terry Allen, Helen Pickersgill and Martin Goldberg.

the amplification and propagation of the signalling cascades (Becskei and Mattaj, 2005). The NPC is an integral component of the NE and suffers as well rounds of disassembly and reassembly on every cell cycle playing a crucial role in the establishment of the nuclear architecture and organization. There is an intrinsic relation between the nuclear transport system and chromatin. At the initiation of mitosis, several components of the NPC and the transport system are relocated to the kinetochores, where they regulate spindle assembly (Belgareh et al., 2001; Kalab et al., 1999; Salina et al., 2003). Furthermore, the interphase NPC can control epigenetic gene expression (Galy et al., 2000; Mendjan et al., 2006). Considering this privileged situation, it is not absurd to implicate NPC components directly in transcription control. In fact, studies in yeast show that production and export of mRNAs are coupled processes and that NPC-promoter interactions are linked to gene activation (Aguilera, 2005; Schmid et al., 2006).

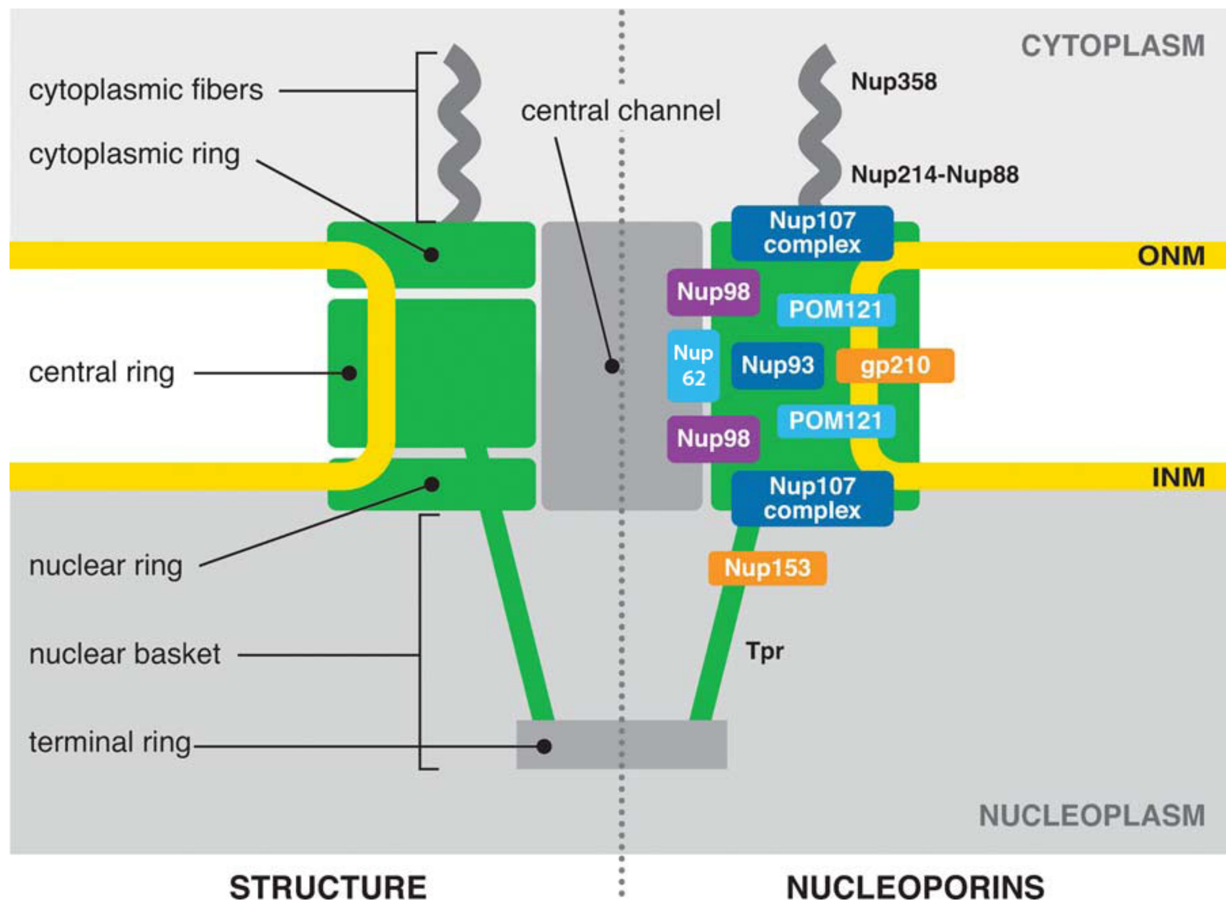


Figure 2. Schematic representation of a cross-section of the NPC showing the main structural features (Left) and the nucleoporin subcomplexes composition (right). Inner (INM) and outer (ONM) nuclear membranes are depicted. Adapted from (Hetzer et al., 2005).

2. NPC structure

Electronic microscopy (EM) techniques have provided very useful structural and functional information of the NPC (Figure 1), from the first images shown in the 1950s (Afzelius, 1955) until the latest published results using modern transmission and scanning electron microscopy, atomic force microscopy and cryoelectron tomography (Akey, 1989; Beck et al., 2004; Goldberg and Allen, 1993; Stoffler et al., 2003). The overall structure and architecture of the NPC (Figure 2) is conserved from yeast to vertebrates diverging only in the size of the complex, whose estimated mass varies from ~60 MDa in yeast to a maximum of ~125 MDa in vertebrates (Cronshaw et al., 2002). A triple ring model of NPC architecture was proposed (Unwin and Milligan, 1982) which presents an 8-fold rotational symmetry (Maul, 1971) and consists, with respect to the NE, on two asymmetrical faces with peripheral structures that

Nucleoporin	Nup subcomplexes	Localization[a]	Motifs[b]		Yeast homologue(s)	Relative abundance[c]	
			FG	RBD		Vertebrates	Yeast[d]
Nup358	?	C	Y	Y	–	8	–
Tpr	Nup98	N			Mlp1p, Mlp2p	16	ND
Nup214	Nup88 Nup62	C	Y		Nup159p	8	8
gp210	POM121	PM			–	16	–
Nup205	Nup188/Nup93	?			Nup192p	16	16
Nup153	Nup107 complex	N	Y		Nup1pc	8	8
Nup188	Nup205/Nup93	?			Nup188p	8	16
POM121	gp210	PM	Y		–	8	–
Nup155	?	C,N			Nup157p, Nup170p	32	32
Nup160	Nup107 complex Nup98 Nup153	N			Nup120p	8	16
Nup133	Nup107 complex Nup98 Nup153	C,N			Nup133p	16	16
Nup96	Nup107 complex Nup98 Nup153	N			C-Nup145p	16	16
Nup107	Nup107 complex Nup98 Nup153	C,N			Nup84p	32	16
Nup98	RAE1 Nup107 complex Tpr	N	Y		N-Nup145p, Nup116p, Nup100p	8	32
Nup93	Nup62 Nup205/Nup188	N			Nic96p	32–48	32
Nup88	Nup214	C			Nup82p	32	8–16
Nup62	Nup62 complex Nup214	C,N	Y		Nsp1p	16	32
Nup75	?	?			Nup85p	16	16
Nup58	Nup62 complex	C,N	Y		Nup49p	48	16
ALADIN	?	?			–	8	–
Nup54	Nup62 complex	C,N	Y		Nup57p	32–48	16
Nup53	NDC1/Nup93	N	Y		Nup53p	16–32	32
Nup50	Nup153	N	Y	Y	Nup2pc	32	ND
Nup45	Nup62 complex	C,N	Y		(Nup49p)	32	(16)
NLPI	?	C	Y		Nup42p	16	8
Nup43	?	?			–	16	–
RAE1	Nup98	N			Gle2p	48	16–32
Seh1	?	?			Seh1p	16–32	16
Nup37	Nup107 complex Nup98 Nup153	?			–	16–32	–
					Sec13p	16–32	ND
NDC1	Nup53/Nup93	PM			Ndc1p	ND	ND

Table 1. Summary of all nucleoporins identified including some relevant characteristics. Contains summarized data from (Allen et al., 2000; Cronshaw et al., 2002; Hawryluk-Gara et al., 2005; Mansfeld et al., 2006; Ryan and Wente, 2000; Vasu and Forbes, 2001).

a C: cytoplasmic, N: Nuclear, PM: pore membrane b FG: phenylalanine- glycine repeats; RBD: Ran binding domain c copies per NPC d (Rout et al., 2000)

anchor to a central spoke-ring complex via a coaxial ring. The peripheral structures form filaments in the cytoplasmic side and baskets in the nuclear side. In vertebrates, the cytoplasmic filaments are ~50nm long (Franke and Scheer, 1970; Franke and Scheer, 1970; Richardson et al., 1988) and the nuclear basket protrudes ~100nm from the NE. The central channel-like feature contains eight spokes sandwiched between the cytoplasmic and nuclear rings with a maximum diameter of 25nm in vertebrates (Cordes et al., 1993; Goldberg and Allen, 1992; Jarnik and Aebi, 1991; Ris, 1997).

Although microscopy techniques provide a static view of the NPC, several studies have been able to discriminate conformational states of the NPC which are thought to reflect structural modifications during the transport process (Beck et al., 2004; Kiseleva et al., 1998; Stoffler et al., 2003). It remains to be established to which, if not all, transport events these conformational changes are associated with.

Very little has been achieved towards the understanding of the NPC structure at the molecular level. There are no reports showing the crystal structure of any complete nuclear pore component. Some studies have shown discrete domains of Nup358 and Nup214 but, although they provided valuable data about their functions individually, they gave very little information about the relation of these proteins with the overall structure of the NPC (Geyer et al., 2005; Pichler et al., 2004; Reverter and Lima, 2005; Vetter et al., 1999; Weirich et al., 2004). *In vitro* reconstitution of self-associating individual components or complete subcomplexes in combination with EM imaging has been used to obtain basic structural information (Buss et al., 1994; Siniossoglou et al., 2000) but the integration of these data in the overall NPC context is difficult and more information is required.

3. NPC composition

The constituents of the NPC are termed nucleoporins or Nups (Table 1). Interestingly, only ~20 different nucleoporins are required to assemble the NPC indicating that every pore contains multiple copies of the same components (Cronshaw et al., 2002; Rabut et al., 2004; Rout et al., 2000). In fact, the molecular architecture of the yeast and vertebrate NPC revealed by immunogold labeling in combination with EM shows that most Nups occupy several positions following the rotational symmetry and, with the exception of the peripheral components, a

symmetric position within the vertical plane of the NE (Fahrenkrog et al., 2000; Rout et al., 2000; Walther et al., 2001; Walther et al., 2002). The exact localization of individual Nups within the NPC has been sometimes controversial due to several reasons such as technical difficulties (antibody specificity, labeling procedure or sample preparation), variability within species or cell lines and mobility of some Nups (Krull et al., 2004).

One major difference between yeast and vertebrate NPC is the mechanical connection to the NE. While yeast NPCs are mobile within the NE (Belgareh and Doye, 1997; Bucci and Wente, 1997), vertebrates have anchored NPCs (Daigle et al., 2001). In relation to their position, physical interactions between neighbor Nups have been studied and mapped (Allen et al., 2002; Huang et al., 2002). Nups associate in subcomplexes prior to incorporation to the NPC (Doye and Hurt, 1997; Ryan and Wente, 2000). Some of these subcomplexes or individual Nups are well conserved between species but others differ widely or have no obvious homologue (Ohno et al., 1998). The dynamic behavior of the NPC components has been systematically studied using Fluorescence Recovery After Photobleaching (FRAP) techniques on GFP-tagged Nups (Rabut et al., 2004). Interestingly, while some components show a high residence time within the NE, others are very mobile revealing that the NPC is highly dynamic and that these individual Nups may have additional cellular functions.

Concerning their primary structure, many Nups present repeated motifs of the sequence FG, FxFG- or GLGF-. These motifs present in Nups, termed in general FG-repeats, are believed to be responsible for the NPC acting as a selective barrier (Bayliss et al., 2000; Bayliss et al., 2002; Stewart et al., 2001; Strawn et al., 2004). FG-repeat containing domains are highly flexible and lack ordered secondary structure (Denning et al., 2003; Rout and Wente, 1994; Ryan and Wente, 2000). Nups containing these repeats could line the translocation channel of the NPC and, as will be discussed below, are thought to play an important role in nuclear transport (Allen et al., 2001; Buss et al., 1994; Denning et al., 2001; Ribbeck and Görlich, 2001; Rout and Aitchison, 2001).

4. Nuclear Transport

Intensive research during the last decade has led to the development of a general model of nucleocytoplasmic transport (Allen et al., 2000; Bayliss et al., 2000; Görlich and Kutay, 1999;

Hetzer et al., 2005; Mattaj and Englmeier, 1998; Weis, 2003; Wente, 2000). While the NPC provides the structure, two major adjacent components govern this process: the transport receptors and the Ran system.

-Transport receptors. It has been well established that certain proteins can interact with FG-repeats containing Nups and cross the NPC barrier. Among them are the transport receptors of the Importin β and NTF2-like families that account for most of the transport events in the cell. They also present an affinity for their binding partners or cargoes which permit their translocation through the NPC. Based on the direction of transport, the mammalian Importin β family members can be subdivided into importins and exportins depending on whether they mediate import into or export out of the nucleus (Allen et al., 2001; Arts et al., 1998; Bayliss et al., 2002; Fornerod et al., 1997; Görlich et al., 1997; Iovine et al., 1995; Mosammaparast and Pemberton, 2004; Ribbeck et al., 1998; Shah et al., 1998; Strawn et al., 2001).

-The Ran system. Ran is a small GTP switch that is mainly maintained in its GTP form in the nucleus and in its GDP form in the cytoplasm. This is achieved by the action of the other components of the Ran system: the chromatin bound Ran exchange factor RCC1 and the cytoplasmic RanGTP hydrolysis stimulators RanGAP and RanBP1/2. This system modulates

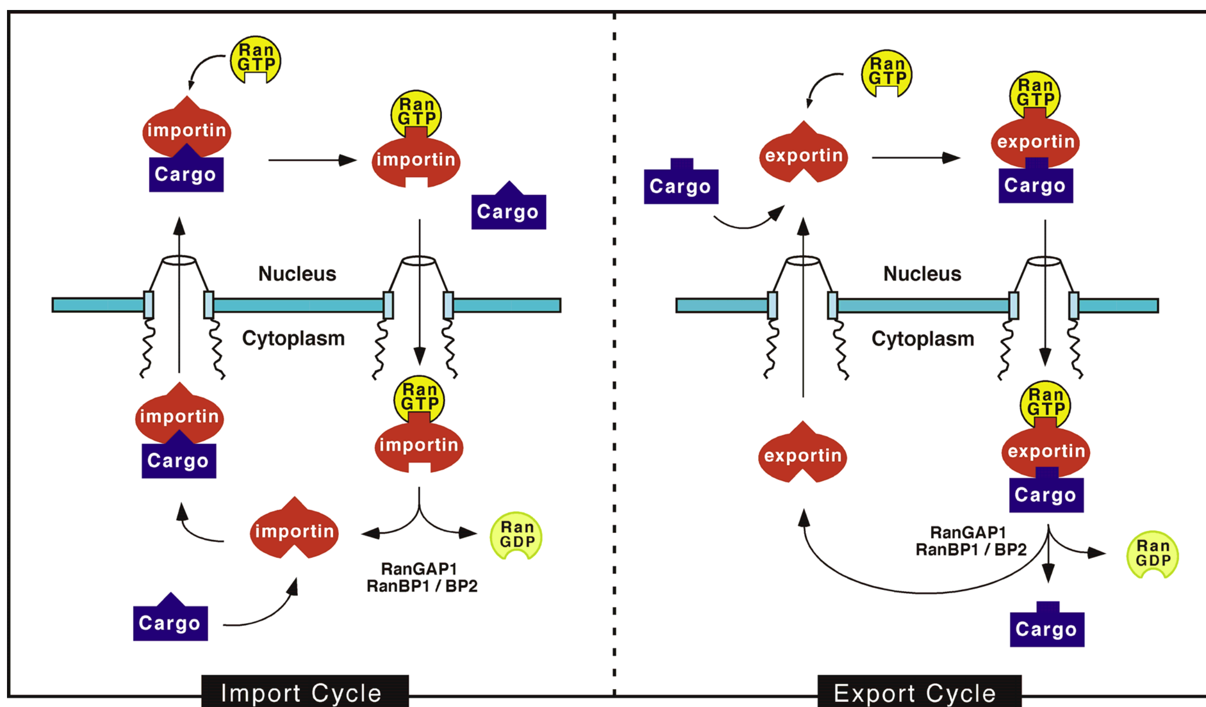


Figure 3. Schematic representation of an import (left) and export (right) cycle. (See full-colour export cycle in cover and animation near page number)

the affinity of most transport receptors for their cargoes defining the directionality of transport and the accumulation of cargos at one side of the NPC (Görlich et al., 1996; Görlich et al., 1996; Izaurralde et al., 1997; Kalab et al., 2002).

Elements destined to be imported contain nuclear targeting signals which bind with high affinity to their import receptors in the cytoplasm (Figure 3). This affinity drops in the nuclear environment where RanGTP levels are high. Export receptors in turn require RanGTP to bind the cargos that need to be exported through the cytoplasmic targeting signals. This trimeric complex is unstable upon translocation to the cytoplasm where RanGTP hydrolysis occurs. Released RanGDP is recycled to the nucleus by NTF2 (Gerace, 1995; Kutay et al., 1997; Lounsbury and Macara, 1997; Nakielny et al., 1999; Ribbeck et al., 1998; Schlenstedt et al., 1997).

4.1. Targeting signals

As it is for transport receptors, the variability of targeting signals present in cargos is a symptom of the diversity of transport pathways adopted by the cell. The best characterized nuclear targeting signals are denominated Nuclear Localization Signals (NLS) and they can be simple or bipartite like those of SV40 large T antigen (SV40 TAg) and nucleoplasmin respectively. Simple NLS are short sequences containing a single cluster of basic amino acids, often preceded by an acidic amino acid or a proline residue. Bipartite NLS are two interdependent clusters of basic amino acids separated by a flexible spacer. Neutral and acidic residues flanking the motif can contribute too (Kalderon et al., 1984; Makkerh et al., 1996; Robbins et al., 1991). The actual NLS receptor is Importin-α which, acting as an adapter, promotes formation of an Importinα/Importinβ heterodimer and translocation of the complex (Görlich et al., 1995).

Nuclear Export Signals (NES) are present in a broad range of substrates. They conform more or less to the consensus $\Phi\text{-}x_{2-3}\text{-}\Phi\text{-}x_{2-3}\text{-}\Phi\text{-}x\text{-}\Phi$ (Φ= L,I,V,F,M; x is any amino acid) and are all translocated to the cytoplasm via the export receptor CRM1 (Fornerod et al., 1997; Kutay and Guttinger, 2005). In contrast to the case of NLSs and Importinα or other exportin-cargo

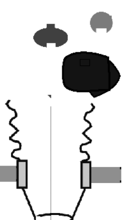
complexes, the affinity of CRM1-RanGTP complex for the NESs is weak (Askjaer et al., 1999; Paraskeva et al., 1999). The biological rationale of this phenomenon has remained unknown.

4.2. RanGTP independent transport. Messenger RNPs

Interestingly, not every transport event follows this general model. As mentioned, other proteins that do not belong to the Importin-β family of transport receptors can directly interact with the FG-repeats of the NPC promoting passage of themselves or their cargos (Asally and Yoneda, 2005; Hetzer and Mattaj, 2000; Vinkemeier, 2004). β-catenin shuttles in and out of the NPC by itself in a RanGTP independent manner (Fagotto et al., 1998; Hendriksen et al., 2005; Yokoya et al., 1999). It has been shown that Importin-α and Importin-β can cross the NPC independently in both Ran dependent and independent manners and, irrespective of that, RanGTP hydrolysis is not required (Kose et al., 1999; Miyamoto et al., 2002).
Another case that requires special study is the export of messenger ribonucleoproteins (mRNPs). Three key components conserved in eukaryotes and unrelated to the Importin β family are recruited to the nascent mRNA: TAP/NXF1 mRNA export receptor (named in yeast Mex67p:Mtr2p), DECD or DEAD-box putative RNA helicases and RNA-binding adaptor proteins like the hnRNP-like ALY(REF1) (named in yeast Yra1p) or the SR proteins. Translocation of the assembled RNP is independent of RanGTP transport and gradient (Huang et al., 2003; Huang and Steitz, 2001; Izaurralde, 2004; Reed and Hurt, 2002; Stutz and Izaurralde, 2003). In fact, it appears that the mRNA export receptor affinities are regulated by cycles of phosphorylation and dephosphorylation on the adaptor proteins (Gilbert and Guthrie, 2004)

4.3. Transport of ribosomal RNPs

Ribosomes are large RNA and protein complexes and their synthesis implicates the sequential coordination of many proteins and snoRNAs (Fatica and Tollervey, 2002). Several import pathways participate cooperatively for the recruitment of all the components to the nucleoli. With some exceptions (Plafker and Macara, 2002), most of the ribosomal proteins can be imported by either of the transport receptors Importin β, transportin, RanBP5 or RanBP7 (Jakel



and Görlich, 1998). In the case of snoRNAs, three different import pathways have been distinguished (Michaud and Goldfarb, 1992) and some of these factors involved in RNA metabolism shuttle between the nucleus and the cytoplasm, suggesting that they play a regulatory role in the maturation process (Leary et al., 2004).

During the last maturation steps, factors implicated in preribosome release and export incorporate to the nucleoli. Ribosomal RNPs are exported via the CRM1 pathway as independent subunits (Andersen et al., 2005). Preribosomes are among the largest cargos crossing the NPC and their export, which is the principal activity of the NPC, requires the action of adaptor proteins and specific mediators. The 60S large subunit utilizes the adaptor protein NMD3, that bridges the interaction with CRM1 providing a NES in trans (Ho et al., 2000; Thomas and Kutay, 2003; Trotta et al., 2003; Warner, 2001). Recent evidence shows that the yeast GTPases Nog1p and Lsg1p regulate the interaction of NMD3 with the rRNP at the nucleus and cytoplasm respectively (Hedges et al., 2005; Kallstrom et al., 2003). Intriguingly, some nucleoporin mutant yeast strains were found to have defective nuclear export of preribosomes while ribosomal maturation is not affected (Gleizes et al., 2001). This finding suggests a fundamental difference between preribosome export and other transport pathways but experimental evidence is lacking.

5. Models of Nuclear Translocation.

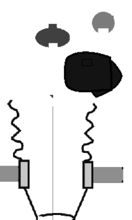
The main paradox of nuclear translocation is that inert molecules bigger than ~40 kDa are not able to cross the NPC indicating that translocation is a size-dependent diffusion event. However, cargo-receptor complexes of 100 kDa have been shown to diffuse through the pore at rates comparable to cytoplasmic diffusion (Ribbeck and Görlich, 2001). Furthermore, the large 60S ribosomal subunit, whose size is 25nm, is efficiently exported. Integrating all experimental evidence to formulate a unique general model of transport seems an arduous task, especially when NPC translocation is able to accommodate a broad range of shuttling elements with very different properties. Traditionally, three models which contribute to a broad concept of transport have been proposed:

- Affinity gradient model (Ben-Efraim and Gerace, 2001). It is based on the findings that transport receptors bind with variable affinities to different FG-containing Nups and that there are some Nups that locate exclusively at either the nuclear or the cytoplasmic side of the NPC. It postulates that transport complexes bind to nucleoporins with progressively increasing affinity though the translocation route.

- Virtual gating model (Rout et al., 2003). It considers the NPC structure as a channel which all molecules encounter but not all can access and NPC translocation as an enzymatic event which can be catalysed by the NPC. Selectivity is accomplished by the Brownian action of the FG-repeats which create a barrier that makes diffusion a thermodynamically complex event. The translocation reaction is then favoured by shuttle interactions with the FG-repeats themselves or by removing the products of the enzymatic reaction which is achieved by the accessory components of the transport machinery.

- Hydrophobic exclusion model (Ribbeck and Görlich, 2002). This model integrates the kinetics of nuclear transport and the NPC structure. It considers the FG-repeats as unstructured domains that can form a hydrophobic meshwork through weak interactions and the transport receptors as the "melting" elements with high surface hydrophobicity. The NPC would not be then a rigid channel but a flexible solution that can adapt to the translocating elements.

The main difficulty with the affinity gradient model is how to explain the directionality of transport and the fact that it can be reversed (Nachury and Weis, 1999; Yang et al., 2004). In fact, some studies excluded that directionality can be driven by differential transport complex-nucleoporin affinities (Becskei and Mattaj, 2003). Nevertheless, the possible biological role of these differential affinities should not be ignored (Fornerod et al., 1997; Kehlenbach et al., 1999; Rexach and Blobel, 1995; Shah et al., 1998). As an alternative, a role of escort during translocation was proposed for the asymmetrically located FG domains of Nup153 and Nup214 which bind with high affinity to transport receptors (Fahrenkrog et al., 2002; Paulillo et al., 2005). Although the physical length, localisation and unstructured nature of FG domains make this model feasible, recent experimental evidence points to another direction: First, imaging of single molecule translocation through the NPC localized the central pore as the location where



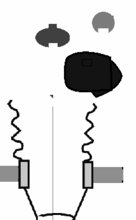
kinetically important interactions take place. It shows shuttling a rather random movement not compatible with an escorted transport (Yang et al., 2004). Second, FG-domain absences on asymmetric nucleoporins do not affect receptor-mediated nuclear transport in yeast (Strawn et al., 2004; Zeitler and Weis, 2004). This finding argues again against an affinity gradient model that would consider FG-domains of asymmetric Nups the most relevant with highest affinities; and against a virtual gating model as well that postulates that these domains play an essential role in creating the selective barrier. Not only asymmetric FG-repeats but up to 50% of the FG mass can be dispensable keeping NPC exclusion diameter and transport unaffected. Except for a minimum central FG-region that remains still required, the importance of most FG-repeats on NPC function is under debate (Strawn et al., 2004). Nevertheless, some specific transport pathways, like mRNPs or rRNPs export, show dependency on the presence of specific Nups, indicating that they do not have redundant functions (Fornerod et al., 1997; Gleizes et al., 2001; Nehrbass et al., 1993).

Very recently, mathematical modeling has shown to be an interesting approach to define nuclear transport in a quantitative manner (Becskei and Mattaj, 2005). They are based on the fact that nuclear translocation can be compared to translocation of solutes across polymers, lipid membranes or protein channels and, therefore, similar mathematical formulations can be applied. Three major classes of quantitative models can be formulated: partitioning, NPC gating and enhanced diffusion. The partitioning model is equivalent to the hydrophobic exclusion model and related to the virtual gating model. It assumes that entering the NPC follows equal dynamics as that of permeation of solutes through lipid membranes (Oren et al., 2004; VanDongen, 2004). The NPC channel, containing FG-domains, would behave as a hydrophobic medium (Allen et al., 2001; Bayliss et al., 2002; Buss et al., 1994; Denning et al., 2001). This model is supported by extensive experimental evidence (Bayliss et al., 2002; Bayliss et al., 1999; Smith et al., 2002). NPC gating and enhanced diffusion are variants that incorporate the capability of the NPC to modify its properties and therefore its permeability by conformational changes that alter the NPC structure (NPC gating) or the shuttle domain interactions within the meshwork (enhanced diffusion). No experimental data has shown to date the capability of the NPC to enhance the diffusion of a complex while it is translocated. In contrast, several studies have shown structural changes that alter the permeability of the NPC

(Jaggi et al., 2003; Shulga and Goldfarb, 2003). Furthermore, EM studies have revealed conformational states of the NPC associated to transport events that may be representative of a functional gating mechanism (Beck et al., 2004; Kiseleva et al., 1998; Stoffler et al., 2003).
In conclusion, it seems that different nucleocytoplasmic transport mechanisms are compatible at the NPC to accommodate passage of many different elements through a unique structure. The mechanism of transport that governs for every shuttling molecule is not only dependent on its own physical properties, which defines its capability to integrate and move through the NPC, but also on the ability of interacting with transport receptors and/or the NPC itself, which provokes the NPC properties to suit the efficient transport of this molecule.

6. Dissecting the NPC

Several approaches have been used to study the functional role of individual Nups or subcomplexes. In yeast, powerful genetics have implicated specific Nups in transport pathways, NPC structure or NE and intranuclear organization (Fabre and Hurt, 1997; Galy et al., 2000; Wente, 2000). In contrast, genetic depletion methods in vertebrates are rarely used due to the essential nature of the NPC components (Smitherman et al., 2000; van-Deursen et al., 1996; Wu et al., 2001). As an alternative to study vertebrate NPCs, *Xenopus* egg extracts are used to promote *in vitro* NE assembly on chromatin templates. These extracts can be submitted to biochemical depletion of single components revealing their relevance in NPC assembly or transport pathways (Finlay and Forbes, 1990; Grandi et al., 1997; Powers et al., 1995; Walther et al., 2003; Walther et al., 2001; Walther et al., 2002).
The outcome of RNA interference technology (RNAi) (Fire, 1999) and its implementation in mammalian cells (Brummelkamp et al., 2002; Elbashir et al., 2001) offers new possibilities for the study of individual components of the transport machinery. RNAi studies combined with immunolocalisation analysis were used to study Nup93, Nup96, Nup98, Nup107, Nup153, Nup205 and the nuclear basket component Tpr (Hase and Cordes, 2003; Krull et al., 2004). Salina and co-workers found using RNAi against Nup358 that it is required for kinetocore function and therefore identified a NPC component as a link between NE breakdown and kinetochore maturation and function (Salina et al., 2003). But to date, none of these studies have revealed functional roles of mammalian Nups on specific transport pathways.



6.1. The putative oncogene Nup214/CAN.

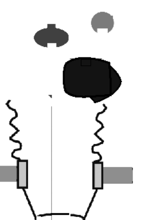
Frequent breakpoints on chromosome 9 in leukemia associated chromosomal translocations raised the interest of the scientific community (Hagemeijer et al., 1990; Kurzrock et al., 1988). Such translocations lead to the aberrant expression of proto-oncogenes or fusion proteins with oncogenic properties, like the well characterized Philadelphia translocation, typically found in Chronic Myeloid Leukemia (CML) and precursor B-cell Acute Lymphoblastic Leukemia (B-ALL), that produces the fusion of *bcr* and *c-abl* genes (De Klein et al., 1986; De Klein et al., 1982). In the early 90's, studies performed on the specific chromosomal translocation (6;9)(p23;q34) of a defined subtype of acute myeloid leukemia (AML) detected and characterized a gene which, due to its proximity to *c-abl,* was originally designated *Cain* or *can*. More translocations were found that implicated this gene in leukemogenesis and therefore the product of this gene, the protein CAN, was proposed to be a putative oncogene (von Lindern et al., 1992; von Lindern et al., 1990; von Lindern et al., 1992). CAN was found to belong to the family of Nucleoporins and was then re-baptized Nup214 (Fornerod et al., 1995; Kraemer et al., 1994). Interestingly, other oncogenic fusions were shown with the nucleoporin gene *NUP98* (Ahuja et al., 1999; Arai et al., 1997; Borrow et al., 1996; Hussey et al., 1999; Nakamura et al., 1996). These data suggest that nucleoporins play an important role in human myeloid leukemia. Kasper and co-workers found that FG-repeats acted as activators of gene transcription by interacting functionally and physically with the transcriptional coactivators CREB binding protein (CBP) and p300. Considering that FG repeats from different Nups elicited similar responses, they proposed that this mechanism may be shared in the pathogenesis of leukemias (Kasper et al., 1999). The possible role of FG-domains in transcription activation remains to be elucidated.

Very recently, a novel mechanism for activation of tyrosine kinases in cancer was found in Acute Lymphoblastic Leukemia (ALL): the formation of episomes resulting in a fusion between NUP214 and ABL1 (Graux et al., 2004). As a consequence, a constitutively phosphorylated tyrosine kinase NUP214-ABL1 is overexpressed. In contrast to previously found chromosomal aberrations, NUP214-ABL1 lacks FG repeats. The role of Nup214 in leukemogenesis remains unknown.

6.2. The cytoplasmic side of the NPC.

To date, three nucleoporins have been exclusively localized to the cytoplamic side of the NPC: Nup88, Nup214 and Nup358. Genetic depletion of Nup214 in mice causes early embryonic death. Embryos showed reduced NLS-mediated protein import, and strong nuclear poly(A) RNA accumulation suggesting that Nup214 is crucial for NPC function and survival (van-Deursen et al., 1996). Nup214 contains a carboxy-terminal FG-repeat domain that binds the export receptor CRM1 and two central coiled coils domains that associate and Nup88 and target Nup214 to the NE (Fornerod et al., 1996; Fornerod et al., 1997). In fact, Nup214 and Nup88 form a stable subcomplex and require each other to localise to the NPC (Bastos et al., 1997; Matsuoka et al., 1999).

Very little is known about the localization and function of Nup88. It was shown to interact with Nup98, a Nup implicated in RNA transport found to shuttle between the nucleus and the NPC (Griffis et al., 2002). Mutants of the *Drosophila* homologue *mbo* show nuclear accumulation of specific proteins due to a defect on the CRM1 export pathway (Roth et al., 2003; Uv et al., 2000). Some studies associate overexpression of Nup88 with aggressiveness in tumors (Agudo et al., 2004). A third Nup214 co-precipitating band of ~66 kDa was detected that may correspond to Nup62 based on the interactions described in their yeast homologues (Bailer et al., 2000; Belgareh et al., 1998). Nup358, also denominated RanBP2, has no yeast homologue and is the biggest Nup (Wu et al., 1995; Yokoyama et al., 1995). It contains FG-repeats, four RanBP1-like RanGTP binding domains that can indeed act as RanGTPase coactivators (Beddow et al., 1995; Bischoff et al., 1995; Richards et al., 1995; Villa Braslavsky et al., 2000), and two Zinc-finger domains that bind RanGDP (Yaseen and Blobel, 1999). It has been shown that Nup358 can interact with the Importin β receptor (Delphin et al., 1997). These data suggest that Nup358 plays a role in nuclear import (Yaseen and Blobel, 1999). The localization and possible role in import of Nup214 and Nup358 was assayed using *in vitro* NE assembly and immuno-EM, Nup358 was found to be the major component of the cytoplasmic fibrils of the NPC while Nup214 is located near the cytoplasmic coaxial ring. Interestingly *in vitro* assembled NPCs deficient in both Nups were still capable of mediating import of proteins (Walther et al., 2002).



7. Molecular dissection of the nuclear pore complex in relation to nuclear export pathways

Several findings presented in this thesis have contributed to the current knowledge of the biology of the Nuclear Pore Complex. Structurally, the hierarchy towards incorporation to the NPC of the cytoplasmic components Nup88, Nup214 and Nup358 and their relevance to nuclear transport has been established. Concerning nuclear transport itself, a supporting role in CRM1-mediated export has been assigned to Nup358 and an explanation to the weak nature of the interaction of CRM1 and its NES-containing cargoes has been elucidated.

This work has amplified as well the concept of nuclear translocation by creating a distinction in transport pathways that, instead of been exclusively dependent on the receptor-NPC and the receptor-cargo interactions, consider the characteristics of the cargo itself. In fact, while showing cargos that can be exported by CRM1 independently of Nup214, we present first *in vivo* evidence of the implication of Nup214 in a NPC gating mechanism for the CRM1-dependent export of preribosomes. Furthermore, this result excludes any implication of the strong CRM1 binding Nup214 FG-domain in this mechanism and in other suggested models of CRM1 export.

Finally, this thesis has provided information concerning the localization and stability of the aberrant product NUP214-ABL that may be of great value for the development of alternative therapies of leukemic diseases.

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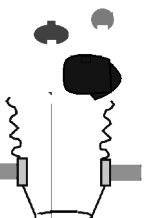

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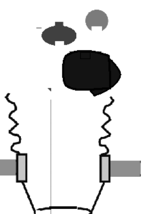

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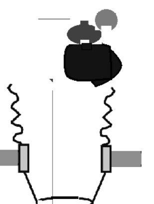

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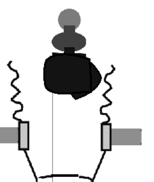

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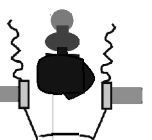
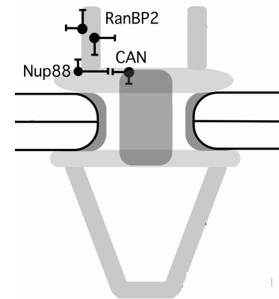


"Born to walk against the wind
Born to hear my name
No matter where I stand I'm alone"

Manowar "Heart Of Steel"



CHAPTER 3

Nup358/RanBP2 attaches to the NPC via association with Nup88 and Nup214/CAN, and plays a supporting role in CRM1-mediated nuclear protein export.

Rafael Bernad, Hella Van Der Velde, Maarten Fornerod and Helen Pickersgill

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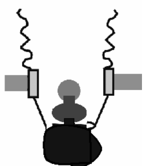

Nup358/RanBP2 attaches to the NPC via association with Nup88 and Nup214/CAN, and plays a supporting role in CRM1-mediated nuclear protein export.

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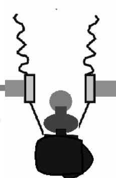
Nuclear pore complexes (NPCs) punctate the nuclear envelope (NE), providing a channel through which nucleocytoplasmic transport occurs. Nup358/RanBP2, Nup214/CAN and Nup88 are components of the cytoplasmic face of the NPC. Here we show that Nup88 localises midway between Nup358 and Nup214 and physically interacts with them. RNA interference (RNAi) of either Nup88 or Nup214 in human cells caused a strong reduction of Nup358 at the NE. Nup88 and Nup214 showed an interdependence at the NPC and were not affected by the absence of Nup358. These data indicate that Nup88 and Nup214 mediate the attachment of Nup358 to the NPC. We show that localisation of the export receptor CRM1 at the cytoplasmic face of the NE is Nup358-dependent, and represents its empty state. Also, removal of Nup358 causes a distinct reduction in NES-dependent nuclear export. We propose that Nup358 provides both a platform for rapid disassembly of CRM1 export complexes and a binding site for empty CRM1 recycling into the nucleus.

Introduction

The nucleus is the defining feature of a eukaryotic cell, and is surrounded by a double membrane known as the nuclear envelope (NE), which prevents free diffusion of macromolecules between the nucleus and the cytoplasm. NPCs are protein channels residing in the NE, through which the active and highly specific transport of RNA and protein between the nucleus and the cytoplasm occurs, a process known as nucleocytoplasmic transport (24, 53, 64). The NPC is a modular and complex structure, displaying 8-fold rotational symmetry (10, 49). It is composed of a series of concentric rings at the plane of the NE, with 80-100 nm filaments extending into the nucleus, distally connected to form a basket structure, and ~50 nm filaments extending into the cytoplasm (22, 29, 49, 52). Approximately 30 proteins termed nucleoporins constitute the vertebrate NPC and contribute to many of its functions (10). Many nucleoporins form subcomplexes, and they collectively afford the structural integrity of the NPC, its assembly and disassembly during mitosis in higher eukaryotes, as well as playing a functional role in nucleocytoplasmic transport (59, 64).

Immuno electron microscopy (EM) studies using a variety of techniques and antibodies have revealed ultrastructural localisations of nucleoporins within the NPC, (e.g. (62). These localisations can be used to explain how certain substructures of the NPC contribute to specific functions. Definitive localisation of nucleoporins has been important for developing models to explain selective translocation through the NPC (8, 50, 53). For example, a subset of nucleoporins containing FG-repeats are thought to generate a hydrophobic barrier at the NPC, permeable only to transport competent macromolecules, which suggests they are localised at accessible regions of the NPCs, lining the translocation route (50, 53).

Three vertebrate nucleoporins are reported to localise exclusively to the cytoplasmic face of the NPC, Nup214/CAN, Nup88 and Nup358/RanBP2. Nup214 has been localised close to the midplane of the NE, possibly as a



component of the cytoplasmic ring (33, 63), and interacts with Nup88 to form a stable subcomplex (6, 20, 40). The mechanism for targeting this Nup88-Nup214 subcomplex to the NPC during nuclear assembly apparently requires both proteins as Nup214 deletion from mouse embryos caused mislocalisation of Nup88 from the NPC, and the Nup214 interaction domain of Nup88 expressed in BHK cells mislocalised Nup214 to the cytoplasm (6, 18, 20). Nup88 is present at an estimated 32 copies/NPC, compared to only 8 copies of Nup214 (10), and the ultrastructural localisation of Nup88 at the NPC is currently unknown. Nup358/RanBP2 is localised to the cytoplasmic filaments of the NPC (63, 66, 68). TEM of purified Nup358/RanBP2 revealed a ~36nm filamentous structure, and Nup358 depletion from *Xenopus* egg extracts caused assembly of NPCs lacking detectable cytoplasmic filaments, indicating Nup358 as a major, and possibly the only nucleoporin constituent of these filaments (12, 63). No nucleoporin binding partners have been found for Nup358, therefore the molecular association of the cytoplasmic filaments with the NPC is unknown. Nup88 and also Nup214 represent possible candidates, although *in vitro* assembled Nup214-depleted NPCs did have cytoplasmic filaments (63).

Soluble transport receptors are carriers that mediate the active transport of macromolecules through the NPC (23, 59). Biochemical studies have shown that for groups of transport substrates there is a specific transport receptor that utilises a subset of nucleoporins to translocate the NPC (45). Many nucleoporins have been shown to bind certain transport receptors *in vitro*, providing primary indication of their roles in specific transport pathways. However the precise roles of these proteins *in vivo* largely remain to be determined, and in vertebrates only a handful of nucleoporins have been shown to play dominant roles in specific transport pathways using model systems, such as *in vitro* nuclear assembly of *Xenopus* egg extracts, the use of antibodies or overexpression in cultured cells and knockout mice (e.g. (5, 57, 62). Nup214 has been shown to interact with several import receptors *in vitro* (44, 67), however Nup214 depletion from *Xenopus* egg extracts resulted in assembly of synthetic nuclei still capable of nuclear protein import (63), illustrating that biochemical evidence is not necessarily indicative of an important functional role. With the advent of new techniques, including RNA interference, more direct functional roles of nucleoporins in specific nucleocytoplasmic transport pathways in vertebrates can be investigated, as has been demonstrated for the role of the Nup107 nucleoporin subcomplex in NPC assembly (26, 60).

Proteins to be exported from the nucleus, including transcription factors and certain shuttling proteins, carry a short and hydrophobic nuclear export signal (NES), which was originally discovered in HIV-1 REV and PKI (16, 65). CRM1 is the transport receptor that recognises NES-containing substrates (1, 19, 21, 47, 55), which belongs to a group of export receptors or exportins that bind their substrates with RanGTP in the nucleus, to form a trimeric export complex (3, 19, 30, 36, 37, 56). Like other nuclear transport receptors, CRM1 is thought to interact directly with specific nucleoporins at the NPC to mediate transport. Immunoprecipitation studies predict that the most stable interaction of CRM1 at the NPC is Nup214, and this complex is more stable in the presence of RanGTP and NES-substrate (4, 32). Nup358 has also been identified in a complex with CRM1, mediated by the zinc finger domains of Nup358 (54), which suggests a role in NES-protein export, however more direct data supporting an important role *in vivo* is lacking.

In this study we investigate the organisation of the three asymmetrically localised cytoplasmic nucleoporins identified in vertebtrates, Nup214, Nup88 and Nup358 *in vivo*, to determine the mechanism of assembly of the cytoplasmic filaments. Using immuno electron microscopy we have localised Nup88 to a position separating Nup358 on the cytoplasmic filaments, and Nup214 near the cytoplasmic ring, and show a novel interaction between Nup88 and Nup358. We show that both Nup88 and Nup214 play a combined role in anchoring Nup358 to the NPC by individually knocking down their *in vivo* expression using RNA interference, and an interdependence for their own stability and NPC localisation. We also show a functional role for Nup358 in CRM1-mediated protein export which elaborates and extends earlier biochemical data.

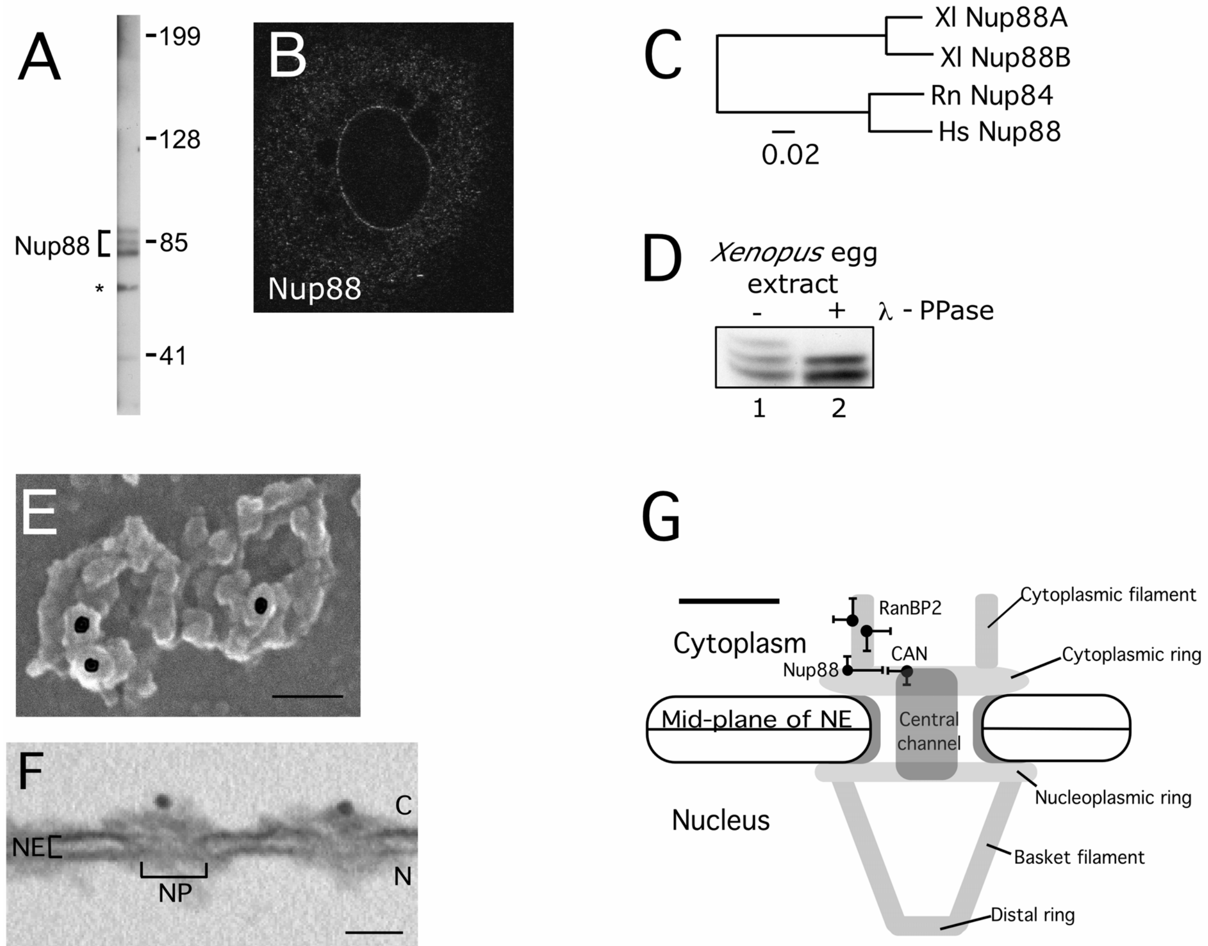
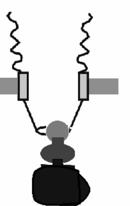


Figure 1: *Xenopus* Nup88 is encoded by two genes and is phosphorylated, and localises adjacent to Nup214/CAN and Nup358/RanBP2. (A) Western blot of *Xenopus* egg extracts probed with anti-XNup88. Asterix represents a non-specific cross-reacting band. (B) Immunofluorescence of *Xenopus* A6 cells probed with anti-XNup88, to show specific staining of the NE. (C) A phylogram to show the divergence of the two genes encoding Nup88 in *Xenopus laevis*, and their conservation with respect to human Nup88 and rat Nup84. (D) Western blot of *Xenopus* egg extracts before (-) and after (+) treatment with lambda protein phosphatase (λ-PPase) probed with anti-XNup88. (E) Representative Scanning electron micrograph (SEM) of an isolated *Xenopus* oocyte NE labelled with anti-XNup88, which was secondary labeled with 10nm colloidal gold. Bar = 50nm (F) Representative Transmission Electron Micrograph (TEM) of a 70nm cross-section through an isolated *Xenopus* oocyte NE labeled with anti-XNup88, and secondary labeled with 10nm colloidal gold. N = nucleus, C = cytoplasm. Bar = 50nm. (G) Summary diagram of the NPC displaying the mean localisation of Nup358, using two separate antibodies directed to C-terminal regions of the protein (see (63), Nup88 and Nup214. Bar = 50 nm. Error bars represent standard deviations of the mean.

Results

Nup88 is a phosphorylated protein encoded by two genes in *Xenopus laevis*, and is localised adjacent to Nup358/RanBP2 and Nup214/CAN on the cytoplasmic face of the NPC

Three vertebrate nucleoporins are known to localise mainly to the cytoplasmic face of the NPC; Nup214, Nup358 and Nup88. Previous studies have determined the ultrastructural localisation of Nup214 and Nup358 at the NPC (33, 48, 63, 66, 68). In order to investigate the



relative organisation of these three nucleoporins, we localised Nup88 on isolated *Xenopus* oocyte nuclear envelopes using immuno-gold electron microscopy (EM). The sequence of *Xenopus* Nup88 was extracted from EST databases using evolutionary conservation to the human and rat Nup88 homologues, and a polyclonal antibody was raised against a purified recombinant C-terminal fragment of *Xenopus* Nup88 comprising amino acids 312 to 741. After affinity purification, the antibody recognised a pattern of three bands at the approximate molecular weight of Nup88 on a Western blot of *Xenopus* egg extracts (Fig. 1A). A fourth band was also observed, which was subsequently found to be non-specific as it was absent when using the XNup88 antibody raised in a different animal (not shown). To determine the specificity of the anti-XNup88 antibody in cells, *Xenopus* A6 cells were fixed, permeabilised and immunostained with anti-XNup88. A punctate staining of the NE was observed (Fig. 1B) characteristic of nucleoporins, which overlapped with monoclonal antibody (mAb)414 (11), which recognises the FG-repeat containing nucleoporins, Nup358, Nup214, Nup153 and p62 (data not shown).

Xenopus laevis is a partially tetraploid organism, having duplicated its genome ~30 million years ago. To determine if the *Xenopus* Nup88 protein is encoded by two divergent genes, which would partly explain the multiple banding pattern we observe, a more detailed analysis of the *Xenopus* EST database was undertaken. Two distinct mRNA species were found, both encoding a protein product homologous to human and rat Nup88, but only 91% identical to each other, too low to be explained by intraspecies variation alone. In addition, the 3’ and 5’ UTR sequences were more divergent than the coding region, which further suggests that Nup88 is encoded by two separate genes in *Xenopus*. The two genes are designated XlNup88A and XlNup88B and a phylogram shows their evolutionary conservation with respect to Human Nup88 and Rat Nup84 (Fig. 1C). XlNup88A encodes a predicted protein product of 726 amino acids, compared to 728 of XlNup88B, and the predicted charge of the two proteins was strikingly different, -17.8 for XlNup88A, and -9.6 for XlNup88B, both of which could contribute to a difference in electrophoretic mobility.

Many nucleoporins are phosphorylated during mitosis, coinciding with NPC disassembly (15, 38, 43, 61) and phosphorylation also affects electrophoretic mobility of proteins. To further investigate the multiple banding pattern of *Xenopus* Nup88, *Xenopus* egg extracts were incubated with a non-specific protein phosphatase from lambda (λ-PPase), before analysis by gel electrophoresis and Western blot. The three banded pattern of Nup88 was reduced to two bands, presumably representing the unphosphorylated forms of the two Nup88 proteins (Fig. 1D). These data suggest that *Xenopus* Nup88 is a phosphorylated nucleoporin and is encoded by two highly homologous but independent genes.

The ultrastructural localisation of Nup88 at the NPC was determined by labelling isolated *Xenopus* oocyte nuclear envelopes with the anti-XNup88 antibody followed by 10 nm gold-conjugated secondary antibody. The labelled envelopes were visualised using field emission scanning EM (FESEM), to image the surface of the NE, and transmission EM (TEM) of 70 nm cross sections through the NE. Representative micrographs from FESEM and TEM are shown in Figs. 1E and F. Using FESEM, the localisation of the gold particles along a radial axis was determined. The mean distance from the centre of the NPC was 39 nm ± 17.4 (n=87). Using TEM, the gold particles were measured distally from the mid-plane of the NE. The mean distance was 30.4 nm ± 7.6 (n=22). The localisation data is summarised in Fig 1G, along with previous localisation of Nup358 and Nup214 using the same method (63). From these labelling data, Nup88 localises, at least in part, to a position in between Nup358 and Nup214.

Nup88 is in a complex with both Nup358/RanBP2 and Nup214/CAN

The immunolocalisation studies position Nup88, Nup214 and Nup358 in close proximity at the cytoplasmic face of the NPC; however only Nup214 and Nup88 have been linked biochemically and nucleoporin binding partners for Nup358 have so far not been identified. To

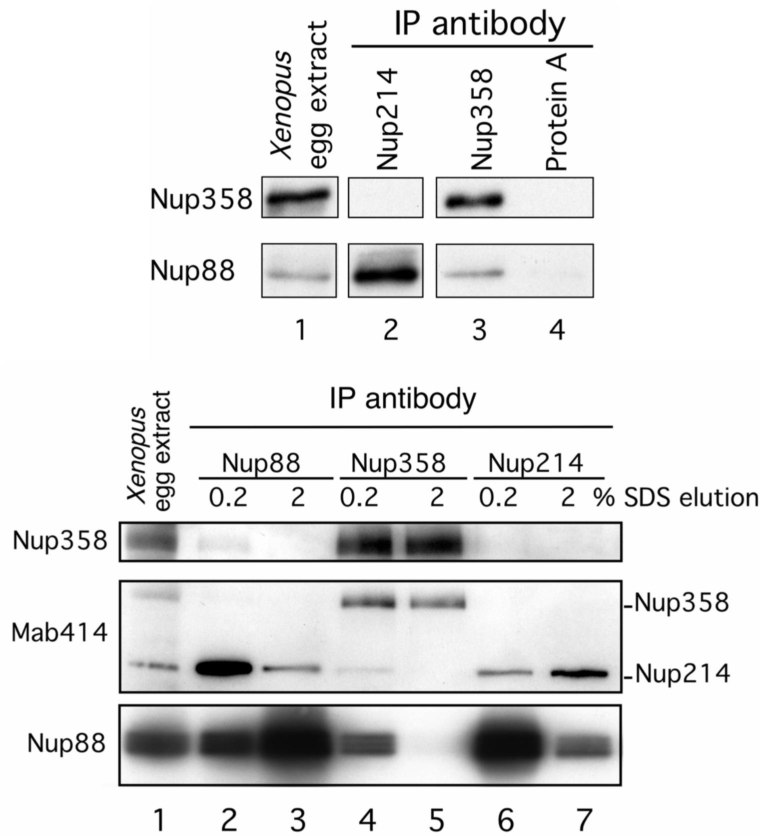
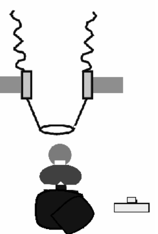


Figure 2: Nup88 is coimmunoprecipitated with both Nup214/CAN, and Nup358/RanBP2. Antibodies to Nup214, Nup358, or Protein A Sepharose were incubated with *Xenopus* egg extract and coimmunoprecipitating proteins analysed by labeling a Western blot with anti-Nup358, or anti-XNup88.

investigate interaction partners of Nup358 we immunoprecipitated Nup358 or Nup214 from fractionated *Xenopus* egg extracts, isolated the bound protein complexes using protein-A sepharose after extensive washing, and analysed the co-immunoprecipitating proteins by gel electrophoresis and Western blotting. Nup88 was found to specifically coimmunoprecipitate with Nup214 (Fig 2, lane 2) as has already been shown (6, 17, 18). Interestingly, Nup358 was also able to co-immunoprecipitate Nup88 from *Xenopus* egg extracts (Fig 2, lane 3). These data show that both Nup214 and Nup358 are interacting partners of Nup88, consistent with their ultrastructural localisation.

RNA interference of Nup88 and Nup214/CAN causes a reduction of Nup358/RanBP2 at the nuclear envelope

Based on immunoelectron microscopy, Nup358 is the most distal cytoplasmic nucleoporin from the midplane of the nuclear envelope, apparently located above Nup88 and Nup214. The immunoprecipitation studies predict Nup88 and/or Nup214 as the sites of interaction through which Nup358 may dock to the NPC. To study the organisation of Nup88, Nup358 and Nup214 we utilised the technique of small interfering RNAs (siRNA, (13) expressed by the pSUPER vector (9) to reduce endogenous expression of each nucleoporin and analyse the localisation of the others using immunofluorescence in human cells. Oligonucleotides containing 19 bases from the mRNA sequences of Nup358, Nup214 and Nup88 were cloned into the pSUPER expression vector as described in Materials and Methods. The pSUPER expression vectors were transfected into HeLa or MCF-7 cells by either electroporation or lipofection as indicated. Empty pSUPER vector was transfected as a negative control in all experiments. After 72 hours, double immunolabellings were performed and nuclear envelope staining intensities of the nucleoporins were quantified in cells which



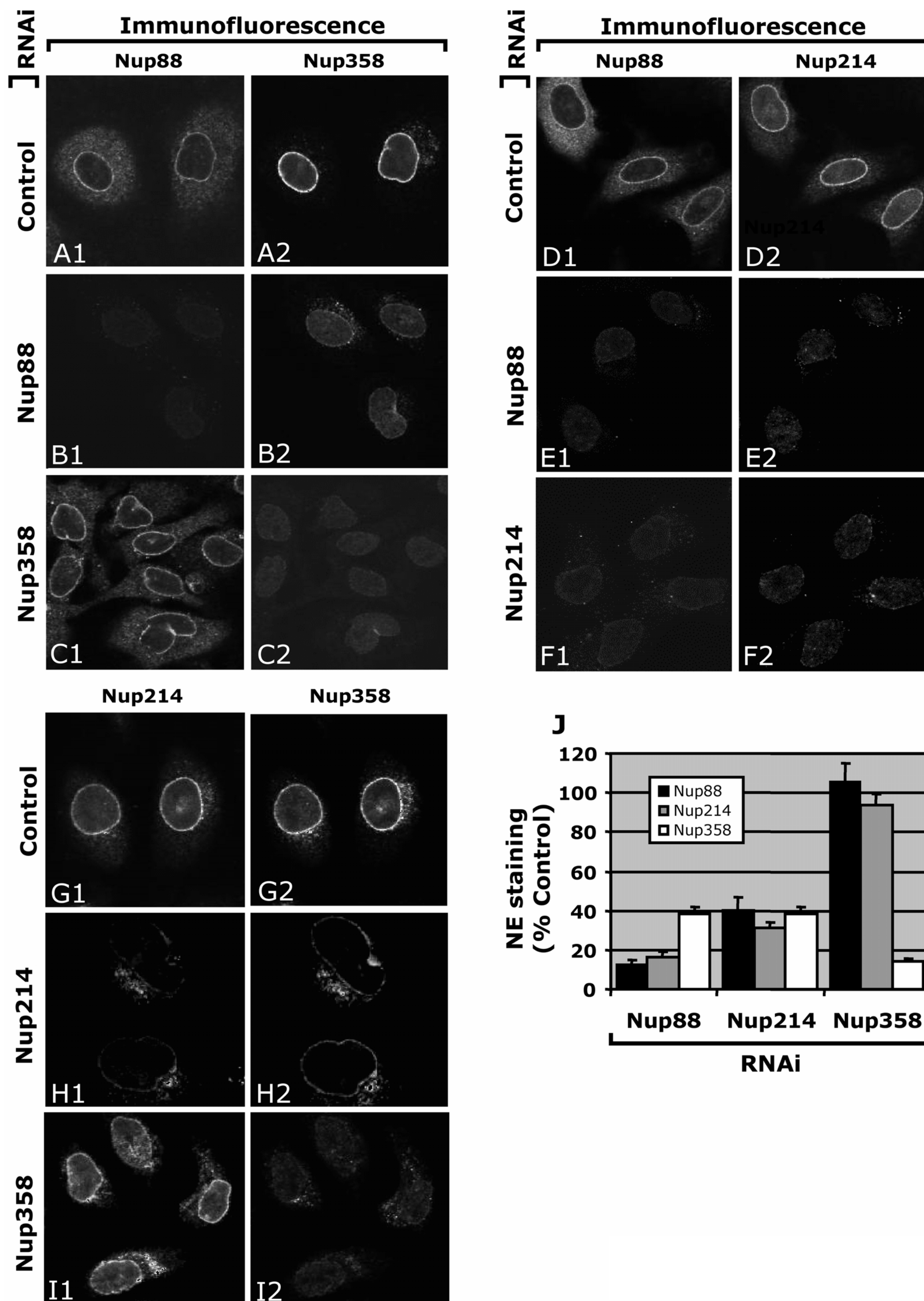


Figure 3: Knockdown of Nup88 or Nup214/CAN causes a decrease in Nup358/RanBP2 at the nuclear envelope (NE). Immunofluorescence of HeLa cells after knockdown of Nup88 (B and E), Nup214 (F and H) and Nup358 (C and I). Cells were fluorescently double-labeled with anti-hNup88 and anti-hNup358F (A, B and C), anti-hNup88 and anti-CAN9977 (D, E and F) or anti-CAN9977 and anti- Nup358V antibodies (G, H and I). A,D and G show control levels 72 hours after transfection with empty pSUPER vector. (J) Graphic representation of the results to show fluorescence levels of Nup88, Nup214 and Nup358 after knockdown of each individual nucleoporin as a percentage of the negative control. Nup358 analysis was performed using two different antibodies leading to similar results.

showed clear reduction of the nucleoporin targeted by RNAi, and compared to the levels in control cells. As shown in Figure 3, antibodies against Nup88, Nup214 and Nup358 decorated the nuclear envelope in the punctate manner characteristic of nucleoporins. Cytoplasmic pools of these nucleoporins were also visible at different intensities. Nup88 antibodies showed more dispersed and higher levels of labelling in the cytoplasm than Nup214 or Nup358, whose cytoplasmic staining was more discrete and concentrated in cytoplasmic bodies (Fig 3, A, D and G). Nuclear envelope staining of each of the three nucleoporins was significantly reduced after transfection with their respective RNAi expression plasmids (Fig 3, B1, E1 for Nup88 RNAi; F2, H1 for Nup214; C2, I2 for Nup358), which was verified by gel electrophoresis and Western blotting (see Fig 4). Interestingly, Nup358 nuclear envelope staining was significantly reduced after RNAi of either Nup88 or Nup214 (Fig 3, B2 and H2 respectively and J). Conversely, Nup358 RNAi had no effect on Nup88 or Nup214 staining (Fig 3, C1 and I1 respectively and J), however RNAi of either Nup88 or Nup214 provoked significant reduction of the other at the NE (Fig 3, E and F respectively, and J). These data suggest that Nup88 and Nup214 codepend to incorporate into the NPC, and Nup358 docking to the NPC *in vivo* requires the presence of both Nup88 and Nup214.

Nup88 RNAi causes an associated decrease in the protein levels of Nup214/CAN but not Nup358/RanBP2.

The specific reduction of Nup358 at the NE after RNAi of Nup88 or Nup214 could also be the result of a decrease in the stability of Nup358 and its subsequent degradation. To study the effect of nucleoporin RNAi on the protein levels of the remaining untargeted nucleoporins, 48, 72 and 96 hours after transfection cells were lysed directly in SDS-sample buffer to minimise breakdown, and the proteins analysed by gel electrophoresis and Western blotting. Transfection of cells with either pSUPER-Nup358 (Fig 4A) or pSUPER-Nup88 (Fig 4B) results in a clear specific knockdown of these proteins within 48 hours, which was stable until the last time point tested at 96 hours, confirming the decrease observed by immunofluorescence. Western blots were also

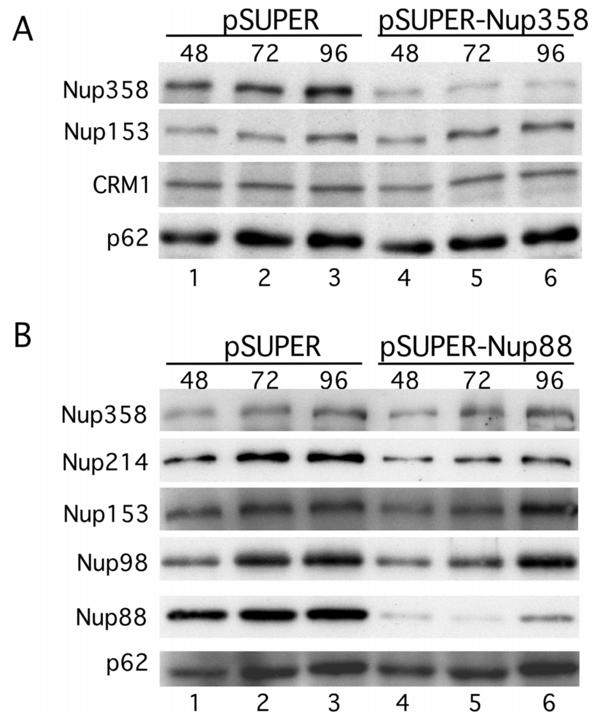
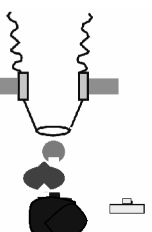


Figure 4: Efficient knockdown of Nup88 and Nup358/RanBP2 using RNAi, and co-reduction of Nup214/CAN on knock down of Nup88. (A) Western blot of MCF-7 cells transfected by electroporation with pSUPER-Nup358 compared to the pSUPER negative control collected 48, 72 and 96 hours post-transfection. The blot was probed with anti-Nup358, Mab414 and anti-CRM1. (B) Western blot of HeLa cells transfected by Fugene with pSUPER-Nup88 compared to the pSUPER negative control collected 48, 72 and 96 hours post-transfection. The blot was probed with anti-Nup358V, anti-hNup88, anti-hNup214, anti-Nup98 and Mab414.

labelled with other nucleoporin antibodies including mAb414. Nup358 RNAi showed no decrease in the levels of Nup214 or Nup88 at the NE as shown by immunofluorescence (Fig. 3), and there was no associated decrease in the protein levels of either Nup153 or p62 (Fig. 4A). Knockdown of Nup88 however, resulted in a significant decrease in the protein levels of Nup214 (Fig. 4B), indicating that Nup214 is less stable in the absence of Nup88. Importantly there was no associated decrease in the protein levels of Nup358, indicating that it is the specific attachment of Nup358 to the NPC which is blocked by Nup88 RNAi and not an effect on protein levels. On knockdown of Nup88 there was no detectable difference in protein levels of either



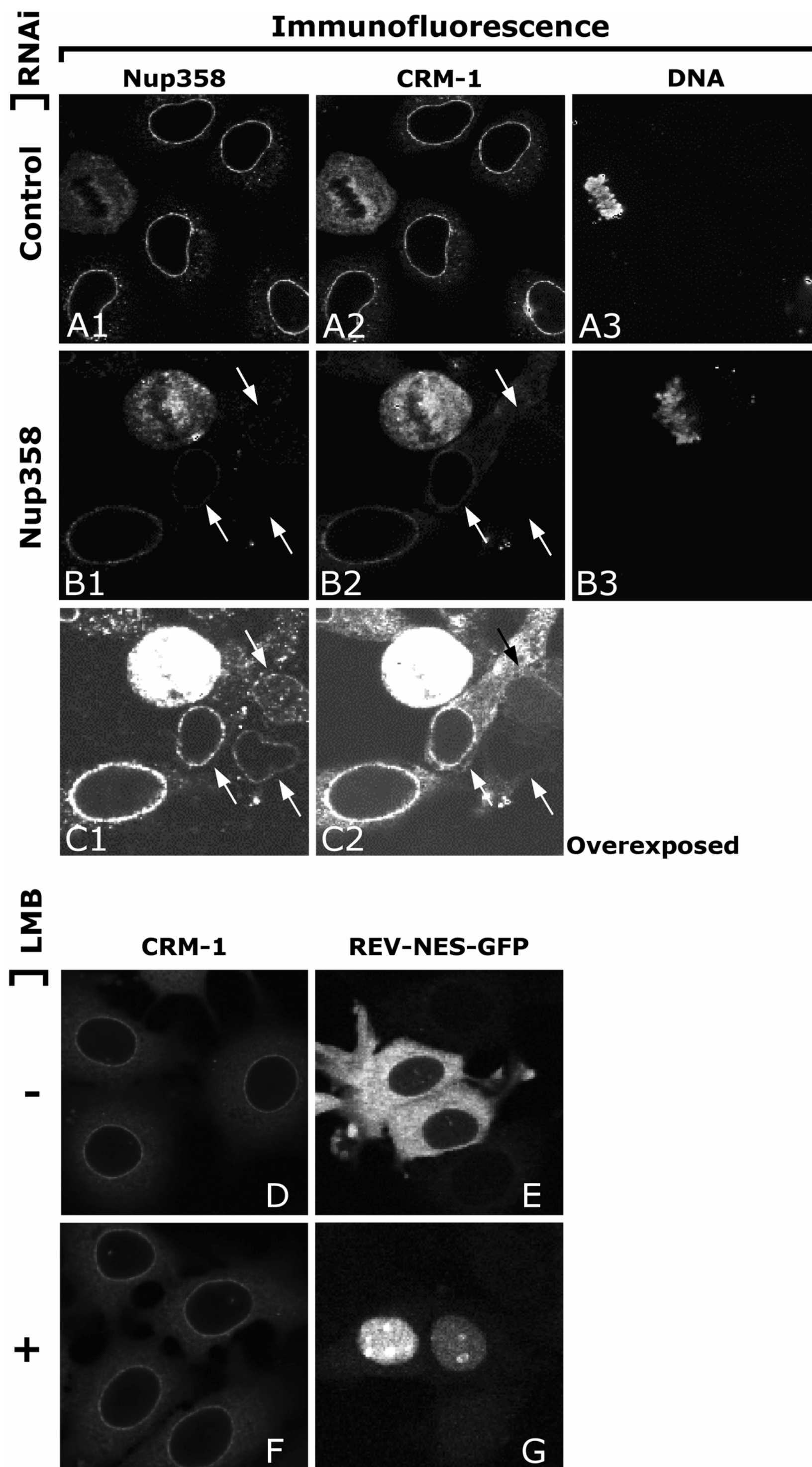


Figure 5: CRM1 is mislocalised from the cytoplasmic side of the nuclear envelope in Nup358 knocked down cells but not when cargo substrate binding is inhibited. HeLa cells were fixed and permeabilised with 0.001% digitonin 72 hours post-tranfection with pSUPER (A) or pSUPER-Nup358 (B and C). Antibodies for Nup358, CRM1 and DNA were applied. (B and C) Nup358 knocked down cells, indicated by arrows, show reduced CRM1 nuclear envelope staining at the accessible side of the nuclear envelope. Under these conditions, DNA antibodies can only access dividing cells (A3 and B3). CRM1 localisation at the cytoplasmic side of the nuclear envelope was not altered on leptomycin B (LMB) treatment of MCF-7 cells (D and F). Treatment was sufficient to abolish CRM1 mediated export of a REV-GFP construct (E and G).

Nup153 or p62, or indeed Nup98, which has also been shown to bind Nup88 *in vitro* (25).

RNA interference of Nup358/RanBP2 mislocalises CRM1

We have shown that Nup358 RNAi specifically reduced its own protein levels without affecting the protein levels of Nup214, Nup88 or CRM1. Immunostaining of CRM1 in mammalian cells shows that this transport receptor is highly concentrated at the nuclear envelope (1, 20, 34), however its binding site(s) are currently unknown, and were still present in Nup214 deficient mouse blastocysts (20). In order to investigate the localisation of CRM1 at the NPC and a possible role for Nup358 in export, we immunolabelled HeLa cells transfected with either empty pSUPER or pSUPER-Nup358. 72 hours after transfection, cells were fixed and digitonin permeabilised to visualize only the cytoplasmic side of the NE. Triple labelling of Nup358, CRM1, and DNA (to verify the digitonin treatment) was performed and images were obtained using confocal microscopy.
As shown in Figure 5A, when the NE is intact and DNA antibodies are unable to enter the nucleus, CRM1 is visible at the cytoplasmic side of the nuclear envelope. However after Nup358 RNAi, CRM1 accumulation is lost (Fig 5, B2 and overexposed C2), clearly indicating that CRM1 localisation at the cytoplasmic side of the nuclear envelope is dependent on Nup358.
To investigate whether the Nup358-dependent localisation of CRM1 at the nuclear periphery represented export complexes or empty CRM1, we treated MCF-7 cells with 100 nM leptomycin B for three hours. Leptomycin B covalently binds to CRM1 and dissociates it from RanGTP and NES substrates (19, 35, 46). Indeed, leptomycin B efficiently blocked CRM1-dependent nuclear export as a Rev-GFP-NES substrate accumulated in the nucleus (Figure 5G). Under these conditions, CRM1 was not reduced at the nuclear periphery (Figure 5F). Leptomycin B treatment also did not change the reduction of CRM1 upon Nup358 depletion (not shown). These data indicate that the Nup358-dependent CRM1 localisation at the nuclear periphery represents CRM1 in its empty state.

Nup358/RanBP2 plays a supportive role in CRM1-mediated NES-protein export

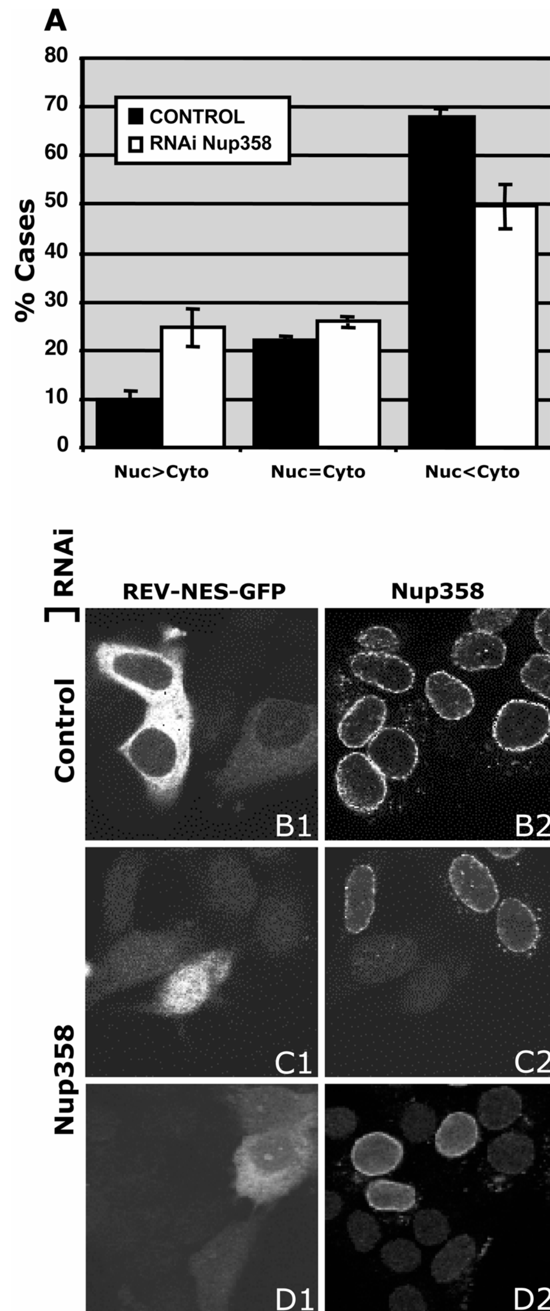
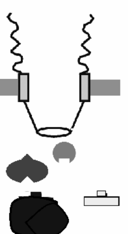


Figure 6: Nup358/RanBP2 knocked down cells are less efficient in CRM1-mediated export of Rev-GFP. (A) Rev-GFP transfected MCF-7 cells were scored for predominant accumulation of GFP in the cytoplasm (nuc < cyto), the nucleus (nuc > cyto), or equal distribution (nuc = cyto) between the two compartments under control (black bars) or Nup358 RNAi (white bars) conditions. The mean distribution in three independent experiments is shown; error bars represent standard errors. Illustrative images of Nup358 (C1 and C2) and empty pSUPER control (B1 and B2) are shown.



To determine whether loss of CRM1 from the nuclear envelope after Nup358 RNAi is functionally significant for CRM1-mediated NES-protein export, export assays were performed in MCF-7 cells by cotransfecting pSUPER with a GFP-linked export substrate that includes both the NLS and NES of the HIV-1 Rev protein (pRev-GFP; (27). Previous studies have shown that under normal conditions Rev-GFP partially accumulates at the nucleoli, but on treatment of cells with actinomycin D the protein is specifically exported and accumulates in the cytoplasm. The localisation of the export substrate was determined 72 hours after transfection using confocal microscopy. Cells were cotransfected with pRev-GFP and either pSUPER empty vector or pSUPER-Nup358. 72 hours after transfection, cells were treated with 5 µg/ml actinomycin D for 3 hours and then fixed and prepared for confocal microscopy. Protein export was quantified in three independent experiments by counting the number of cells (n>100) with predominantly cytoplasmic accumulation of GFP, a predominantly nuclear accumulation, or an even distribution between the nucleus and cytoplasm. Under control conditions, Rev-GFP was localised predominantly in the cytoplasm in 68% ± 2, consistent with it being efficiently exported (Fig. 6A and B1). 22% ± 1 of cells had Rev-GFP dispersed evenly between the nucleus and cytoplasm, and the remaining 10% ± 2 of cells showed nuclear accumulation. However, after Nup358 RNAi, the localisation of Rev-GFP was significantly redistributed towards the nucleus (Fig. 6A and C1). 50% ± 5 of cells now accumulated Rev-GFP in the cytoplasm, 26% ± 1 had an even distribution throughout the cell, and 25% ± 4 accumulated Rev-GFP predominantly in the nucleus (Figure 6C1). These results show that Nup358 plays a contributory role in CRM1-mediated export of NES cargoes.

Discussion

We have investigated the organisation of three cytoplasmically orientated nucleoporins, Nup358, Nup88 and Nup214 at the NPC. Previous studies have localised Nup358 and Nup214 to specific NPC substructures (33, 48, 63, 66, 68). Here we immunolocalise Nup88 and study the role of each protein in assembling mature NPCs. We also study in detail the function of the cytoplasmic filaments in CRM1-mediated nuclear protein export.

Characterisation of *Xenopus* Nup88
Nup88 was originally identified as an interacting partner for Nup214 (6, 20). Here we studied Nup88 initially in *Xenopus laevis*. and found it to be encoded by two divergent genes. The two genes, designated XlNup88A and XlNup88B, are 91% homologous, and gene A was found to be more abundant in the EST database, indicating that it is the more common form. We don't currently know which of the two bands identified by the antibody on Western blots of dephosphorylated *Xenopus* extracts represents which form of Nup88. It is also unclear at present whether the two Nup88 forms possess redundant functions. *Xenopus* Nup88 was also found to be phosphorylated, which is a common feature for many nucleoporins, and is thought to be linked to nuclear envelope breakdown and reassembly after mitosis (15, 38, 43, 61).

Assembly of the cytoplasmic filaments
Using a combination of approaches we find that the association of Nup358, which is predicted to be a predominant component of the cytoplasmic filaments from EM structural analysis and studies in *Xenopus* (12, 63), is dependent on interactions with and between both Nup214 and Nup88, providing evidence of a structural collaboration between these three nucleoporins to assemble mature NPCs. Firstly we have localised *Xenopus* Nup88 to a position in close proximity to both Nup214, a known interacting partner, and Nup358, placing it in a position to physically interact with the cytoplasmic filaments, possibly with the N-terminal leucine-rich domain of Nup358 that is suggested to be located at the more central position (63). We show that Nup88 can be coimmunoprecipitated with both Nup358 and Nup214 indicating that the three proteins do indeed interact *in vivo*. It remains possible that the Nup88-Nup358 interaction is bridged by additional nucleoporins. The levels of Nup88 that were co-immunoprecipitated with Nup358 were significantly less than with Nup214, however Nup214 is known to form a soluble and stable subcomplex with Nup88 in the cytoplasm (6, 20), and it is possible that the interaction of Nup358 with Nup88 occurs predominantly at the NPC. To

show that the observed interactions and ultrastructural organisation of these three proteins is relevant to the organisation of the *in vivo* NPC we showed, using an RNAi approach, that the association of Nup358 with the NPC was specifically dependent on the presence of both Nup214 and Nup88. We also found that localisation of Nup214 and Nup88 at the NPC occurred only in the presence of each other, indicating a codependence of NPC localisation. This interdependence is consistent with the absence of Nup88 in Nup214 knock-out blastocysts (58) and a mislocalisation of Nup159, the closest yeast homologue of Nup214, in Nup82-Δ108 cells, that carry a mutation in the yeast homologue of Nup88 (28).

We have previously shown that the cytoplasmic filaments and Nup358 were still present in *in vitro* synthesised NPCs that lack Nup214 (63), again suggesting that it is Nup88 that makes a physical connection to Nup358 rather than Nup214. Indeed we were unable to detect co-precipitations between Nup214 and Nup358. The precise mechanism of this interdependence remains to be elucidated, although it may partially be explained by protein stability, as reduction of Nup88 caused a significant decrease in the protein levels of Nup214.

Nup358/RanBP2 and NES-mediated export

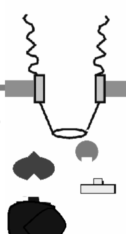
Using an *in vivo* export assay that was previously used to measure differences in nuclear export signal strength (27), we found a significant decrease in CRM1-mediated nuclear export when expression of Nup358 was substantially reduced i.e., by 90% or more. In experiments where RNA interference was less efficient, hardly any reduction in nuclear export could be observed. This may explain why RNA interference of Nup88 did not affect NES-mediated export, as Nup358 levels, even though strongly reduced, were not as low.

Hydrolysis of GTP on Ran is not required for a single round of NES-mediated export (14, 51), however continued export is predicted to be dependent on recycling of both Ran and CRM1. In addition, it appears that rapid export complex disassembly by RanGTP hydrolysis close to the cytoplasmic face of the NPC is required for overall export efficiency by enhancing the directionality of export (7). Biochemical experiments have indicated that CRM1-NES-RanGTP export complexes bind stably to Nup214 and are disassembled by the combined action of RanGAP and either RanBP1 or RanBP1-like domains of Nup358 (4, 32). A main candidate to provide these activities is Nup358, as it associates with RanGAP, contains RanBP1-like domains and is located close to the cytoplasmic exit of the NPC (39, 41, 42, 66, 68). The presented results showing a decrease in export due to Nup358 depletion therefore provide direct *in vivo* support of these models. We do not presently have *in vivo* evidence for which is the terminal binding site of CRM1 containing export complexes. The strong *in vitro* interaction with Nup214 makes this a likely candidate. However, we do not observe accumulation of export complexes at the nuclear periphery when Nup358 is depleted, suggesting that interaction with Nup214 is at least not rate limiting. Conversely, we find a strong decrease of CRM1 at the nuclear periphery when Nup358 is absent. This Nup358-dependent nuclear rim localisation does not represent export complexes because it is not affected by leptomycin B, which is known to dissociate CRM1 from RanGTP and cargo (19, 35). Our data therefore indicate that Nup358 both provides a platform for rapid disassembly of CRM1-export complexes and a binding site for empty CRM1 recycling into the nucleus.

Materials and Methods

Antibodies

To generate specific antibodies against *Xenopus* Nup88 (anti-XNup88) a C-terminal fragment spanning amino acids 312-741 of the XNup88B was expressed as an N-terminal His_6-tagged fusion protein in pRSET A (Qiagen) in BL21 (DE3) CodonPlusRIL (Stratagene), isolated from inclusion bodies, and dialysed against PBS/8.7% glycerol to generate soluble protein. To generate antibodies against Human Nup214 (anti-hNup214), a cross-linked C-terminal peptide (amino acids 2076–2090) was synthesised. Antibodies were raised in rabbits and affinity purified against the antigen crosslinked to an Ultralink™ Iodoacetyl gel (Pierce). Anti-hNup358/RanBP2 antiserum, anti-hNup358V and



anti-hNup358F, were generously provided by Dr. V. Cordes (Karolinska Institute, Stockholm, Sweden), and Drs. A. Gast and F. Melchior (Max Planck Institute for Biochemistry, Munich, Germany) respectively. Anti-hCRM1 (20), anti-hNup88 (BD Transduction laboratories), anti-CAN9977 (Fornerod et al., 1995), monoclonal antibody (mAb) 414 (Eurogentec/Babco), anti-DNA 2C10 antibody, gift from Drs. Yoshiuki Kanai and Tetsuo Kubota (University of Tokyo, Japan) and anti-hNup98 (31) were previously published.

EM Immunolocalisation

Nup88 immunolocalisation on isolated *Xenopus* oocyte nuclear envelopes using TEM and FESEM was carried out as previously described (62). Briefly, antibodies were diluted 1:100 with PBS and incubated with isolated *Xenopus* oocyte NEs. The primary antibody was labelled with a 1:20 dilution of 10nm gold-conjugated anti-rabbit secondary antibodies (Amersham) and the samples prepared for analysis by FESEM or TEM. Negative controls minus primary antibody were performed and revealed the secondary antibodies to be specific (data not shown). For FESEM analysis, isolated NEs were visualised at 100kx to 300kx magnification and the position of gold-labelled antibodies were quantified in relation to the centre of the NPC. For TEM quantification the distance of the gold particles from the midplane of the NE was determined. All measurements were calculated using AnalySIS software (SIS, Munster, Germany).

***Xenopus* egg extracts and Immunoprecipitation**

Fractionated *Xenopus* egg extracts were prepared as previously described (62). For dephosphorylation of Nup88, 400units Lambda protein phosphatase (New England Biolabs) was added to 50 µl *Xenopus* egg extract according to the manufacturer's instructions, and incubated at 30°C for 15min. For immunoprecipitation, extracts were diluted 1:4 in binding buffer (200 mM NaCl, 20 mM Hepes-KOH, pH7.9, 1 mM β-mercaptoethanol, Complete Protease Inhibitor Cocktail (Roche), 8.7% glycerol) and incubated with 4 µg antibody for 1 hour at 4°C. 10µl bed-volume Protein-A Sepharose beads (Pharmacia Biotech) was added and incubated for 1 hour at 4°C. Beads were collected by centrifugation, washed three times in binding buffer, once in binding buffer supplemented with 500mM NaCl, and eluted in 2% SDS. Samples were mixed with SDS protein sample buffer, boiled for 5 minutes and analysed by SDS-PAGE and immunoblotting.

Cell Culture

HeLa cells (ATCC CCL-2) and MCF-7 cells were grown in DMEM supplemented with 10%FBS (GibcoBRL) and antibiotics, at 37°C and 5% CO_2 in a humidified incubator. *Xenopus* A6 Cells were cultured in L-15 (LEIBOVITZ) medium with Glutamax-1 W/ L-amino acids (Gibco) supplemented with antibiotics, at 20°C in a humidified incubator.

RNA interference of Nucleoporins

The oligonucleotides used for silencing of Nup88 (TGCTTTGTTGAACACATCC), Nup214 (TTGCCCAAGGAACGCTCGA) and Nup358 (CGAGGTCAATGGCAAACTA), were purchased from Sigma (UK), and cloned into the pSUPER vector as previously described (9). Empty pSUPER vector was used as a control. MCF-7 cells or low passage HeLa cells were transfected at an estimated efficiency of 50-95%, with either 3-4µg pSUPER plasmid using electroporation as described previously (2) or 2 µg pSUPER plasmid in 6 cm dishes using Fugene-6 (Roche) according to the manufacturers instructions. 48, 72 and 96 hours post-transfection, cells were either fixed for immunofluorescence or lysed directly in boiling SDS-sample buffer, and knockdown efficiency was analysed by SDS-PAGE and Western blotting. Nuclear export assay using pRev-GFP was performed as described (27), except that 240 ng of plasmid DNA was electroporated along with 4 µg of pSUPER plasmid and expression was allowed to proceed up to 72 hours.

Immunofluorescence Microscopy and Image Analysis

Cells were fixed for 15 minutes in fresh 3.7% formaldehyde and permeabilised with either 0.001% digitonin for 10 minutes (15 minutes for *Xenopus* cells) at room temperature or 0.2% Triton-X100 for 10 minutes at room temperature. For immunofluorescence, cells were blocked in

blocking buffer (1% skimmed milk in PBS) for 15 minutes at room temperature and incubated in primary antibody diluted in blocking buffer for 3 hours at room temperature. Cells were washed 3 times in blocking buffer and incubated in fluorescently-conjugated secondary antibody (Molecular Probes). For double immunolabellings, cells were incubated simultaneously in the two antibodies, except for Nup88 and Nup214 double immunolabelling where cells were incubated for 2 hours in anti-hNup88 followed by addition for 1 hour of anti-CAN9977. Cells were washed once in blocking buffer and once in PBS, then mounted in Vectashield (Vector Laboratories). Images were recorded with a Leica TCS SP2 confocal microscope. For quantification of immunofluorescence at the nuclear envelope confocal images were analysed using Image J software. Nuclear rim intensity measurements at four points per cell were averaged and subtracted from the intranuclear intensity and intercellular background. To exclude possible experimental error due to staining variability between samples, non-knocked down cells in the same imaged field were used as internal controls.

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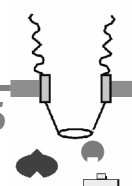

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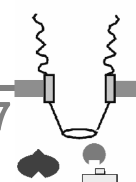

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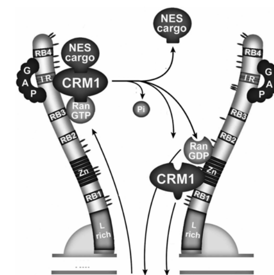
"I wear my sunglasses at night
So I can, so I can
Keep track of the visions in my eyes"

Corey Hart "Sunglasses at Night"



CHAPTER 4

Supraphysiological nuclear export signals bind CRM1 independently of RanGTP and arrest at Nup358

Dieuwke Engelsma, Rafael Bernad, Jero Calafat and Maarten Fornerod

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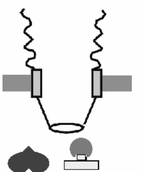

Supraphysiological nuclear export signals bind CRM1 independently of RanGTP and arrest at Nup358

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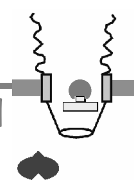
Leucine-rich nuclear export signals (NESs) mediate rapid nuclear export of proteins via interaction with CRM1. This interaction is stimulated by RanGTP but remains of a relatively low affinity. In order to identify strong signals, we screened a 15-mer random peptide library for CRM1 binding, both in the presence and absence of RanGTP. Under each condition strikingly similar signals were enriched, conforming to the NES consensus sequence. A derivative of an NES selected in the absence of RanGTP exhibits very high affinity for CRM1 *in vitro* and stably binds without the requirement of RanGTP. Localisation studies and RNA interference demonstrates inefficient CRM1-mediated export and accumulation of CRM1 complexed with the high-affinity NES at nucleoporin Nup358. These results provide *in vivo* evidence for a nuclear export reaction intermediate. They suggest that NESs have evolved to maintain low affinity for CRM1 to allow efficient export complex disassembly and release from Nup358.

Introduction

Nucleocytoplasmic transport occurs through large protein complexes that fenestrate the nuclear envelope (NE), termed nuclear pore complexes (NPCs) (reviewed in (39, 46, 48). Nucleocytoplasmic transport is accomplished by soluble transport receptors that interact with both cargo and NPC. Importins mediate import of several different classes of proteins, while exportins mediate nuclear exit of proteins, tRNAs, U snRNAs and other RNAs, with the exception of mRNAs (reviewed in (19).

The small GTPase Ran functions as a switch that governs directionality of importin and exportin-mediated transport (reviewed in (19). Nuclear Ran is predominantly bound to GTP, whereas cytoplasmic Ran is loaded with GDP. Nuclear RanGTP dissociates importin/cargo complexes providing direction to nuclear import (20). Exportin/cargo heterodimers require the cooperative binding of RanGTP as RanGTP/exportin/cargo heterotrimeric complexes are several orders of magnitude more stable (13, 26, 27). After translocation through the nuclear pore complex, export complexes are destabilised by RanBP1 or RanBP1-like domains in RanBP2/Nup358. The export reaction is completed by hydrolysis of Ran-bound GTP, stimulated by RanGAP1 (reviewed in (19).

One well-characterised example of the exportin class is CRM1/exportin1, which exports proteins exposing a leucine-rich NES (13, 16, 44). Similar to other importin β-like receptors, CRM1 has been suggested to translocate through the NPC by multiple low-affinity hydrophobic interactions with FG-repeat containing nucleoporins. (38, 39). In addition to these weak interactions, the FG repeat region of Nup214/CAN, a nucleoporin located at the cytoplasmic side of the NPC, forms a particularly strong interaction with CRM1, which is further stimulated by RanGTP and NES-cargo (2, 14, 24). Another nucleoporin that associates with CRM1 is Nup358/RanBP2. This protein forms large fibrillar structures that emanate from the NPC into the cytoplasm (Wu et al., 1995; Yokoyama et al., 1995; Delphin et al., 1997; Walther et al., 2002). CRM1 binds Nup358 in an empty state, which suggested that Nup358 serves as docking site for recycling CRM1 (3, 42). The majority of importins and exportins mediate nuclear transport of one or a few structurally related substrates (for



examples see (19). Notable exceptions are the importin α/β heterodimeric import receptor and CRM1, which transport a great variety of proteins and ribonucleoprotein particles across the NPC. This promiscuity in transport substrates likely evolved because these receptors recognise short ubiquitous peptide sequences. The leucine-rich NES recognised by CRM1 was first identified in the viral HIV-1 Rev protein (11) and in the cellular protein A phosphorylation inhibitor (PKI) (52). Both sequences contain a stretch of 4 regularly spaced leucines. Numerous studies have contributed to the definition of a leucine-rich NES consensus sequence as: $\Phi\text{-}X_{2\text{-}3}\text{-}\Phi\text{-}X_{2\text{-}3}\text{-}\Phi\text{-}X\text{-}\Phi$ (Φ: L, I, F, V, M; X: any amino acid)(6, 21, 28, 56). The presence of leucine residues is not a prerequisite for NESs and several NESs have been identified that diverge from this postulated consensus sequence (see (12) for review). Following the currently ill-defined NES consensus sequence, most proteins are predicted to harbour NES consensus sequences. This hampers the annotation of valid export signals and their characterisation *in vivo*.

Although currently characterised NESs differ to some extent in their capacity to bind CRM1, each possesses a rather low affinity for CRM1 (2, 36). This is not characteristic of all exportins, as exportin-t and CAS/exportin 2 bind their cargo in the low nM range (26, 27). Two strong CRM1-interactors have been reported: NMD3 and snurportin 1, which bind CRM1 with 100-fold higher affinity as compared to the well-studied Rev protein (36, 47). The interaction with snurportin 1 is mediated through a large domain of at least 159 amino acids, while the domain for strong interaction of Nmd3 is unknown.

In order to select for strong NESs and to chart NES diversity, we have screened a 15-mer random peptide library for CRM1-binding peptides. Surprisingly, both in the presence and absence of RanGTP, highly similar sequences were selected. *In vitro*, a derivative of one of these NESs bound CRM1 with high affinity, bypassing the requirement for RanGTP. *In vivo*, nuclear export of this signal was ineffective, as it accumulated at Nup358. We suggest that physiological NESs must maintain a low affinity for CRM1 to allow efficient disassembly from CRM1 and release from Nup358.

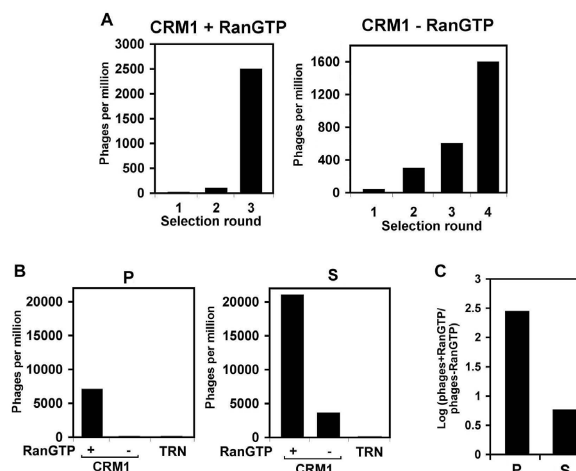


Figure 1. Selection of CRM1-binding peptides from a random peptide library. (A). A phage library displaying 15-mer random peptides was affinity selected on CRM1 columns in the presence or absence of RanGTP. The number of selected phages in each recursive selection round is expressed as colony forming phages selected per million of input. (B). Purified P (P0) and S (S0) phages were affinity selected on CRM1 and transportin 1 (TRN) columns in the presence or absence of RanGTP as indicated. Selected phages were compared as above. C. S phages are less responsive to RanGTP than P phages. Log ratios of phages selected on CRM1 columns in presence (phages+RanGTP) and absence (phages-RanGTP) of RanGTP are calculated for P (P0) or S (S0) phages.

Results

In vitro selection of synthetic NESs

To identify high-affinity peptide interactors of CRM1 we screened a fUSE5 15-mer random peptide library (35) with a complexity of $2*10^{8}$. Z-tagged CRM1 was immobilised on IgG-sepharose columns and affinity selections of $1*10^{9}$ infectious phage were performed in the presence or absence of RanGTP. To increase selectivity, phages selected in the presence of RanGTP were eluted through the combined action of RanGAP and RanBP1. Three to four selection rounds were performed through recursive cycles of phage amplification and affinity selection. As shown in Figure 1A, clear increases in the number of affinity-selected phages were apparent under both selection conditions. Sequence analysis revealed strong enrichment of a unique signal under each selection condition. A single phage bearing the

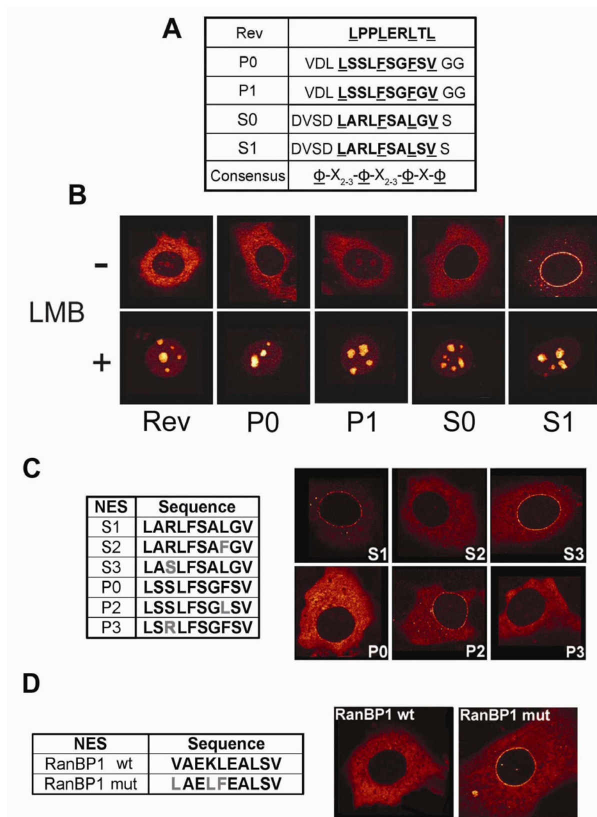
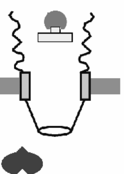


Figure 2. Permutations of selected peptides mediate distinct CRM1-mediated subcellular localisation. A. Amino acid sequences of P0, P1, S0 and S1 15-mer peptides compared to the HIV-1 Rev NES and the NES consensus sequence. NES sequences are in bold and consensus hydrophobic amino acids (ϕ) are underlined. One letter amino acid abbreviations are used. B. Shuttling reporter proteins containing GFP, the export incompetent Rev(1.4) variant and peptide sequences from (A) were transiently expressed in MCF7 cells and subcellular localisation was detected by green fluorescence 24 hours post transfection. The effect of 50 nM LMB was monitored 3 hours after addition. C. Identification of the critical amino acid residues in S1 NES. Positions 3 and 8 in P0 and S1 NESs were interchanged (left), and subcellular localisation of the GFP reporter plasmids were analysed as in (B) (right). D. Mutagenesis of the natural RanBP1 NES. A peptide corresponding to the natural RanBP1 NES was mutated to conform the high affinity NES consensus (left). Subcellular localisation was analysed as above (right).

peptide sequence VDLLSSLFSGFSVGG was enriched to near-homogeneity after three selection rounds in the presence of RanGTP and was termed Powerphage or P phage. This phage contained the NES consensus sequence LSSLFSGFSV, hereafter to be referred to as P0. After four selection rounds in the absence of RanGTP a single phage was highly enriched, termed Starphage or S phage, bearing the sequence DVSDLARLFSALGVS. Surprisingly, as NES peptides were not expected to bind in the absence of RanGTP, this peptide contains an NES consensus sequence LARLFSALGV very similar to P0 (Fig. 2A). This signal will hereafter be referred to as S0. Next, purified P phages and S phages were tested individually for their ability to specifically bind CRM1 in the absence or presence of RanGTP. No binding of either P phages or S phages was observed to z-tagged transportin 1 columns (Fig. 1B), confirming the specificity of the phage display selection. As expected, P phages bound CRM1 in the presence of RanGTP, and S phages in the absence of RanGTP (Fig. 1B). Binding of S phages was enhanced 6-fold by RanGTP, compared to a stimulation of approximately 250-fold for P phages (Fig. 1C). These data suggest that both in the presence and absence of RanGTP, leucine-rich-type NESs were affinity-selected on CRM1 from a random peptide pool.

In vivo activities of synthetic NESs

Even though the potential NESs P0 and S0 conform to the "3-2-1" spacing of hydrophobic amino acids, we noted an unusual glycine residue between the third and the fourth hydrophobic amino acid of the S0 sequence (Fig. 2A). A glycine at this position is known to abolish activity of the Rev NES (56). We therefore mutated this glycine into a serine in S0 and replaced in P0 the serine in this position for a glycine (Fig. 2A). We named these second-generation NES sequences S1 and P1, respectively. To test the export activities of the peptides *in vivo*, we inserted these into a reporter construct that has previously been used to compare NES activity (21). This reporter consists of green fluorescent protein (GFP) fused to a mutant form of the HIV-1 Rev protein, Rev(1.4), that provides importin ß-mediated import and nucleolar retention but lacks export activity.
When fused to the strongest NESs, this reporter localises completely to the cytoplasm (21). The HIV-1 Rev NES sequence was used as a positive control. As shown in Figure 2B, both P0 and S0



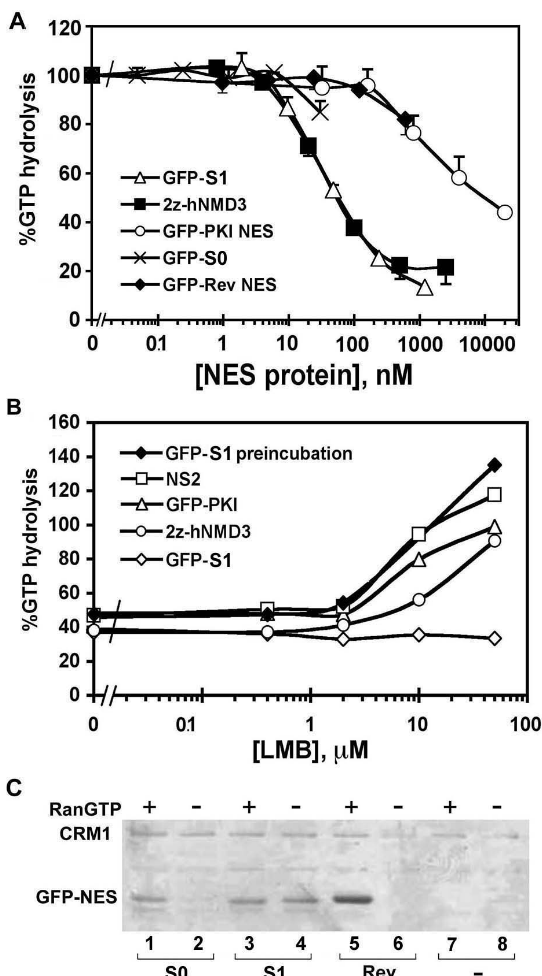


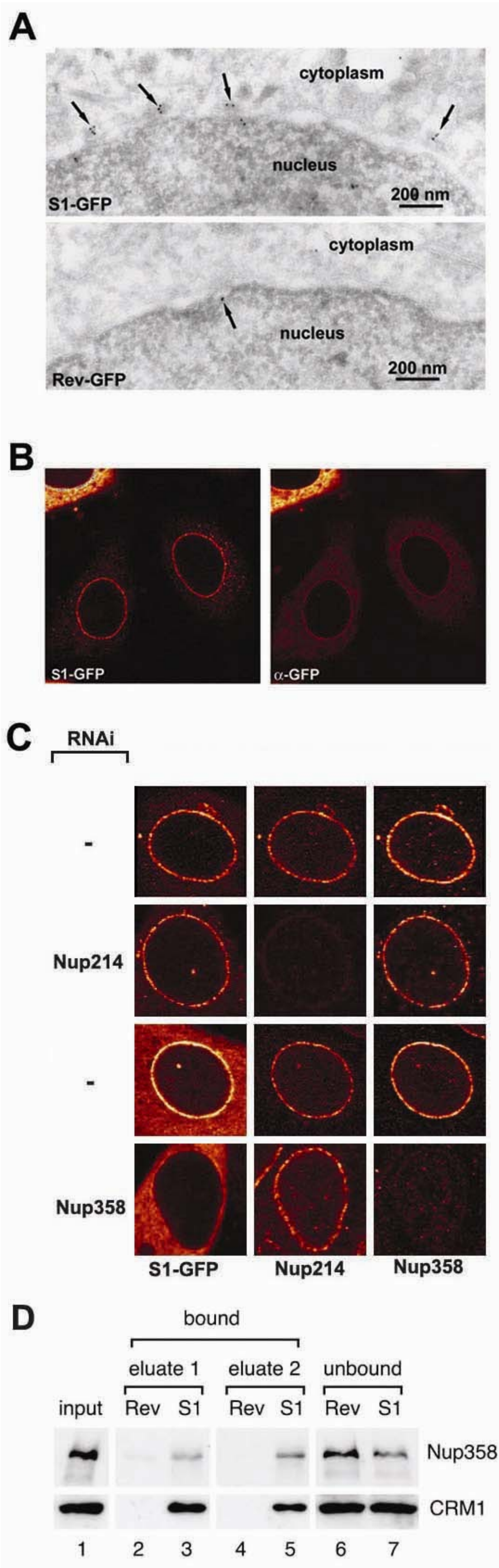
Figure 3. S1 NES binds to CRM1 with high affinity and independent of RanGTP. (A). CRM1 affinities of S0 and S1 peptides in recombinant GFP_3 fusion proteins were measured using the CRM1 RanGAP assay, which measures their ability in the presence of CRM1 to protect RanGTP from RanGAP-stimulated hydrolysis as a function of NES concentration. Regular strength NESs of PKI and HIV-1 Rev, and the high-affinity interaction of 2z-Nmd3 served as references. Error bars denote standard errors of three independent experiments. (B). Differences in LMB sensitivity of export complexes. Different concentrations of LMB were added to RanGTP/CRM1 complexes containing GFP_3-S1, GFP-PKI, the MVM NS2 peptide or 2z-Nmd3, and stability was measured using the CRM1 RanGAP assay as above. (C). RanGTP independent binding to CRM1 of S1 NES. IgG sepharose columns containing 1.5 μM z-tagged CRM1 were incubated in the absence (-) or presence (+) of 4.5 μM RanGTP as indicated, and in the absence (-) or presence of 1 μM GFP_3-S1 (S1), GFP_3-S0 or GFP_3-Rev (Rev). Bound fractions are visualised using SDS PAGE and Coomassie staining. A fraction of z-tagged CRM1 is co-eluted and indicated on the left.

localise the reporter protein only to the cytoplasm, indicating they confer strong export capacities, as the export signals completely overcome import activity as well as nucleolar retention. P1-GFP exhibits faint nucleolar staining similar to the Rev control NES. Interestingly, S1-GFP exhibited a prominent staining at the NE. This localisation is also appreciable for S0-GFP, although to a lesser extent. All NES fusion proteins accumulate in the nucleus upon treatment with the CRM1 inhibitor leptomycin B (LMB) (Fig. 2B). This demonstrates that the cytoplasmic localisation mediated by the P0, P1, S0 and S1 sequences, as well as the nuclear rim staining of S0-GFP and S1-GFP are CRM1-dependent.

The difference in localisation of the S1 and P0 NESs could be explained by their penultimate hydrophobic position, a critical position in Rev-type NESs. To determine if this is the case, we mutated this position into leucine in P0, creating P2, and into phenylalanine in S1, creating S2 (Fig. 2C). When introduced in the GFP reporter plasmid and expressed in cells as above, P2 mediated a clear nuclear rim staining, whereas S2 did not (Fig. 2C). No effect was observed when the arginine of S1 was changed to serine (S3), or the corresponding serine in P0 was changed to arginine (P3) (Fig. 2C). These data indicate that the amino acid sequence LXXLFXXLSL can mediate CRM1-dependent NE localisation. When the hydrophobic residues of a naturally occurring NES present in RanBP1 (57) were mutated conforming this consensus, this NES promoted clear nuclear rim localisation (Fig. 2D).

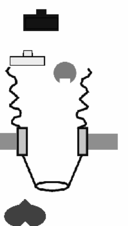
S1 NES binds to CRM1 with high affinity and stably binds without the requirement of RanGTP

In order to understand the striking localisation of the S1-like NESs, we analysed the CRM1 binding characteristics of this NES in RanGAP and pull-down assays. We expressed NES peptides as GFP fusion proteins in bacteria and purified the proteins using affinity chromatography. The CRM1 RanGAP assay is based on the fact that RanGTP is protected from RanGAP-mediated hydrolysis when present in export complexes, and can therefore be used to compare NES affinities (2, 26, 36). As references for standard NES



strength, we used GFP fused to the PKI or Rev NES (11, 52), whereas the z-tagged form of Nmd3 was used as a reference for a high-affinity CRM1 interaction in this assay (47). As shown in Figure 3A, the protein containing the S1 NES showed an approximately 100-fold higher affinity for CRM1 than the standard NESs. In fact, the affinity of S1 for CRM1 was comparable to the 2z-Nmd3 protein. The S0 NES showed an affinity in between S1 and the standard NESs. To further evaluate the affinity of S1 for CRM1, we tested the LMB sensitivity of the S1/CRM1/RanGTP complex. Increasing concentrations of LMB were added to preformed NES/CRM1/RanGTP complexes and subjected to RanGAP stimulated RanGTP hydrolysis. Under these conditions, CRM1 complexes containing the S1 NES were resistant to LMB in contrast to standard NESs from PKI or MVM NS2 (Fig. 3B). The sensitivity of 2z-Nmd3-containing complexes was intermediate. When CRM1 was preincubated with LMB before addition of the S1 NES, the protective effect was lost (Fig. 3B). In this assay, the high concentration of RanGAP (100 nM)

Figure 4. S1 NES localises at Nup358. (A). Immunoelectron microscopy. Cryosections of MCF-7 cells transfected with S1-GFP or RevNES-GFP containing reporter constructs (see Figure 2) were labelled with anti-GFP antibodies followed by protein A gold. In cells expressing S1-GFP protein gold (arrows) decorates the outer aspect of the nuclear envelope at NPCs. (B). Immunofluorescence. Cells as in (A), upper panel, were permeabilised with low concentrations of digitonin such that the nuclear membrane remained intact and labelled with anti-GFP antibodies. Anti-GFP antibodies stain the NE and colocalise largely with the signal from GFP. (C). Knockdown of Nup358 by RNAi removes S1-GFP from the NE. HeLa cells were cotransfected with a plasmid expressing shRNAs targeting Nup358 or Nup214 and a S1-GFP containing reporter plasmid. Cells were analysed 72 hours post transfection for Nup358 and Nup214 levels and S1-GFP by indirect immunofluorescence and direct GFP fluorescence, respectively. A strong knock-down of Nup358, but not of Nup214 reduces S1-GFP from the NPC. (D). S1 NES physically interacts with Nup358. Proteins from Xenopus interphase egg extracts were affinity selected on immobilised biotinylated Rev or S1 NES peptides. Starting material (lane 1) as well as bound (lanes 2-5) and unbound (lanes 6 and 7) fractions were analysed for the presence of Nup358 and CRM1 by western blotting.



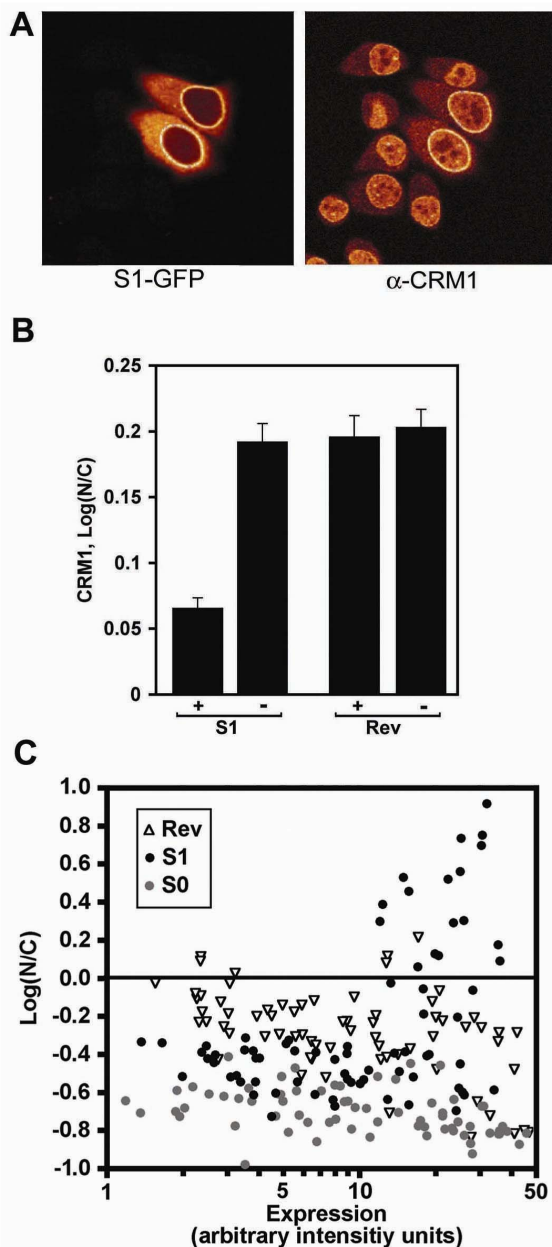


Figure 5. S1 NES sequesters CRM1 at the NE (A) and in the cytoplasm (B) and can inhibit its own export (C). (A). HeLa cells were transfected with the S1-GFP containing reporter construct as before and GFP was detected together with CRM1 with direct GFP fluorescence and indirect immunofluorescence respectively. (B). Cells were transfected with the S1 or RevNES (Rev) containing GFP reporter plasmids as in (A) and nuclear and cytoplasmic CRM1 immunofluorescence signals were measured in confocal sections of 15 transfected (+) or untransfected (-) cells. Log ratios of the means are significantly reduced in S1-GFP expressing cells, not in RevNES-GFP expressing cells. Error bars denote standard errors. (C). Single cells expressing different levels of S0 (black circles), S1 (grey circles) and RevNES (white triangles) GFP reporter proteins were analysed for nuclear and cytoplasmic GFP levels. S0 NES and RevNES mediate cytoplasmic localisation irrespective of expresion level. In contrast, the S1 NES promotes nuclear export at low expresion levels but not at high expression levels.

ensures that once RanGTP is released from CRM1, RanGTPase is immediately activated before RanGTP can rebind to CRM1 (Bischoff and Görlich, 1997). Therefore the assay mainly measures off-rates, indicating that high affinity binding of S1 to CRM1 is accomplished by a slow off-rate.

To assess the capability of S1 to bind CRM1 in the absence of RanGTP, a CRM1 pull- down assay was performed. CRM1-columns were incubated with various GFP-tagged NESs in the presence or absence of RanGTP, after which eluted fractions were analysed by Coomassie staining (Fig. 3C).While binding of S0 and Rev NES-containing proteins to CRM1 was greatly stimulated by RanGTP, GFP_3S1 bound both in the presence and absence of RanGTP (Fig. 3C). From these data, we conclude that the S1 NES exhibits a "supraphysiological" affinity (i.e. greater or stronger than normally present in the cell) for CRM1 such that stable binding to this export receptor takes place in the absence of RanGTP.

S1 NES localises at Nup358

To further investigate the prominent NE signal of the S1 NES reporter protein, we determined the localisation of S1-GFP. Fixed cells were permeabilised with digitonin, which permeabilises the cell membrane but leaves the NE intact. Anti-GFP antibodies continued to stain the NE, albeit weaker than direct GFP fluorescence (Fig. 4B). In cells permeabilised with Triton X-100, allowing antibody access to the inside of the NE, the nuclear rim staining was the same (data not shown) suggesting that antigen accessibility explains the difference with direct GFP fluorescence. To study S1-GFP localisation at higher resolution, S1-GFP and RevNES-GFP were localised by immunogold staining on ultrathin cryosections using anti-GFP antibodies and 10 nm protein A conjugated gold. As shown in Figure 4A, S1-GFP predominantly localises to the cytoplasmic side of the NPC, at the position of

the cytoplasmic filaments of the NPC. RevNES-GFP did not show significant NPC localisation. Considering the EM localisation of S1-GFP, we selected Nup358 as a candidate for mediating S1-GFP accumulation. Short hairpin interfering RNA to Nup358 were expressed in HeLa cells together with S1-GFP. Cells were analysed 72 h after transfection when Nup358 protein levels are reduced by up to 90% (3). As illustrated in Figure 4C, upon knockdown of Nup358, S1-GFP disappeared almost completely from the NE. Control transfections showed no reduction of NPC-associated S1-GFP. Another nucleoporin that could mediate S1/CRM1 interaction at the cytoplasmic face of the NPC is Nup214 (Kehlenbach et al., 1999, Askjaer et al., 1999). However, removal of Nup214 from the NPC by RNAi did not effect S1 localisation (Fig. 4C). To confirm that the S1/CRM1 complex physically interacts with Nup358, we affinity selected proteins from *Xenopus* interphase egg extract on immobilised S1 or Rev NES peptides. In these extracts Ran is almost exclusively in the GDP bound form. Under these conditions, a significant fraction of Nup358 stably associates with CRM1 to the S1 NES affinity column, not to the Rev NES column (Fig. 4D). We conclude that S1 NES accumulation at the NPC is directly mediated by Nup358.

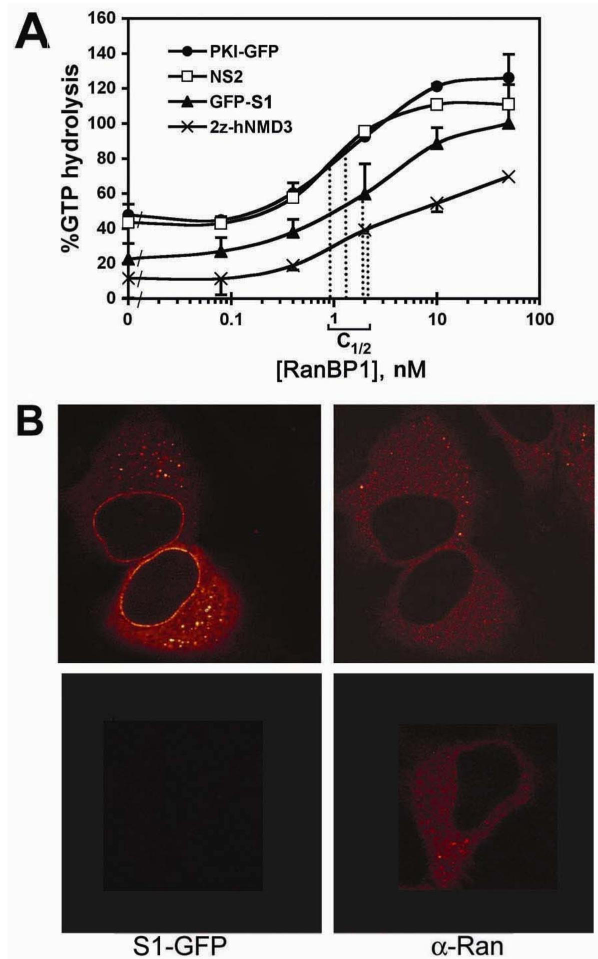
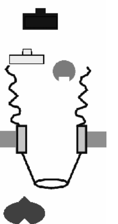


Figure 6. RanGTP can leave the S1/CRM1/RanGTP complex at Nup358. **(**A). GFP_3S1/CRM1/RanGTP complexes display normal sensitivity to RanBP1. Trimeric NES/CRM1/RanGTP complexes were assembled and incubated with increasing concentrations of recombinant RanBP1, and CRM1 RanGAP assays were performed as in Figure 3. Regular strength NESs of PKI and Rev and the high-affinity interaction with 2z-Nmd3 served as references. RanBP1 concentrations of half maximum release of protection of GTP hydrolysis ($C_{1/2}$) are indicated. (B). Ran does not accumulate at the NE in S1-GFP expressing cells. HeLa cells were transiently transfected with the S1-GFP containing reporter construct and permeabilised with low concentrations of digitonin to ensure intactness of the nuclear membrane. S1-GFP and Ran were detected by direct GFP fluorescence (left) and indirect immunofluorescence (right) respectively.

The S1/CRM1 complex arrests at Nup358 and S1 is an inhibitor of CRM1

We have recently shown that CRM1 localises to Nup358 *in vivo* in a LMB-insensitive way (3) and proposed this could represent empty CRM1 before recycling into the nucleus. Conceivably, S1 NES attaches to this population of Nup358-bound CRM1. Alternatively, the S1-NES/CRM1 complex could attatch *de novo* at Nup358. In this case additional CRM1, stoichiometric to the S1 cargo, would be expected to localise at the NE. Therefore, we investigated whether CRM1 would accumulate with GFP in S1-GFP transfected cells. Untransfected cells show a predominantly nuclear and NE CRM1 staining (Fig. 5A). Expression of S1-GFP at the NE in transfected cells, causes a clear NE accumulation of CRM1 (Fig. 5A). To assess changes in nucleocytoplasmic distribution of CRM1, staining intensities in nuclear and cytoplasmic compartments were determined in S1-GFP or Rev-GFP expressing cells. Transfection of S1-GFP induced a 35% increase of cytoplasmic CRM1 (Fig. 5B). CRM1 localisation was not influenced by expression of RevNES-GFP. These data indicate that the



S1/CRM1 complex arrests at Nup358 upon NPC translocation and that S1 remains bound to CRM1 in the cytoplasm.

The sequestering of CRM1 by the S1 NES suggests that expression of the S1 could lead to an inhibition of CRM1 function. To test this, we expressed S0, S1 and Rev-GFP proteins transiently for 24 h in MCF-7 cells and measured their subcellular localisation as a function of cellular protein expression level. As shown in Figure 5C, S0 and Rev NESs can promote nuclear export of the shuttling GFP reporter, irrespective of the expression level. In contrast, S1-GFP only promotes cytoplasmic accumulation when expressed at low to moderate levels, whereas at high expression S1-GFP accumulates in the nucleoplasm (Fig. 5C). This indicates that by sequestering CRM1, the S1 NES acts as an inhibitor of CRM1 function.

S1/CRM1/RanGTP complexes display normal sensitivity to RanBP1

Our data suggest that the S1 NES remains bound to Nup358 as a consequence of its ability to bind CRM1 without RanGTP. Alternatively, S1/CRM1/RanGTP complexes could fail to dissociate at Nup358 because they are insensitive to RanBP1. In a CRM1 RanGAP assay, low concentrations of RanBP1 strongly promote RanGTP hydrolysis (2), presumably by loosening the RanGTP/CRM1 interaction (4). As shown in Figure 6A, all NESs tested in this assay responded similarly to RanBP1 addition after export complex formation. The RanBP1 concentration at which RanGTP hydrolysis has recovered by 50%, diverged no more than 3-fold from S1 NES to the standard NESs. These data predict that, unlike CRM1 (Fig. 5A), Ran does not accumulate at the cytoplasmic face of the NPC upon S1-GFP expression. To allow visualisation of a potential Ran enrichment at the cytoplasmic side of the nuclear pore, S1-GFP transfected cells were permeabilised with digitonin and stained with an anti-Ran antibody. As shown in Figure 6B, Ran was not enriched at the NE upon expression of S1-GFP, nor was it increased in the cytoplasm.

Discussion

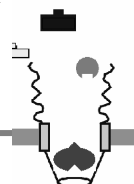
In this study, we identify signals exhibiting high-affinity interactions with the widely-studied export receptor CRM1. The results presented here bear relevance to the understanding of the mechanism of CRM1-mediated export and the evolution of leucine-rich NES motifs.

Supraphysiological NESs provide *in vivo* evidence for a novel nuclear export intermediate

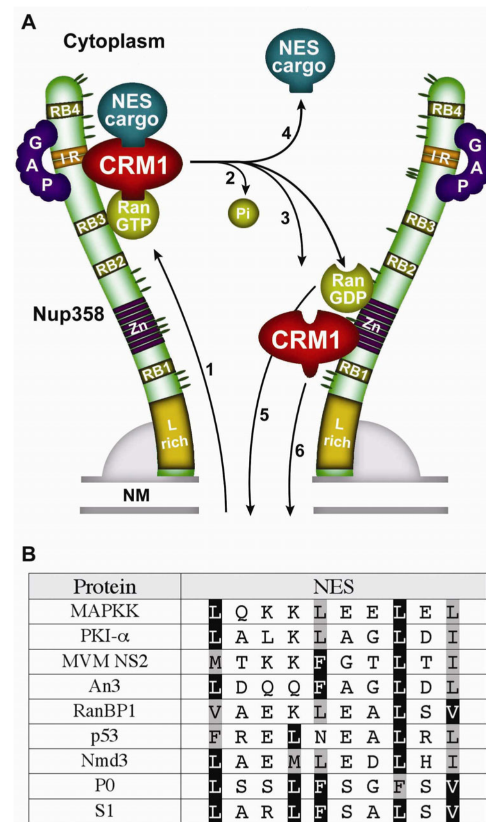
An unexpected outcome of our peptide selection was that a shuttling substrate containing the highest affinity S1 NES accumulated at the NE. This accumulation represents CRM1/NES export complexes because the localisation is LMB sensitive and CRM1 accumulates with the S1 NES. In addition, immuno-electron microscopy showed S1 NES accumulation at the cytoplasmic face of the NPC. Since Nup214/CAN binds strongly to CRM1 *in vitro* in a RanGTP and NES stimulated way, this nucleoporin represented a likely candidate to mediate this NPC localisation (2, 15, 24). However, RNAi experiments showed that the NE localisation was dependent on Nup358. This effectively rules out a potential role of Nup214 in the NE accumulation of S1/CRM1 as Nup214 is not affected by removal of Nup358 (3).

A LMB-insensitive interaction between CRM1 and Nup358 has been reported *in vitro* (42) and *in vivo* (3) and most likely represents the empty state of CRM1. The S1 NES dependent CRM1/Nup358 interaction is cargo dependent and LMB sensitive, and must therefore represent a different binding site. Co-immunoprecipitation of CRM1 with Nup358 from Xenopus egg extracts is greatly stimulated by RanQ69LGTP, a non-hydrolysable form of RanGTP, in contrast to importin β, importin 5 or importin 7 (50). This observation supports the idea of a cargo-dependent CRM1 interaction site on Nup358.

Why does the high-affinity NES accumulate at Nup358, while standard NESs do not show this behaviour? Biochemical analyses revealed that S1 possesses an affinity for CRM1 two orders of a magnitude higher than standard NESs. Our *in vitro* data further show that S1, unlike standard NESs, is able to interact stably with CRM1 in the absence of RanGTP. Thus, a likely explanation for the accumulation of S1 at Nup358 is that this reflects a failure of the S1/CRM1/RanGTP complex to dissociate, thereby keeping CRM1 in the export complex

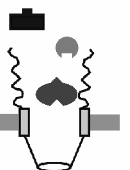


conformation. Because Nup358 contains four RanBP1-like RanGTP binding domains (RBDs) the S1/CRM1 accumulation might be bridged by RanGTP. However, our biochemical data indicate that S1/CRM1/RanGTP complexes are fully sensitive to destabilisation by RanBP1. In addition, no accumulation of Ran was observed at the cytoplasmic face of the NE indicating that Ran is able to leave the S1/CRM1/RanGTP complex. We conclude that the S1/CRM1 complex remains bound to Nup358 via CRM1, mimicking an export complex just prior to RanBP1-like RBD and RanGAP assisted complex disassembly. As illustrated in Figure 7A, we propose that Nup358 functions as the CRM1 export complex disassembly site at the NPC. To facilitate this process Nup358 contains binding sites for export complexes, RanGTP hydrolysing cofactors as well as binding sites for RanGDP and empty CRM1. An additional effect of the export complex binding site of Nup358 would be to decrease reverse export of CRM1 export complexes that would form in the cytoplasm. Under normal conditions, these complexes are unlikely to form because of the cytoplasmic activity of RanGAP and RanBP1 (Görlich et al., 2003; Becsksei and Mattaj, 2003). However, under conditions where cytoplasmic RanGTP is relatively high, for example by decrease of RanGAP activity at lower temperatures (Görlich et al., 2003), capture of cytoplasmic export complexes may contribute to nuclear exclusion of NES proteins. Consistent with this idea, NES cargoes and CRM1 accumulate prominently at the nuclear rim when added in vitro to permeabilised cells in combination with RanQ69L (Kehlenbach et al., 1999; (33). Proteins containing NESs such as S1 that bind to CRM1 without RanGTP are predicted to be more susceptible to reverse export. However, S1 is clearly absent from the nuclear compartment, indicating that export is more efficient than reverse export. This most likely reflects the higher affinity of S1 NES for CRM1 in the nucleus through the cooperative binding of RanGTP. We have previously shown that reduction of Nup358 leads to a moderate reduction of Rev-NES mediated nuclear export (Bernad et al., 2004), that we have proposed are due to a decrease in CRM1 recycling to the nucleus. We have not measured the effect of Nup358 depletion on cytoplasmic accumulation of



Protein	NES
MAPKK	L Q K K L E E L E L
PKI-α	L A L K L A G L D I
MVM NS2	M T K K F G T L T I
An3	L D Q Q F A G L D L
RanBP1	V A E K L E A L S V
p53	F R E L N E A L R L
Nmd3	L A E M L E D L H I
P0	L S S L F S G F S V
S1	L A R L F S A L S V

Figure 7. (A). Model of CRM1 export complex disassembly at the cytoplasmic face of the NPC. Nup358 is depicted as a filamentous protein (8) with the different domains indicated. The orientation is suggested by immuno EM studies (51) and the localisation of the ALK-Nup358 oncoprotein (30). 1. NES/CRM1/RanGTP complexes are translocated through the core NPC and bind to a cargo-dependent CRM1-binding site on Nup358. This binding may be cooperative with RanGTP/Ran Binding Domain (RBD, RB1-4) interaction. 2. RanGTP hydrolysis stimulated by Nup358-bound RanGAP (31, 32) and the RBDs of Nup358 (49, 53, 55). 3. CRM1 can be released into the cytoplasm, as is the NES cargo protein (4) or bind to the LMB insensitive CRM1 binding site on Nup358 that is located in the zinc finger region (3, 42); likewise RanGDP may bind to the zinc finger Ran Binding Domain (54); 5. RanGDP and (6) CRM1 recycle to the nucleus. NM, nuclear membranes; Zn, zinc finger domains; L-rich, leucine rich domain (See full-colour figure in cover). (B). Natural NESs deviate at hydrophobic residues from highest affinity NES sequence. Alignment of the sequence of previously identified natural NESs from MAPKK (17), PKI (52), MVM NS2, Xenopus An3 (2) RanBP1 (37) p53 (45) and Nmd3 (47) and the artificial P0 and S1 NESs. Consensus hydrophobic residues are shaded, hydrophobic residues identical to the S1 NES are boxed.



S1 NES cargoes, but it is conceivable that at low expression levels the S1 NES is able to efficiently compete for limiting amounts of CRM1, making its nuclear export relatively unaffected by Nup358 depletion.

High-affinity NESs are "too good to be optimal"

We set out to identify signals exhibiting high-affinity interactions with CRM1 by screening a library of 15-mer peptide motifs in a phage display setup. The number of representations for a random 15-mer peptide encompasses $3*10^{19}$ unique sequences. The complexity of the 15-mer peptide library employed was many orders of magnitude smaller at $2*10^{8}$ unique sequences. The probability of retrieving a consensus NES, defined by 4 hydrophobic amino acids spaced in 3-2-1, 2-3-1 or 2-2-1 (without intervening hydrophobic amino acids) in our library is approximately 0.02. Therefore, roughly $4*10^{6}$ different consensus NES sequences are expected in the library. Remarkably, under RanGTP selection conditions, which favoured export complex formation, a unique signal was enriched after just three rounds. This phage contained the highly active P0 NES that conforms to the consensus NES sequence. This indicates that the phage display selection conditions allowed us to enrich high-affinity NESs and that these are rarely encountered in the library. In the absence of RanGTP, a unique signal was selected, which displays a robust NES sequence of a striking similarity to the P0 signal. This experimental outcome advocates that CRM1 contains one major peptide binding site, which corresponds to the NES binding site.

We obtained a quantitative measure of CRM1 interaction by comparing the S1 NES to a z-tagged version of Nmd3 (47). This protein displays a high affinity for CRM1 comparable to that of snurportin 1 (see below). S1-GFP and 2z-Nmd3 possessed a similar affinity for CRM1 that was approximately 100-fold higher than standard NESs. Even though a short Rev-type NES has been proposed in human Nmd3 that is required for CRM1 interaction, this is unlikely to be sufficient for the high affinity binding, as the untagged version of Nmd3 has a much lower affinity for CRM1 (see Thomas and Kutay, 2003 for discussion). Interestingly, CRM1 is less sensitive to LMB when bound to S1 as compared to standard NESs or 2z-Nmd3. LMB covalently binds to Cys_{528} of hCRM1 (25, 34),suggesting that access to Cys_{528} is masked by a tight NES interaction.

In vitro measured affinities between CRM1 and NES cargoes are low in comparison to interactions of other exportins with their cargo (2, 26, 27). Snurportin 1, a natural high affinity cargo for CRM1, does not contain a short Rev-type NES but requires a large domain for CRM1 interaction (36). This was taken to suggest that high-affinity CRM1 interaction could not be accomplished by small leucine-rich type NESs, and that CRM1 required a co-factor RanBP3 to boost NES-CRM1 affinity (9, 29). In contrast, our data now demonstrate that high-affinity CRM1 binding can be accomplished by leucine-rich NESs, but is ineffective *in vivo*, because high-affinity NESs interact with CRM1 without RanGTP. As a consequence, illustrated by the S1 NES, export complexes accumulate at Nup358 and in the cytoplasm. This suggests that the large CRM1 interaction domain of snurportin 1 and perhaps Nmd3 are required for efficient release from CRM1. Sequence alignment of 58 published high-confidence NESs display a high level of variation, even within the consensus hydrophobic amino acids (la Cour et al., 2003). As illustrated in Figure 7B, natural NESs only show a subset of the hydrophobic residues of the high-affinity NESs. When we replaced the consenus hydrophobic residues of a natural NES, derived from RanBP1 protein, into the high-affinity NES hydrophobic residues, the mutated version showed high-affinity behaviour, as it was targeted to the NPC. This strengthens the idea that natural Rev-type NESs are selected to bind CRM1 but counter-selected to bind with high affinity.

In conclusion, selection and analysis of high-affinity NES sequences provides clues to understanding the low-affinity nature and complexity of natural NESs. The highest affinity NESs are novel inhibitors of the CRM1 pathway and may be useful for structural characterisation of the CRM1/NES complex. The approach to select supraphysiological cargoes for nuclear transport receptors could be more widely applicable to study discrete steps of nucleocytoplasmic communication *in vivo*.

Materials and Methods

Antibodies
Anti-GFP antibodies for immunofluorescence were from Abcam, for immunoelectron microscopy from Roche. Antibodies to Nup358 (Walther et al., 2003) CRM1 (14), Nup214 (Bernad et al., 2004) and Ran (22) were described previously.

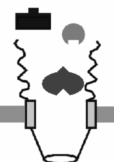
Plasmid construction
For *in vivo* transport assays, phage inserts were provided with BglII and AgeI sites and inserted into the AgeI and BamHI sites of Rev(1.4)-GFP (21). Second generation mutations were introduced by PCR. For bacterial expression three copies of GFP were placed in front of the NES, and introduced into the XmaI/PstI sites of pQE30 (Qiagen). pSuper-214, the shRNA expression plasmid to Nup214 targets nt 3828-3850 of the Nup214 ORF.

Recombinant protein expression and purification
Z-tagged CRM1 and transportin 1 were expressed as previously described (2, 18). Ran, RanBP1, Rna1p were expressed and purified according to Izaurralde et al. (23). Ran was loaded with GTP according to the method described previously (5), 2z-hNMD3 was a gift from U. Kutay (47). NS2 peptide was described previously (2). GFP_3-NESs, GFP-PKI (41) and CRM1 (10) were purified on Ni-NTA agarose (Qiagen). CRM1 was further purified on a Resource Q column. zz-CRM1 and zz-transportin columns were prepared as published (Askjaer et al., 1999).

Phage display
The 15-mer phage library (kind gift from T. Schumacher) represents a secondary amplification of a library initially created by Nishi et al. (35) using the filamentous phage vector fUSE5 (40). Zz-CRM1 columns were blocked for 2 h at 4˚C in phage binding buffer (PBB; TBS, 0.01% Tween 20, 1 mM $MgCl_2$) containing 1% BSA. After 2 washes with PBB, 5 µl phage library containing $1*10^9$ infectious phages were added to CRM1 columns, either in presence or absence of 4.5 µM RanGTP in 50 µl PBB plus 0.1 %BSA. Phages were bound for 2 h at 4˚C. Columns were washed three times with PBB and eluted for 5 min at RT in 50 µl PBB containing 180 nM RanBP1 and 430 nM Rna1p or PBB alone. Selected phage pools were amplified by using *E. coli* K91-Kan as previously described (43). After each selection round 0.5 - 1 µl eluted phages and 1 µl of input phages were used for titration as described (43). After each selection round starting from the second round, phages were isolated and amplified as described. 0.75µl phage suspension was directly used for sequencing, using primer 5’-TGAATTTTCTGTATGAGG.

CRM1 RanGAP assays
CRM1 RanGAP assays were performed as described (2). For LMB assays, increasing concentrations of LMB in 5 µl Ran buffer were mixed with 10 µl of 100 nM Rna1 and added to assembled complexes containing 1 µM CRM1, 200 pM Ran[γ-^{32}P]GTP and either 480 nM GFP-S1, 380 nM 2z-hNMD3, 20 µM PKI-GFP or 5 µM NS2 present in 35 µl Ran buffer. Pre-incubation of LMB was performed by addition of LMB to 1 µM CRM1 and 200 pM Ran[γ-^{32}P]GTP, 5 min prior to addition of 480 nM GFP_3-S1. Assays testing RanBP1 sensitivity were performed by incubation of export complexes containing 360 nM CRM1 and concentrations as described for other GAP assays. Increasing concentrations of RanBP1 in 5 µl PBS/8.7 % glycerol together with 10 µl 100 pM Rna1 in Ran buffer were used for Ran[γ-^{32}P]GTP hydrolysis.

Pull down assays
To detect recombinant CRM1/NES interaction, z-tagged CRM1 columns were incubated with 1 µM GFP-NES protein and 2.5 µM RanGTP when indicated. Binding reactions were performed in 50 mM HEPES-KOH pH 7.9; 200 mM NaCl; 8.7% glycerol (buffer B) containing 0.1 mM DTT. After slowly shaking for 2 h at 4˚C, beads were washed three times with buffer B and eluted for 10 min at RT with 50 µl buffer B. Samples were fractionated on SDS-polyacrylamide gels and visualised by Coomassie staining. To detect interaction with Nup358, 0.5 µmol of biotinylated S1 or Rev (GVPLQLPPLERLTLDC) NES peptide was immobilised on 5 µl streptavidin agarose beads (Sigma). NES beads were blocked for 1 h in 1% BSA and incubated for 3 h with 100 µl of Xenopus interphase egg extract diluted 1:1 with 10 mM HEPES pH 7.4; 100 mM KOAc;



3mM MgOAc; 5 mM EGTA; 150 mM sucrose; 1 mM DTT (acetate buffer). Beads were washed three times with acetate buffer and bound proteins were eluted in 0.2% and 2% SDS. Bound and unbound fractions were separated on 6% SDS-PAGE and blotted.

Cell culture and transfections

MCF-7 cells were transfected using electroporation as previously described (1). HeLa cells were transfected by using Fugene-6 (Roche) according to manufacturer's instructions. Both cell lines were transfected with 1 μg of pRev(1.4)-GFP plasmids on glass coverslips in 35 mm diameter dishes. Cells were fixed 24 h post transfection. When required, 50 nM LMB was added 3 h prior to fixation. For RNAi assays 1 μg of pSuper-358 (Bernad et al., 2004) or pSuper-214 was co-transfected and cells were cultured for 72 h before analysis.

Immunofluorescence stainings and image analysis

Indirect immunofluorescence was performed as previously described (3). Images were recorded with a Leica TCS SP2 confocal microscope. For CRM1 localisation analysis Image J software was used to measure the nuclear and cytoplasmic intensities of 15 cells. For measuring nuclear export as function of GFP-NES expression level, total cellular and nuclear GFP signals were recorded using large pinhole confocal microscopy. To cover the complete range of expression, fields of cells were recorded with different PMT settings (250 to 550 V), and pixel values were combined using PMT to pixel value calibration curves.

Cryoimmunogold Electron Microscopy

Transfected MCF-7 cells were fixed, sectioned, immunolabeled and imaged as described (7).

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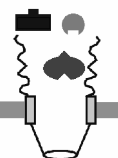

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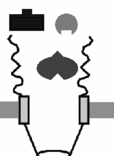
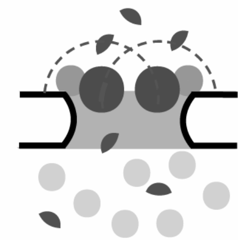


“Was many years ago that I left home and came this way
I was a young man, full of hope and dreams
But now it seems to me that all is lost and nothing gained
Sometimes things ain't what they seem”

Iron Maiden. “Stranger in A Strange Land”



CHAPTER 5

The Nup214/Nup88 nucleoporin subcomplex is required for CRM1 mediated 60S preribosomal nuclear export

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The Nup214/Nup88 nucleoporin subcomplex is required for CRM1 mediated 60S preribosomal nuclear export*

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Running title: CRM1 NPC transport pathways

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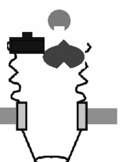
The nuclear pore complex (NPC) conducts macromolecular transport to and from the nucleus and provides a kinetic/hydrophobic barrier composed of phenylalanine-glycine (FG) repeats. Nuclear transport is achieved through permeation of this barrier by transport receptors. The transport receptor CRM1 facilitates export of a large variety of cargos. Export of the ribosomal 60S subunit follows this pathway through the adaptor protein NMD3. Using RNAi, we depleted two FG-containing cytoplasmic oriented NPC complexes, Nup214/Nup88 and Nup358, and investigated CRM1-mediated export. A dramatic defect in NMD3-mediated export of preribosomes was found in Nup214/Nup88 depleted cells, while only minor export defects were evident in other CRM1 cargos or upon depletion of Nup358. Derivatives of Nup214 lacking the FG-repeat domain rescued the NMD3 export defect. We show that this domain is not accessible to freely diffusing molecules from the nucleus, indicating that it does not conduct cargo through the NPC. The coiled-coil region of Nup214 is sufficient for stabilizing the Nup214/Nup88 subcomplex and NMD3 export. We propose that Nup214 plays independent roles in NPC function by participating in the kinetic/hydrophobic barrier through its FG-rich domain and by enabling NPC gating through association with Nup88.

INTRODUCTION

Selective communication between the nucleus and the cytoplasm in eukaryotes occurs through nuclear pore complexes (NPC), multiprotein assemblies that transverse the nuclear envelope (NE) (1-3). Each NPC is composed of ~30 proteins, collectively termed nucleoporins (4), and displays an 8-fold horizontal rotational symmetry in relation to the NE (5). The general shape of the NPC is conserved from yeast to humans (4,6,7), but individual nucleoporins differ widely in sequence. A large subset of nucleoporins contains long phenylalanine-glycine dipeptide containing domains (FG-repeats) which are thought to form a hydrophobic/kinetic meshwork creating a barrier to most macromolecules while allowing passage of transport receptor complexes (2,8). These complexes are thought to pass the NPC by interacting with FG-repeats, thus permeating the NPC core (8-11). To achieve nuclear transport, proteins and RNAs bind transport-competent receptors, either directly or indirectly via adaptor proteins (12-16).

Directionality of transport through the NPC is determined by the Ran GTP/GDP gradient which exists between the nucleus and the cytoplasm (17-19) and/or the presence of specific high affinity binding sites for transport receptors located at either the nuclear or cytoplasmic faces of the NPC (20). Although the general NPC architecture is symmetric, the localisation of several nucleoporins is restricted to either the nuclear or the cytoplasmic face of the NPC (7), supporting

*We thank Ulrike Kutay for the generous gift of GFP-NMD3 and rpL29-GFP, Erik Wiemer (Erasmus University Rotterdam, The Netherlands) for the kind gift of pEW103, Reuven Agami for pSUPER, Rene Bernards (NKI Amsterdam, The Netherlands) for pSUPER-GFP, Beric Henserson and Josean Rodriguez for plasmid reagents, Volker Cordes, Frauke Melchior and Andrea Gast for antibody reagents, Lauran Oomen and Lenny Brocks for valuable assistance in confocal microscopy, Jacques Neefjes group for discussions and Bernike Kalverda for critically reading the manuscript. R.B. is supported by a grant from The Netherlands Science Foundation Earth and Life Sciences. H.P. is supported by a Marie Curie European Community Training and Mobility Fellowship.



the idea that nucleocytoplasmic asymmetry might be established by asymmetric distribution of specific binding sites at the NPC. Indeed, preferential interactions between several transport receptors and specific FG-containing nucleoporins have been described in vitro (21-24). In contrast, recent studies in yeast show that receptor-mediated nuclear transport is not affected when FG-repeats of asymmetric nucleoporins are absent (25,26), indicating that they are not essential for directional transport and that the FG-domains of different nucleoporins may be functionally redundant. Furthermore, imaging of single molecule translocation through the NPC shows that the most kinetically important interactions during nuclear translocation take place in the central pore and that these interactions exhibit the characteristics of unbiased diffusion indicating that there is no directionality within the NPC itself (27).

Next to permeation, gating is a second proposed mode of energy-dependent NPC translocation. It involves conformational changes of the NPC to achieve transport (28) and it is supported by conformational states that have been detected under various conditions (29-32). The role of individual nucleoporins in this process is unknown.

The Nup214/ Nup88 subcomplex is localized to the cytoplasmic face of the NPC (33). Nup214 is dispensable for *in vitro* NPC assembly and protein import (34) but it is essential in vertebrate cells and its depletion causes a strong mRNA export defect (35). Nup214 contains two central coiled coils known to interact with Nup88 (21,33) and a long C-terminal FG-repeat that interacts strongly with the transport factor CRM1 *in vitro* in a RanGTP and cargo stimulated fashion (21,36). These data suggest that Nup214 plays an essential role in CRM1 mediated export. But it remains to be elucidated if and how NPC asymmetry influences transport processes that are initiated on the opposite side of the NPC (37). Recent studies proposed that the FG-rich domain of the asymmetric nuclear Nup153 and cytoplasmic Nup214 can cross the NPC providing a binding site to transport receptors and escorting transport complexes through the NPC (38,39).

CRM1 mediates the nuclear export of proteins bearing a nuclear export signal (NES) by binding cooperatively with RanGTP (13,16,40,41). The nature of NES-containing cargos differs widely. The large subunit of the ribosome is exported to the cytoplasm via the CRM1 pathway, assisted by the transport adaptor NMD3 (42-44).

We have compared the roles of the Nup214/Nup88 and Nup358 complexes in different CRM1 export pathways. We show that the CRM1-mediated export of the 60S ribosomal subunit is dependent on the Nup214/Nup88 subcomplex while that of small NES cargos remains relatively unaffected. We show that the central domain of Nup214 is required for 60S export and Nup88 targeting to the NPC, while the FG repeats are dispensable.

EXPERIMENTAL PROCEDURES

Antibodies–Anti-hNup358/RanBP2 antiserum, anti-hNup358V, and anti-hNup358F were generously provided by V. Cordes (Karolinska Institute, Stockholm, Sweden), A. Gast and F. Melchior (Max Planck Institute for Biochemistry, Munich, Germany), respectively. Antibodies to Nup214 (45) anti-hNup88 (BD Transduction Laboratories), monoclonal antibody (MAb) 414 (Eurogentec/Babco) and anti-HA (12CA5) were previously described.

Plasmid construction–pSuper-358 (45), pSuper-214, Rev-S1-GFP (24) and HA-Nup153 (46) were described previously. The NLS-eGFP-NES insert containing the SV40 NLS and PKI NES was subcloned from pBSSK (47) into the pcDNA3 vector (Stratagene) using the *Hin*dIII *Not*I restriction sites. The NLS-eGFP insert was amplified by PCR from pBSSK using F primer 5' CCCCTCGAGGTCGACGGTATC 3' and R primer containing a *Not*I site 5' ATATATATAGCGGCCGCTTAGTTTCTAGAC TTGTACAGCTC 3' and subcloned into pCDNA3 by digestion with *Hin*dIII and *Not*I. GFP-NMD3 and rpL29-GFP were a kind gift from U. Kutay (43). To create a RNAi insensitive Nup214 expressing plasmid, *Dpn*I mediated site-directed mutagenesis was performed on pBluescriptKS(-)CAN (48) creating four silent mutations in the target sequence TCACATCCGCTAGCAACAC. Wild type and mutated Nup214 coding sequences were subcloned into the *Eco*RI sites of pcDNA3 (Stratagene). A DNA oligo, which contained

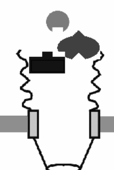
*Age*I, *Sac*II and *Fse*I sites, was cloned into the RNAi insensitive Nup214 *Xcm*I sites, located at positions 6157 and 6253 of the ORF, leading to the parental construct. The *Age*I and *Fse*I sites were used to perform unidirectional deletions using the ExoIII/S1 Deletion Kit (Fermentas). To create Nup214-FRB, the FRB domain lacking the HA1 tag from the plasmid pC_4-R_HE (Regulated Heterodimerization Kit, Argent) was PCR amplified and cloned in-frame into the parental construct using *Age*I and *Sac*II sites. To create pcDNA3 HA-Nup214(585-832), HA-Nup214(804-1058) and HA-Nup214(585-1058), Nup214 regions were PCR amplified on pBluescriptKS(-)CAN (48) and cloned into pcDNA3-HA (49). FKBP lacking the HA1 tag and SV40 NLS was PCR amplified from the plasmid pC_4EN-F1E (Regulated Heterodimerization Kit, Argent) to clone into pRev(1.4)-GFP (50) using *Bam*HI and *Age*I sites; and into GST-NLS-GFP from plamid pEW103 (kind gift of Erik Wiemer, Erasmus University Rotterdam, The Netherlands) using *Bsr*GI and *Sac*II sites. All constructs were sequenced for confirmation.

Cell culture and transfections–Low passage HeLa cells and MCF-7 cells (ATCC CCL-2) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (GibcoBRL) and antibiotics at 37°C and 5% CO2 in a humidified incubator. Transfections were performed using electroporation on MCF-7 cells as described previously (51) or Fugene-6 (Roche) on HeLa cells according to the manufacturer's instructions. For all RNAi assays in HeLa cells, experiments proceeded for 72 hours and the amount of pSUPER plasmids transfected was 106ng/cm^2 on 70-80% confluent cells. pSUPER-GFP was a generous gift from Rene Bernards, NKI Amsterdam. For immunofluorescence experiments, NLS-GFP, NLS-GFP-NES and GFP-NMD3 were co-transfected at a maximum of 47ng/cm^2, pRev-NES-GFP as described (45), pRev-S1-GFP at 21.7ng/cm^2, rpL29-GFP at 1.2ng/cm^2, pRev-FKBP-GFP at 1ng/cm^2 and GST-NLS-GFP-FKBP at 2.3ng/cm^2. For all rescue experiments pcDNA3 derived plasmids were co-transfected at 5.3ng/cm^2 except for the heterodimerization assays where Nup214-FRB was co-transfected at 7.3ng/cm^2 maximum. For western blot analysis, pcDNA3 and pcDNA3-HA derived plasmids were co-transfected at 26.5ng/cm^2. The non-immunosupresive rapalog AP21967 (Regulated Heterodimerization Kit, Argent) was used at 500nM for 3 h prior to fixation except for Nup214 overexpression, which was at 250nM. Leptomycin B was used for 2 hours at a concentration of 100nM.

Immunofluorescence stainings and image analysis–Indirect immunofluorescence was performed as previously described (45). Images were recorded with Leica TCS NT2 and SP2 confocal microscopes and analysed using ImageJ Software. For subcellular distribution studies, a minimum of 100 cells per condition were scored. Presence of Nup214 at the NE (Figure 3A, Nup214 expression) was defined as positive when rim intensities were 3-fold higher than average cytoplasmic intensities, determined using Image J Software. Nup88 NE staining analysis was performed as previously described (45) on 25 cells per sample.

RESULTS

The Nup214/Nup88 subcomplex is dispensable for basic NES-mediated nuclear export– The strong *in vitro* interaction between Nup214 and CRM1 (21,36), suggests that this nucleoporin has an important role in NES-mediated nuclear export (20,36). To test this, we depleted Nup214 by expression of Nup214-specific shRNAs in human cells and recorded nucleocytoplasmic localisation of NES-reporter proteins. To confirm efficient depletion, Western blot analyses were performed on knocked-down cells lysates. As shown in Fig.1E, Nup214-shRNA resulted in strong depletion of Nup214 (lane 1), while shRNA directed to Nup358 (lane 2) or GFP (lane 3) had no effect. As expected from previous studies, knockdown of Nup214 caused a strong depletion of Nup88, indicating that the stability of these two nucleoporins is co-dependent (45). We first tested a NES-reporter protein consisting of the NES derived from PKI (52) fused to GFP. Import activity of this protein is provided by an SV40 nuclear localization signal (NLS). In control cells, this reporter protein is excluded from the nuclei, indicating that the NES is active and prevails over the NLS activity. Nuclear accumulation of this protein was detectable when cells were treated with the CRM1 inhibitor Leptomycin B, indicating that NLS-GFP-NES is exported via



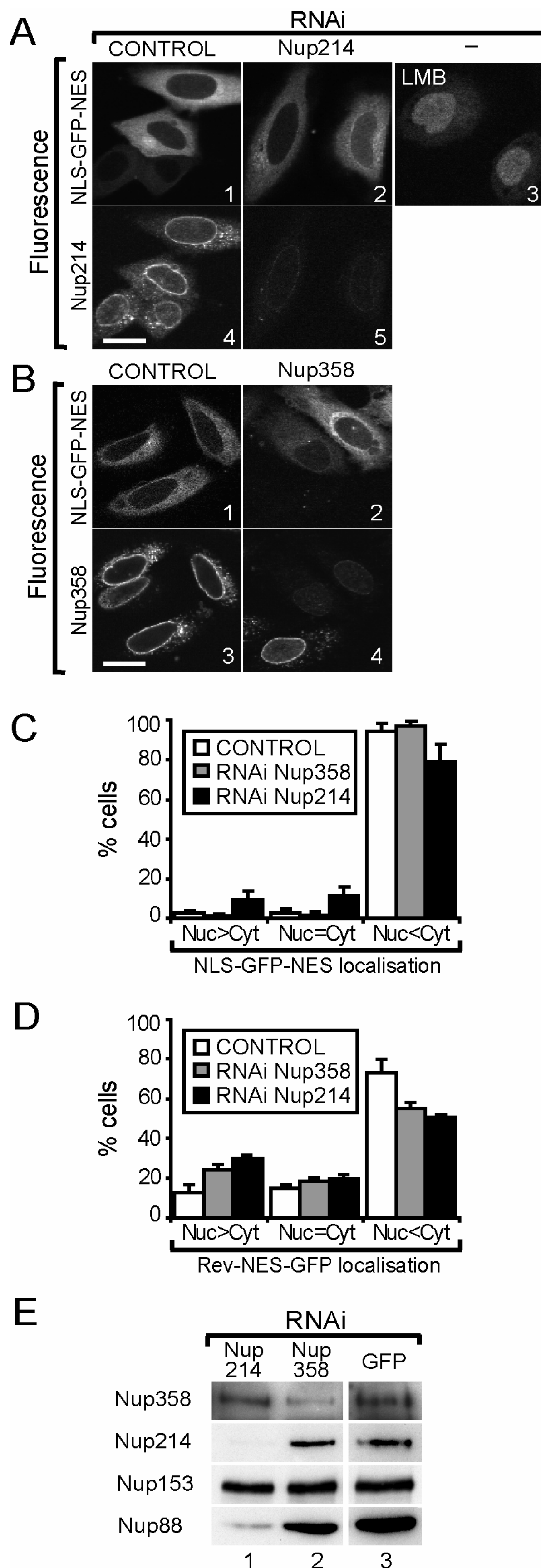


Figure 1. Wild type levels of the Nup214/Nup88 subcomplex are not required for basic NES-mediated nuclear export. Subcellular distribution of the NES reporter proteins NLS-GFP-NES (A-C) and Rev(1.4)-NES-GFP (D) depleted for Nup214/Nup88 (A,C and D) or Nup358 (B,C and D) in HeLa and MCF-7. Cells were immunolabelled with anti-hNup214 (A2 and A4) and anti-hNup358F (B2 and B4) primary antibodies and Texas Red labelled secondary antibodies. Scale bars represent 20μm. (C), (D). Quantification of the subcellular distribution of NLS-GFP-NES and Rev(1.4)-NES-GFP on knocked down cells. 100 fluorescent cells per condition were scored for predominantly nuclear (Nuc>Cyt), equal (Nuc=Cyt) or predominantly cytoplasmic (Nuc<Cyt) GFP fluorescence intensity. The mean distribution is shown and error bars represent standard errors. (E). Western Blot of HeLa cells transfected with pS-Nup214 (lane 1), pS-Nup358 (lane 2) and pS-GFP (lane 3). Blots were probed for Nup358, Nup214, Nup153 and Nup88 using anti-Nup358V, anti-hNup214, MAb 414 and anti-hNup88 respectively. Note that knock-down protein levels are an underestimate of true knock-down efficiency, due to incomplete targeting of the cell population by transient transfection, especially noticeable for Nup358.

CRM1 pathway (Figure 1, A3). Energy depletion of transfected cells by chilling on ice resulted in an even distribution of the protein between the nucleus and the cytoplasm, indicating that the reporter protein is small enough to slowly diffuse through the NPC (data not shown). Surprisingly, depletion of the Nup214/Nup88 subcomplex caused only a small export defect (Fig. 1A and C). Nuclear export of the NES-GFP-NLS reporter in cells depleted of Nup358 was unaffected (Fig. 1B and C). We have previously shown that depletion of Nup358 caused a small reduction in export of a Rev(1.4)-GFP-NES reporter protein (45), which is targeted to the cytoplasm and sensitive to LMB (24,50). In addition to an NLS, the Rev(1.4) protein also provides nuclear retention activity which permits a more stringent assessment of nuclear export. We repeated the above experiments using this reporter protein. In this case, depletion of either Nup358 or the Nup214/Nup88 subcomplex resulted in a moderate reduction of export efficiency (Fig. 1D). As the Nup214/Nup88 subcomplex is required for anchoring of Nup358 to the NPC (45), the

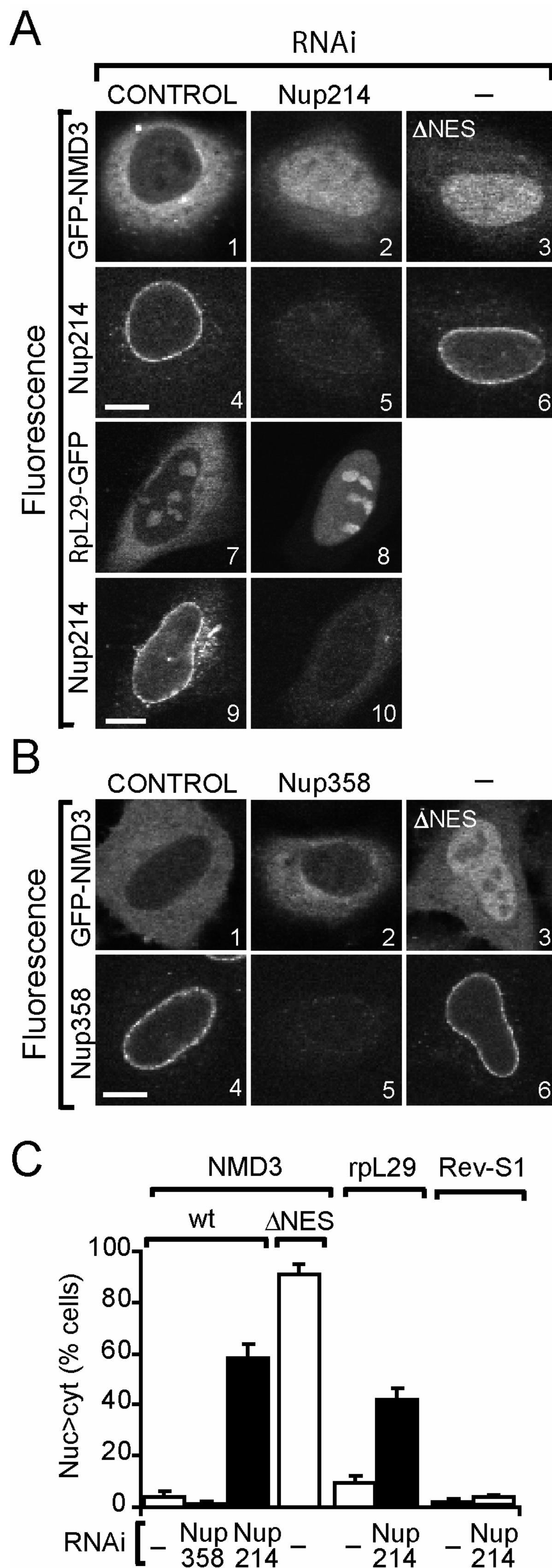
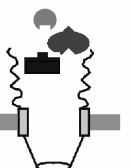
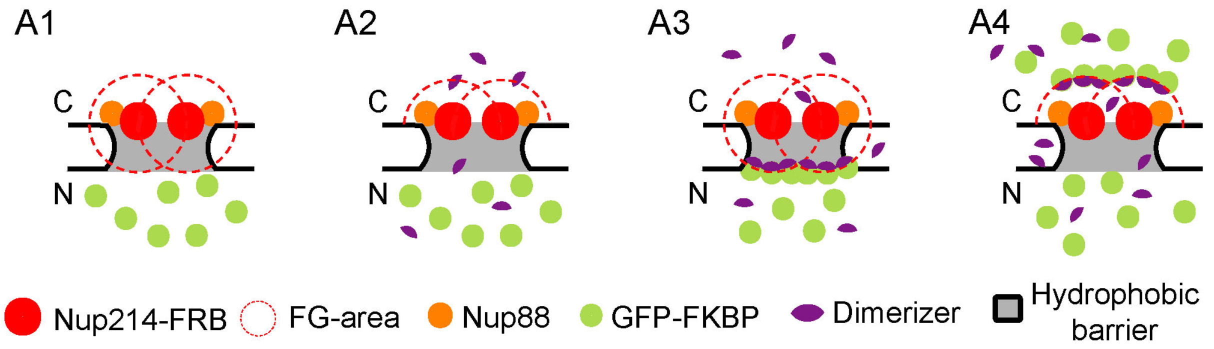


Figure 2. The Nup214/Nup88 subcomplex is required for CRM1-mediated 60S preribosome export. (A), (B). Subcellular distribution of GFP-NMD3 and RpL29-GFP expressing HeLa cells 72 hours after co-transfection with pSUPER control (A1:2, A7:8 and B1:2) or shRNAs expression plasmids targeting Nup214 (A3:4 and A9:10) or Nup358 (B3:4). Cells were fluorescently labelled with anti-hNup214 (A2, A4, A6, A8 and A10) and anti-hNup358F (B2, B4 and B6) primary antibodies and Texas Red labeled secondary antibodies. HeLa cells expressing GFP-NMD3$^{\Delta NES}$ represent maximum nuclear accumulation (A5, and B5). Scale bars, 10µm. (C) Quantification of results illustrated in (A) and (B) showing percentages of cells presenting nuclear accumulation (Nuc>Cyt) of GFP-NMD3, GFP-NMD3$^{\Delta NES}$ and rpL29-GFP as well as those for the supraphysiological NES reporter Rev(1.4)GFP-S1. Error bars represent standard errors.

reduction of nuclear export by depletion of Nup214/Nup88 may be largely attributable to co-depletion of Nup358 from the NPC. We conclude that the Nup214/Nup88 subcomplex does not have a major role in NES-dependent nuclear export of these cargos.

The Nup214/Nup88 subcomplex is required for CRM1-mediated 60S preribosome export– Considering the discrepancy between the strong binding *in vitro* between CRM1 and Nup214 and the weak effects of Nup214 depletion on NES-mediated export *in vivo*, we hypothesized that Nup214 might be required for certain classes of CRM1-dependent nuclear export substrates. It has been previously shown that the large 60S preribosome subunit is exported via CRM1 and the transport adaptor NMD3 (43,53). We therefore investigated the role of the Nup214/Nup88 subcomplex in 60S preribosomal nuclear export by studying the localisation of GFP-tagged NMD3 (43).
In control cells, GFP-NMD3 was largely excluded from the nucleus (Fig. 2A and C). This cytoplasmic localisation of NMD3 was strictly dependent on its NES (Fig. 2A and 2C). Depletion of the Nup214/Nup88 subcomplex resulted in a striking nuclear redistribution of GFP-NMD3 (Fig. 2A and C). In contrast, Nup358 deficient cells showed no difference to the control (Fig. 2B and C). The localisation of the NMD3$^{\Delta NES}$ remained unchanged in Nup214-shRNA or

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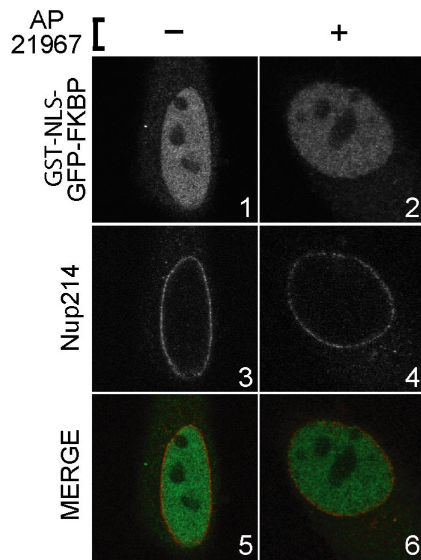
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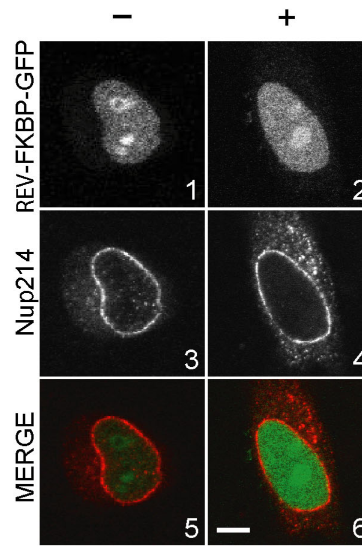
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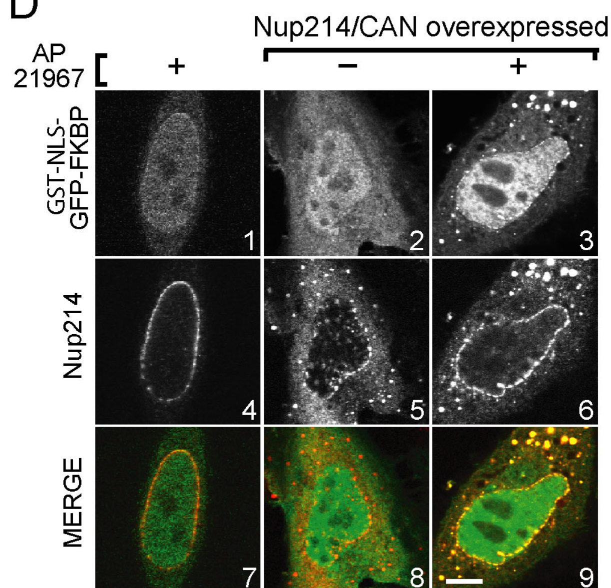
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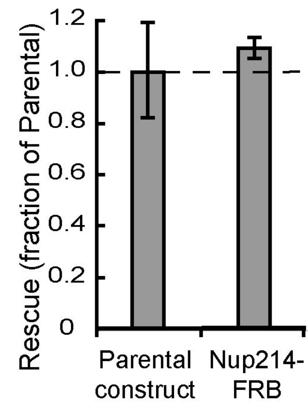
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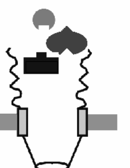
Figure 3. The FG-repeat domain of Nup214 does not access the nucleus. (A) Schematic representation of a system to assess nuclear presence of the FG repeat domain of Nup214. Nucleoporins Nup214-FRB and Nup88 are represented as red and orange circles respectively. The dashed red circle around Nup214 represents the minimum reach area required of an unstructured Nup214 FG-repeat domain to access the nucleus. After addition of the rapamycin dimerizer (purple ovals; A2), nuclear GFP-FKBP (green circles) would accumulate at the NE only if the Nup214 C-terminus has access to the nucleus (A3). Cytoplasmic GFP-FKBP would accumulate at the NE if the FG repeat domain of Nup214 has access to the cytoplasm (A4). N, nucleus; C, cytoplasm. (B, C and D) Subcellular localization of GST-NLS-GFP-FKBP (B, D) or Rev(1.4)-FKBP-GFP (C) after knockdown of endogenous Nup214 and exogenous expression of Nup214-FRB. Note that in D1:3 GST-NLS-GFP-FKBP is present in nucleus and cytoplasm. Nup214 is detected by anti-hNup214 and Texas Red labelled secondary antibody. Rapamycin derived heterodimerizer (AP21967) is added as indicated. Scale bars, 10 µm. (E) FRB-tagged Nup214 is active in promoting 60S preribosomal export. Rescue of GFP-NMD3 nuclear export in Nup214-depleted cells by parental (left) or FRB-fused Nup214 proteins. Methods as in Fig. 4. (Full-colour images shown in cover flap).

Nup358-shRNA expressing cells, indicating that NMD3 nuclear import was not impaired by depletion of the Nup214/Nup88 subcomplex or Nup358 (data not shown). To confirm that nuclear accumulation of GFP-NMD3 reflected a 60S preribosome export defect, the localisation of the preribosomal component rpL29 was assessed in wild-type or Nup214/Nup88 depleted cells. Apart from the accumulation at the nucleoli, which is also observed in control cells, depletion of Nup214/Nup88 resulted in a strong nuclear accumulation of GFP-tagged rpL29 (Fig 2A8, 10 and C). *In vitro*, certain recombinant versions of NMD3 possess a very high affinity for CRM1 (43), approximately 100-fold higher than regular NESs. To test whether the export defect due to depletion of Nup214/Nup88 was specific for high affinity NESs, we determined the nuclear export driven by an NES of a similar affinity, the supraphysiological S1 NES (24). Depletion of Nup214/Nup88 did not induce nuclear accumulation of this reporter protein, indicating that the effects were not related to high-affinity CRM1 binding of NMD3 (Fig 2C).

The FG-repeat domain of Nup214 cannot access the nucleus– It has been proposed recently that the FG-domain of Nup214 could access the nuclear compartment providing a binding site for export complexes. This mechanism would explain how a cytoplasmic localised nucleoporin can mediate export (38,39). In order to examine this possibility (Fig. 3A1), we have tested accessibility of the carboxy-terminal domain of Nup214 to the nuclear compartment *in vivo*. For this, we used a rapamycin-dependent heterodimerizer system (54). The small (95 amino acids) FRB protein, one of the two heterodimerizing components, was fused to the C-terminus of the RNAi-insensitive Nup214. The other heterodimerizing component, FKBP, was fused to either GST-NLS-GFP or the NES-deficient Rev(1.4)-GFP, two constitutively nuclear proteins that cannot freely diffuse across the NPC. As depicted in Figure 3A2-3, rapamycin-dependent dimerization would occur only if the C-terminal FRB containing domain of Nup214 could reach the nuclear compartment.
Expression of Nup214-FRB rescued the effects of shRNA-induced Nup214/Nup88 depletion in Nup214 expression (Fig 3 B3,4 and C3,4) and NMD3 nuclear export (Fig 3 E). This indicates that the Nup214-FRB RNAi-insensitive derivative of Nup214 is correctly expressed, targeted to the NE and functional. Previous studies have shown that a fusion protein consisting of a FRB and Rev NES is small enough (~11.5 kD) and capable to diffuse freely through the NPC, indicating that the FRB component would not prevent the capacity of Nup214 C-terminus to cross the NPC (55). When Nup214-FRB and GST-NLS-GFP-FKBP were highly overexpressed (Fig 3D1:6), the two proteins colocalized in cytoplasmic dots in a rapamycin-dependent manner (Fig 3D2-3,5-6,8-9), providing a control for rapamycin-induced heterodimerization. Furthermore, small amounts of cytoplasmic GST-NLS-GFP-FKBP were sufficient to induce a visible colocalization with Nup214 (Fig. 3D1,4,7). However, no rapamycin-induced heterodimerization was detected when the nuclear reporter proteins were confined to the nucleus, using either GST-NLS-GFP-FKBP or Rev(1.4)-GFP-FKBP protein (Fig 3B2,4,6 and Fig 3C2,4,6). These results indicate that the FG-repeat



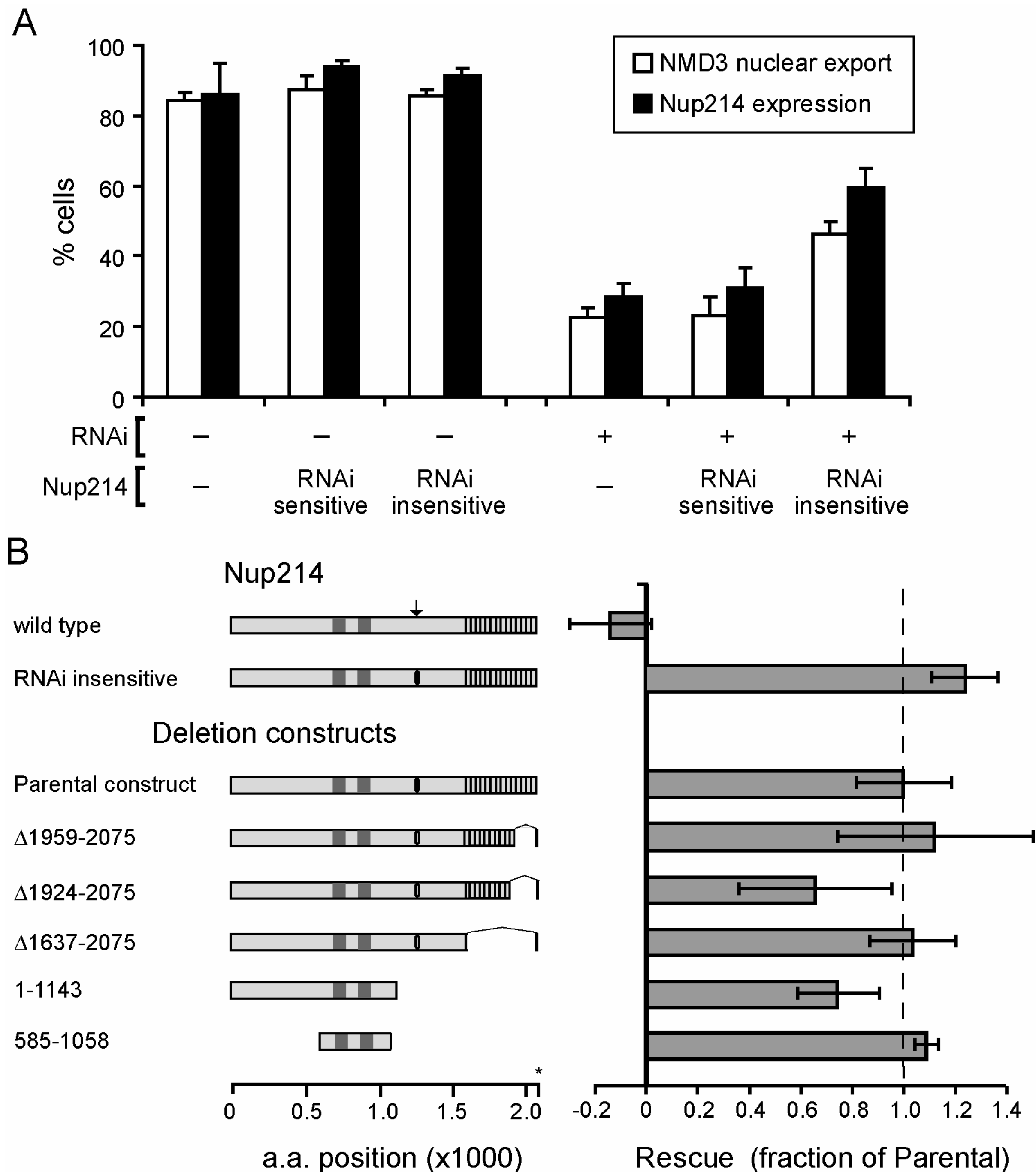


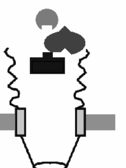
Figure 4. Nup214 FG repeats are dispensable for 60S preribosomal nuclear export. (A) Rescue of GFP-NMD3 nuclear export and Nup214 expression in Nup214/Nup88 depleted HeLa cells by exogenous Nup214. HeLa cells were transfected with Nup214-shRNA expression plasmids (right) or control plasmids (left) and co-transfected with RNAi sensitive or insensitive versions of a Nup214 expression plasmid as indicated below the graph. White bars represent percentages of cells showing cytoplasmic GFP-NMD3 staining greater or equal to nuclear staining. Black bars represent percentages of cells showing Nup214 staining at the nuclear envelope. Error bars represent standard errors. (B) Rescue of GFP-NMD3 nuclear export in Nup214/Nup88 depleted HeLa cells by Nup214 deletion mutants. Nup214 deletion constructs are represented as horizontal bars. Dark boxes indicate central coiled coil domains; vertical bars, FG repeats; arrow, RNAi target. A black oval denotes a mutated RNAi target. Amino acid positions of Nup214 are shown at the bottom 1-2090 (asterisk). Bars graph at the right show the percentage of rescue obtained for each construct relative to the parental deletion construct (dashed line). Error bars represent standard error

domain of Nup214 is not able to access the nuclear compartment from its cytoplasmic site.

Nup214 FG repeats are dispensable for 60S preribosomal nuclear export–In order to determine which region of Nup214 was required for preribosomal nuclear export, we designed several deletion constructs of Nup214 and expressed them in Nup214/Nup88 depleted cells. In order to ensure expression of the re-introduced Nup214 proteins, we designed four silent point mutations in the nucleotide sequence that is targeted by the Nup214 shRNA. To determine the extent of rescue that can be obtained in this setup, we transfected HeLa cells expressing GFP-NMD3 under normal or Nup214/Nup88-depleted conditions with plasmids expressing either RNAi sensitive or insensitive Nup214 (Fig. 4A). In control cells, efficient nuclear export of NMD3 was found in 84% cells and 86% of the cells showed a clear NE staining of Nup214. These scores were not significantly altered when wild type or RNAi-insensitive Nup214 were exogenously expressed. Upon depletion of Nup214/Nup88, only 23% of cells showed efficient nuclear export of GFP-NMD3. Concomitantly, the presence of Nup214 at the NE was reduced to 28% of cells. Neither GFP-NMD3 export nor the expression of Nup214 were significantly enhanced when an RNAi-sensitive Nup214 mRNA was overexpressed, indicating that the exogenous Nup214 transcript was recognised and degraded by the RNAi machinery. In contrast, when the RNAi-insensitive version was re-introduced, 46% of cells were able to export GFP-NMD3. This coincided with a significant increase of Nup214 expression (Fig. 4A). These data indicated specific rescue of shRNA-mediated depletion of Nup214/Nup88 by exogenous Nup214 DNA constructs and defined the dynamic range of the assay to be roughly from 25 to 50% of wild-type.

We next tested GFP-NMD3 nuclear export of Nup214/Nup88 depleted cells that lacked FG-repeats to a varying extent (Fig. 4B). These derivatives were created by ExoIII deletion from a parental construct which had a small deletion from amino acid position 2055 to 2076 of the Nup214 sequence. This parental construct was able to rescue NMD3 export to levels comparable to the full length rescue plasmid. Interestingly, most FG-repeat deletions rescued to similar levels as the parental construct, and two constructs encoding Nup214 versions lacking the entire FG repeat domain (Nup214$^{\Delta 1637\text{-}2075}$ and Nup214$^{1\text{-}1143}$) containing the CRM1 binding site were not significantly different in their capacity to rescue than the parental construct. Expression of Nup214$^{\Delta 1637\text{-}2075}$, Nup214$^{1\text{-}1143}$ or HA-Nup153 did not rescue expression of endogenous Nup214 (Fig 5A, B and not shown). We conclude that Nup214, but not its FG-repeat region, is essential for 60S preribosome export.

Nup214 central coiled coil domains are sufficient for 60S preribosomal nuclear export–We have shown that Nup214 domain can not access the nuclear compartment. In addition, we have excluded the possibility that Nup214 function in 60S preribosomal export is mediated by any carboxy-terminal mediated interaction. These facts suggest that Nup214 does not interact directly with the 60S export complex. In order to further test this possibility, we have expressed three versions of the central Nup214 coiled coil domains. These domains are required to mediate interaction with Nup88 and with the NPC (56,57). Incorporation of the HA1-tagged coiled coil domains into the NPC was analyzed by immunofluorescence and confocal microscopy imaging in Nup214-depleted HeLa cells. As shown before (56), while the first and second Nup214 coiled coils showed no or low NE staining (Fig 5A, 14 and 15 respectively), the protein containing both domains was targeted to the NE very efficiently (Fig 5A, 16). Analogously, Nup88 NE staining was found as high as wild type levels only when the construct containing both coiled coils of Nup214 was expressed (Fig 5C, D). Next, we analyzed NMD3 export by confocal microscopy imaging. While cells expressing the first or second coiled coil domains (Fig 5B) of Nup214 elicited no or little rescue on NMD3 export assays (Fig 5A6,7), cells expressing the complete central domain (585-1058) rescued NMD3 export capacity to the same extent as the Nup214 RNAi insensitive construct (Fig 4B and 5A8). Western blot analysis of HeLa cell extracts expressing shRNAi targeting Nup214 and co-transfected with HA-Nup153, Nup214$^{\Delta 1637\text{-}2075}$, Nup214$^{585\text{-}832}$, Nup214$^{804\text{-}1058}$ or Nup214$^{585\text{-}1058}$ (Fig 5B, lanes 2-6 respectively)



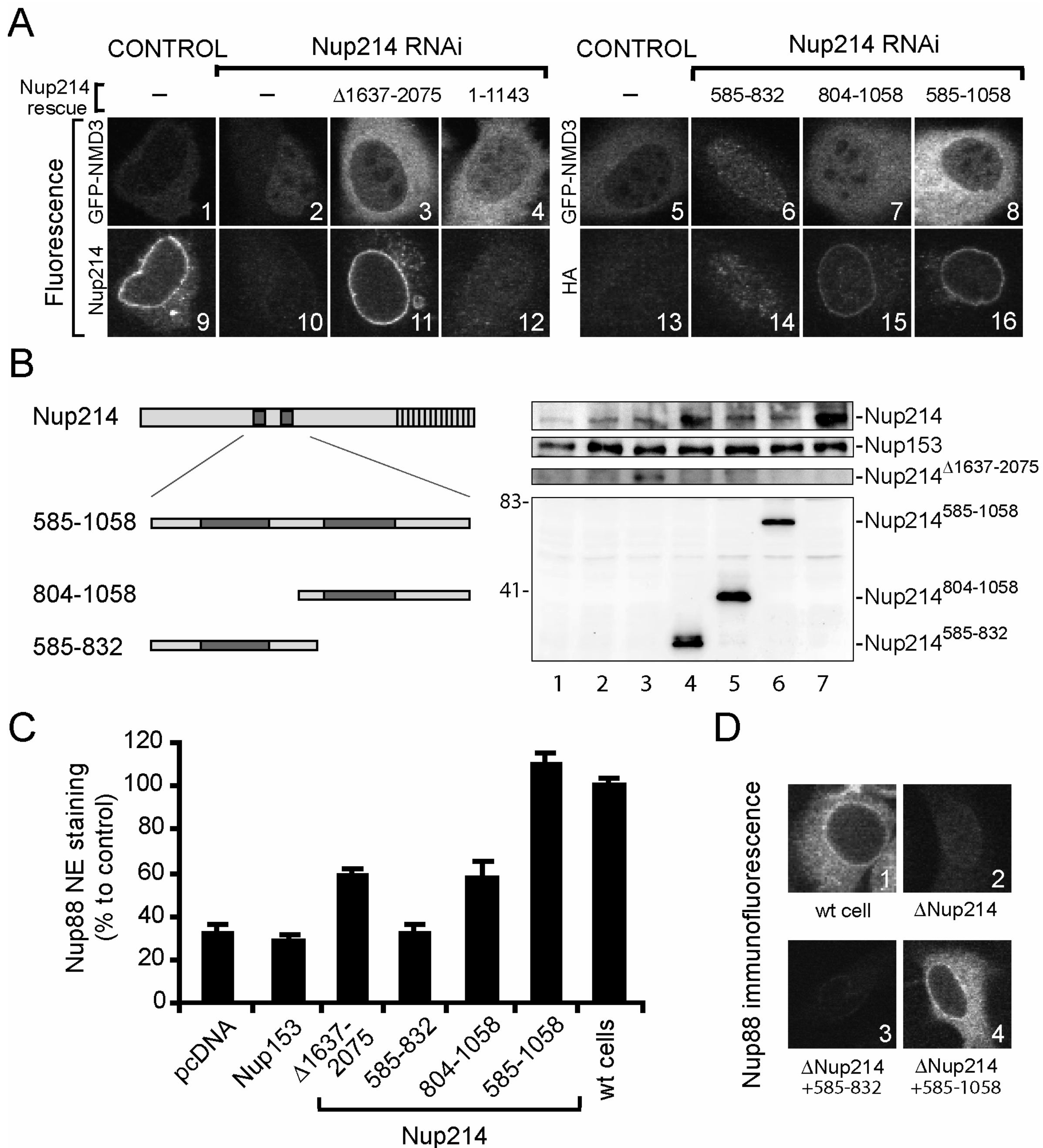


Figure. 5. Nup214 central coiled coil domains are sufficient for 60S preribosomal nuclear export. (A). Subcellular distribution of GFP-NMD3 expressing HeLa cells 72 hours after co-transfection with pSUPER control (A1,9 5,13) or pS-Nup214; and with empty pcDNA (A2,10) or Nup214 rescue plasmids expressing $Nup214^{\Delta 1637\text{-}2075}$ (A3,11), $Nup214^{1\text{-}1143}$ (A4,12), $Nup214^{585\text{-}832}$ (A6,14), $Nup214^{804\text{-}1058}$ (A7,15) and $Nup214^{585\text{-}1058}$ (A8,16). Cells were fluorescently labelled with anti-hNup214 (A9:12) and anti-HA 12CA5 (A13:16) primary antibodies and Texas Red labeled secondary antibodies. (B). Western Blot of HeLa cells transfected with pSUPER empty (lane 7) or pS-Nup214 (lanes1:6). HA-Nup153 (lane 2), $Nup214^{\Delta 1637\text{-}2075}$ (lane 3), $Nup214^{585\text{-}832}$ (lane 4), $Nup214^{804\text{-}1058}$ (lane 5) and $Nup214^{585\text{-}1058}$ (lane 6) were co-transfected. Blots were probed for Nup214, Nup153 using MAb 414, for $Nup214^{\Delta 1637\text{-}2075}$ using anti-hNup214 and for HA1 using 12CA5. (C) Nup214 coiled-coil region is sufficient to target Nup88 to the NPC. Graphic representation showing fluorescence levels of endogenous Nup88 as a percentage of the empty pSUPER negative control after knockdown of Nup214 and co-expression of the indicated plasmids. Representative images are shown in (D); note that absence of Nup214 reduces endogenous levels of Nup88 (see also Fig. 1E).

showed that endogenous Nup214 levels continued to be significantly reduced, indicating that their expression did not interfere with Nup214 RNAi. HA-Nup153 expression also did not rescue NMD3 export (data not shown). These results indicate that Nup214 function in 60S export is mediated by the central domain of Nup214, which interacts with Nup88.

DISCUSSION

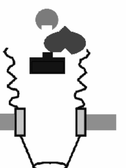
In this study we have assessed the role of the Nup214/Nup88 complex in CRM1-mediated nuclear export. Human CRM1 was first identified as a Nup214 co-precipitating protein that interacted specifically with the C-terminal FG-repeat of this nucleoporin (21). *In vitro*, this interaction is enhanced by RanGTP and cargo, suggesting a role in translocation through the NPC or disassembly of export complexes (36). It was therefore surprising that depletion of the Nup214/Nup88 subcomplex had little or no effect on CRM1-dependent nuclear export of simple export cargos. In yeast, a relatively strong *in vitro* interaction between Nup159 and Xpo1 exists as well (26), suggesting that this interaction has an important evolutionary conserved function. However, removal of the high affinity domain in Nup159 does not significantly affect nuclear export of an NES-GFP-NLS reporter protein (26) or cell viability. Furthermore, studies in yeast indicate that a significant fraction of FG-repeats can be removed from the NPC before cell viability is compromised (25). Therefore, the *in vivo* significance of the high affinity CRM1/Nup214 interaction remains unknown.

In addition to contributing to the hydrophobic inner core of the NPC (8), the FG-repeat region of Nup214 has recently been proposed to move cargo through the NPC from the nuclear to the cytoplasmic face of the NE (38,39). In fact, the FG-repeat region of Nup214 is able to cross the NPC by itself (58), is predicted to be unstructured (59), and long enough to cross the NPC from a cytoplasmic anchoring point. In addition, overexpression of Nup214 results in a presence of this nucleoporin at both sides of the NPC (60). But, irrespective of such a system operating, our Nup214 depletion data indicate that it is not essential for CRM1-mediated nuclear export in cultured cells. To examine whether the C-terminal tail of Nup214 reaches the nuclear face of the NPC *in vivo*, we provided GFP reporter proteins and the C-terminal tail of Nup214 with rapamycin-dependent heterodimerizing tags. Using this system, we only detect NPC localization of the reporter proteins when they are in the cytoplasm, indicating that the FG repeat region of Nup214 can access the cytoplasm but not the nucleoplasm.

We found that the presence of the Nup214/Nup88 subcomplex was required for CRM1-mediated nuclear export of 60S pre-ribosomal subunits. This indicates that different cargos served by the same transport receptor have different nucleoporin requirements, which complements earlier observations that different nucleoporins serve distinct nucleocytoplasmic transport pathways (61-64). Because depletion of Nup214 has no general effect on nuclear protein import (34), or export (this study), the observed preribosomal nuclear export defect is likely not a consequence of pleiotropic effects on other nuclear transport pathways.

The mechanism by which large ribonuclear protein complexes translocate though the NPC remains largely unknown. The size of a 60S preribosomal particle (25 nm) is approximately 100-fold a GFP molecule and close to the upper NPC size limit for a non-deformable cargo (65) suggesting that a significant conformational change of the NPC should occur during its translocation. Recent analysis using cryoelectron tomography of functional *Dyctiostelium* NPCs has revealed distinct structural states correlating with a variable central volume that likely represented large cargo in transit (32).

In yeast, nuclear export of 40S and 60S preribosomes was reported to require the Nup159p/Nup82p/Nsp1p subcomplex (37), which is the proposed yeast homologue of the vertebrate Nup214/Nup88/Nup62 subcomplex (57). Both Nup214/Nup88/Nup62 and Nup159/Nup82/Nsp1 subcomplexes are associated through interactions of coiled-coil domains (56,57) and these domains in Nup159p and Nsp1p are necessary and sufficient for cell viability (66,67). We find that in vertebrate cells, the requirement of the Nup214/Nup88 subcomplex for 60S preribosomal export is dependent on the central coiled coils domain that contains the Nup88 and possibly



Nup62 interaction domains but not on its N-terminus nor its large FG repeats region. Because of the structural characteristics of the Nup214 central domain and its role in correctly positioning the cytoplasmic Nup214/Nup88 subcomplex, it is likely part of the cytoplasmic structures of the NPC. Furthermore, this region lacks FG repeats, thought to be essential for CRM1 interaction. Therefore, we consider it unlikely that a direct interaction between Nup214 and the 60S preribosomal export complex is required for 60S export. We rather propose that the Nup214/Nup88 core domain plays a structural role in large scale conformational changes required for 60S preribosome export, perhaps functioning in a hinge-like manner. This is consistent with deletion analysis in yeast, indicating that only the coiled-coil domain of Nup159, the closest yeast homologue of Nup214, is required for 40S preribosomal nuclear export (37). Further evidence that the Nup214/Nup88 subcomplex is required for nuclear export of large cargoes is the strong mRNA export defect of depletion of Nup214 and Nup88 (35,68), or their yeast equivalents Nup159 and Nup82 (61,62,69).

In conclusion, we have shown that the Nup214/Nup88 subcomplex is required for CRM1-mediated export of a specific cargo, the 60S preribosome, in a process independent of strong CRM1-FG interactions.

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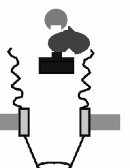

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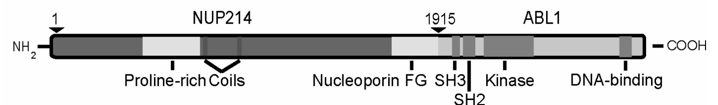
"La Biblia cuenta una historia,
que un dios terrible dictó.
El drama de dos hermanos,
el justo y el traidor.
Abel mezquino y cobarde,
el siervo de su señor.
Caín que no entró en el juego,
y que se rebeló.
[...]
Quizá los hombres seamos,
a un tiempo Abel y Caín.
Quizá algún día destruya,
lo oscuro que hay en mí.
El destino no está marcado al nacer.
Yo he elegido ser lo que siempre seré.
Hijo de Caín"

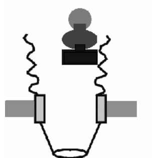
Barón Rojo "Hijos de Caín"



CHAPTER 6

Characterization of the NUP214-ABL onco-protein associated with acute lymphoblastic leukemia

Rafael Bernad,Kim de Keersmaecker, Maarten Fornerod and Jan Cools



Characterization of the NUP214-ABL onco-protein associated with acute lymphoblastic leukemia

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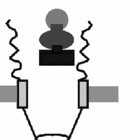
Abstract

Overexpression of an aberrant fusion between the nucleoporin Nup214 and the protein tyrosine kinase Abl1 is frequent in T-cell Acute Lymphoblastic Leukemias (T-ALL). We have studied the localization, nuclear pore complex (NPC) interaction and function of the protein encoded by the translocation product *NUP214-ABL* in order to increase our understanding in the role of this nucleoporin fusion in leukemogenesis. We have found that NUP214-ABL1 interaction with the NPC is equivalent to that of Nup214. NUP214-ABL1 deletions that contained the central coiled coils were sufficient for NPC incorporation but not for tyrosine kinase activity auto-activation and transformation, Abl1 downstream targets were not phosphorylated and an undescribed phosphorylation of Nup358 was found. We propose that the NPC provides a platform that permits, under optimal conditions, proximity autophosphorylation and activation of NUP214-ABL1.

Introduction

Chromosomal translocations are implicated in many human malignancies. One well characterized example is the Philadelphia translocation, a BCR-ABL fusion protein with constitutive protein kinase activity that alters the signaling pathways that control the proliferation, survival, and self-renewal of hematopoietic stem cells. It is typically found in Chronic Myeloid Leukemia (CML) and precursor B-cell Acute Lymphoblastic Leukemia (B-ALL) (De Klein et al., 1986; De Klein et al., 1982). This translocation is rare in T-cell Acute Lymphoblastic Leukemias (T-ALL) (Pui et al., 2004). Very recently, a novel transcript product of the fusion of the oncogenes *NUP214* and *ABL1* was described in T-ALL patients and cell lines (Graux et al., 2004). The recurrent rearrangement leads to the expression of a



constitutively phosphorylated tyrosine kinase. As is the case for *ABL1*, *NUP214* translocations have been described previously in leukemias (von Lindern et al., 1992; von Lindern et al., 1990; von Lindern et al., 1992). These data suggest that this nucleoporin may play an important role in the development of this disease. In contrast to other translocation products that contain Nups, NUP214-ABL1 lacks most of the characteristic FG repeats domain, thought to be implicated in oncogenic transformation (Ahuja et al., 1999; Arai et al., 1997; Borrow et al., 1996; Hussey et al., 1999; Kasper et al., 1999; Nakamura et al., 1996).

Our study focused on the characterization of NUP214-ABL1. We have studied its localization and function in T-ALL cells lines and in transfected cells. We show that this fusion localizes to the NPC and interacts with Nup358, Nup88 and Nup62. The NPC localization and nucleoporin interaction is dependent on the Nup214 coiled coils domain, which is however not sufficient for activation of Abl1 downstream targets. Also other Nup214 domains were not sufficient in these respects, suggesting that multiple Nup214 domains contribute to Nup214-Abl1 oncogenic activity. In addition, a Nup214-Abl1-dependent tyrosine phosphorylation site was found on Nup358. We propose a proximity mechanism, based on the structure and composition of NPC, for NUP214-ABL1 auto activation and oncogenicity.

Materials and Methods

Antibodies– Anti-hNup358V and anti-hNup358F were generously provided by V. Cordes (Karolinska Institute, Stockholm, Sweden) and F. Melchior (Max Planck Institute for Biochemistry, Munich, Germany), respectively. Antibodies to Nup214 (Bernad et al., 2004), anti-CAN9977 (Fornerod et al., 1995), anti-hNup88 (BD Transduction Laboratories), Anti-Nup62 (BD Transduction Laboratories), Anti-Nup358 (Santa Cruz, N-20), monoclonal antibody (MAb) 414 (Eurogentec/Babco), Anti-ABL1 K-12 (Santa Cruz), Anti-ABL1 8E9 (BD Transduction Laboratories), Anti-phospho-ABL1 Tyr245 (Sigma), Anti-phospho Tyr(4G10) (Upstate), Anti-ERK2 (Santa Cruz), Anti-phospho-ERK1/2 (Cell Signaling) and anti-HA (12CA5) were previously described.

Cell culture and retroviral transduction–HEK 293T and Ba/F3 cells were cultured, transfected and transduced as described previously (Cools et al., 2003). Transduced Ba/F3 cells were selected with puromycin (2.5µg/ml) or neomycin (600µg/ml medium) and grown in the presence of IL-3 (1ng/ml) when required. For growth curves, 10^5 Ba/F3 cells were seeded in 1 ml medium and viable cells were counted on 4 consecutive days. K-562, JURKAT, BE-13, PEER and ALL-SIL cells were cultured in

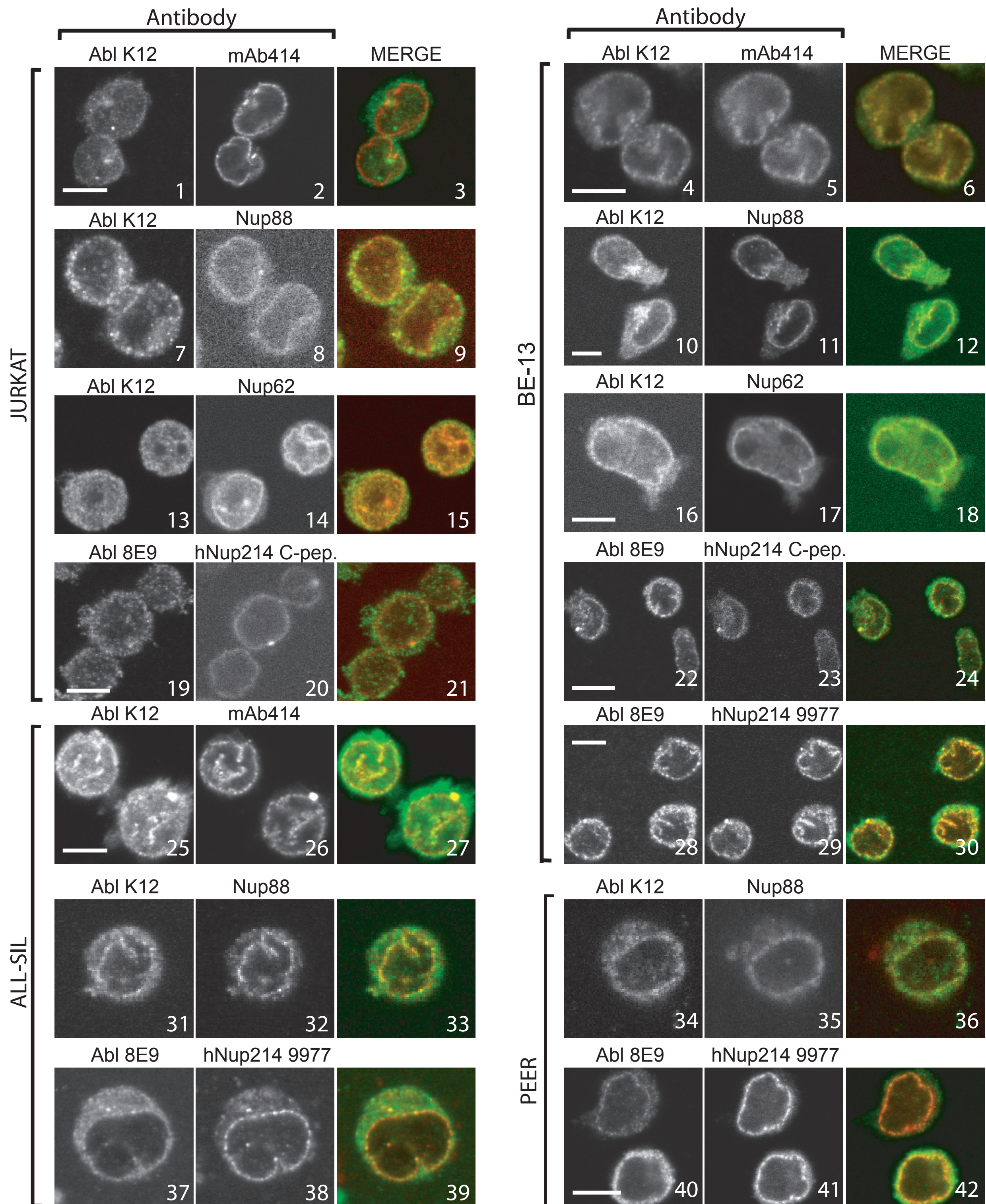


Figure 1: Immunofluorescence of ALL cell lines JURKAT, PEER, BE-13 and ALL-SIL. Note that all cell lines, except JURKAT, are NUP214-ABL positive. Cells were fluorescently double-labeled with anti-Abl K12 in combination with mAb414, anti-hNup88, or anti-Nup62; or anti-Abl8E9 in combination with and anti- hNup214 or anti-CAN9977. (Full-color merges shown in cover flap).

RPMI-1640 + 20% FCS.

Immunoprecipitations-10[7] cells were lysed for 30 minutes in ice, spun and incubated for 1-2 hours with pre-clear Protein-G-Sepharose in lysis buffer. After centrifugation, pre-cleared lysate was incubated with antibody-coupled beads for 4 hours and washed with lysis buffer. For loading on SDS gel, beads were resuspended in Nupage SDS loading buffer supplemented with reducing agent.

Immunofluorescence stainings and image analysis–Indirect immunofluorescence was performed as previously described (Bernad et al., 2004). Cells were spun at 1200 rpm for 1 min prior to fixation. Images were recorded with Leica TCS NT2 and SP2 confocal microscopes.

Results and Discussion

NUP214-ABL1 is targeted to the NPC. Nup214-ABL1 fusion proteins contain the central coiled-coil region of Nup214 that mediate targeting to the NPC (Chapter 5 and (Belgareh et al., 1998; Fornerod et al., 1996). To determine whether Nup214-Abl1 is localized to the NPC, we used immunofluorescence and confocal imaging on several malignant T-cell lines: JURKAT, ALL-SIL, BE-13 and PEER. With the exception of the JURKAT cells, all of them expressed Nup214-ABL1 fusion (Graux et al., 2004). As shown in Figure 1, only *NUP214-ABL* positive cell lines showed nuclear envelope localisation of Abl1 epitopes, presumably representing the fusion product. To confirm that NPC localization of Nup214-Abl1 is mediated by the central coiled-coil region of Nup214, we performed immunolocalization assays on Ba/F3 cells expressing either full length NUP214-ABL1 or deletion constructs (Fig. 2, A). The NUP214-ABL1 deletion that contained the coiled coils incorporated to the NPC (Figure 2, B), whereas constructs that lacked the coiled-coils were located in the cytoplasm. This result indicates that Nup214 central coiled coils are required for NUP214-ABL1 incorporation to the NPC.

NUP214-ABL1 interacts with Nup62 and Nup88. NUP214-ABL1 NPC localisation seen by immunofluorescence prompted us to study the capacity of this fusion to interact with Nup214 NPC interaction partners Nup62 and Nup88 (Belgareh et al., 1998; Fornerod et al., 1996). We performed immunoprecipitation assays on ALL-SIL (containing NUP214-ABL1, (Graux et al., 2004) and K-562 (containing BCR-ABL1, (Wu et al., 1995) cell lines and we were able to co-precipitate Nup62 and Nup88 using ABL1 antibodies only in ALL-SIL (Figure 3, A and not

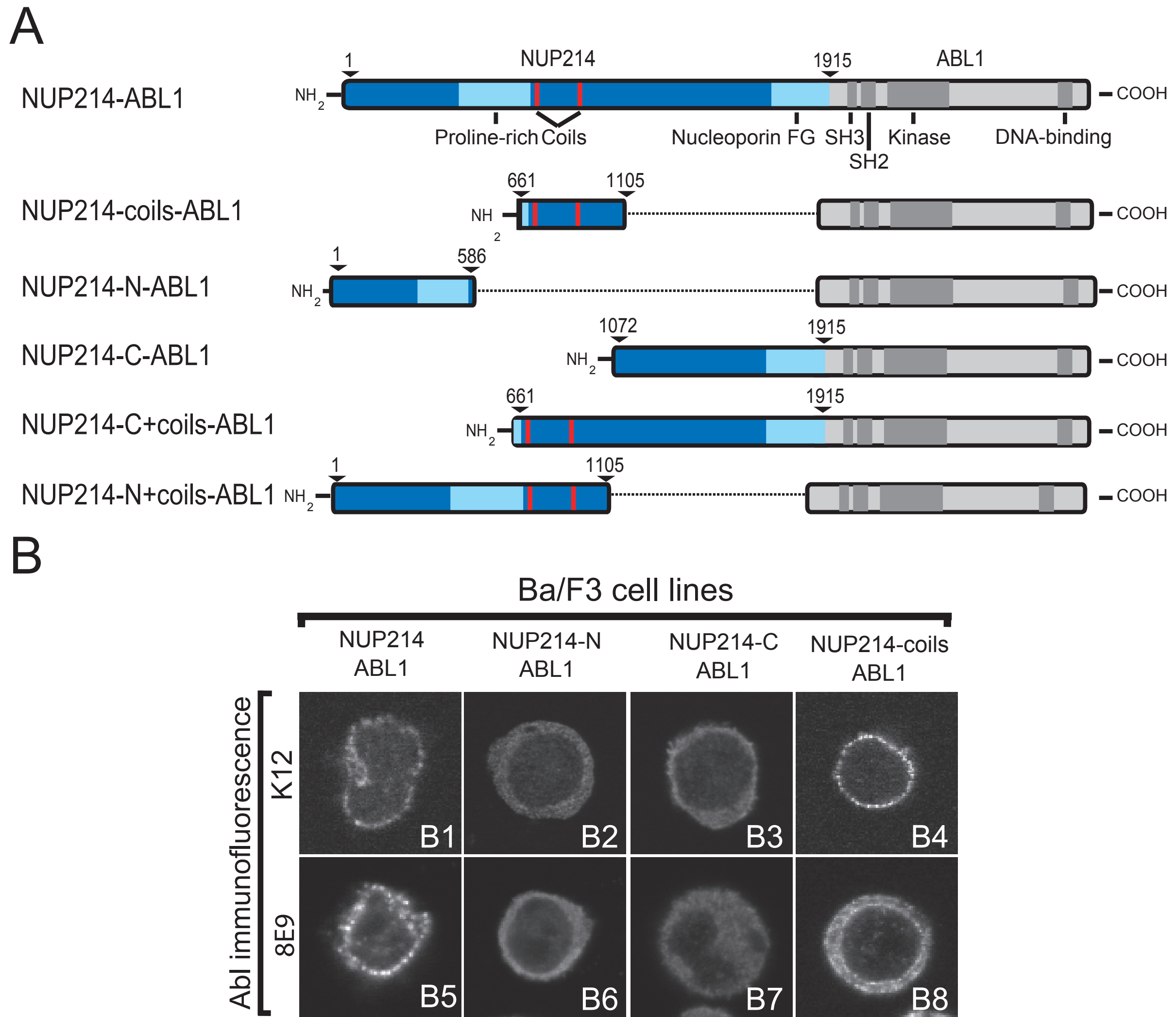


Figure 2(A) Schematic representation of NUP214-ABL1 fusion products and deletion derivatives developed. Nup214 aminoacid positions are marked. FG: Phenylalanine glycine. (B) Immunofluorescence of Ba/F3 cell lines expressing Nup214-ABL1, Nup214-N-ABL1, Nup214-C-ABL1 and Nup214-coils-ABL1 (left to right). Cells were fluorescently double-labeled with anti-Abl K12 (B1:4); or anti-Abl8E9 (B5:8).

shown). Also, we could detect ABL1 using Nup62 antibodies as bait (Figure 3, B). To test the capacity of NUP214-ABL1 deletion constructs to interact with Nup62, we transduced Ba/F3 cells and performed IPs using ABL1 antibodies. As expected, only the deletion that contained the coiled coils domain showed interaction with Nup62 (Figure 3, C). These results further confirm that NUP214-ABL1 interaction with the NPC is analogous to that of Nup214.

Deletions of NUP214-ABL1 suppress Ba/F3 transformation capacity. Oligomerization of fusion kinases is a recurrent mechanism of transformation (Golub et al., 1996). Nup214 contains two central coiled coils (Belgareh et al., 1998; Fornerod et al., 1996). In order to test

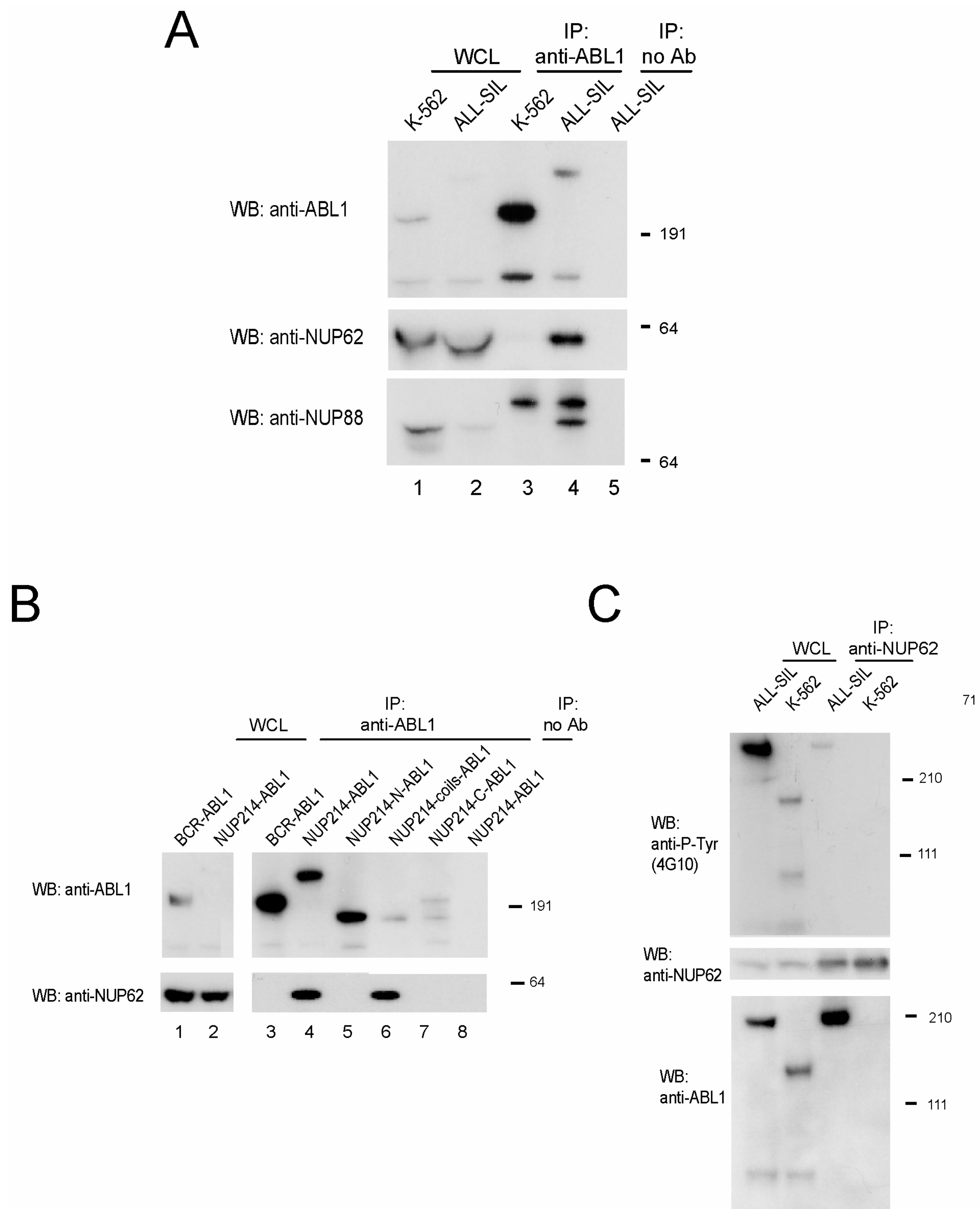


Figure 3: (A and B) Immunoprecipitations of leukaemya cell lines K562 and ALL-SIL. Note that K562 contains a BCR-ABL1 traslocation and ALL-SIL a NUP214-ABL translocation. Antibodies to ABL1 (A), or Nup62 (B) couple to protein G sepharose were incubated with cell extract and coimmunoprecipitating proteins analysed by labelling a Western blot with anti- Nup62 or anti-Nup88 (A); or anti-P-Tyr 4G10 or anti-ABL1 (C). ABL1 immunoprecipitation on extracts from Ba/F3 cells expressing BCR-ABL1, Nup214-ABL1, Nup214-N-ABL1, Nup214-C-ABL1 and Nup214-coils-ABL1 (left to right). WCL: Whole Cell lysates.

whether the Nup214 central coiled coils domain is sufficient to induce transformation, we performed factor-independent growth assays on Ba/F3 cells expressing either BCR-ABL1, full length NUP214-ABL1 or the deletion derivatives shown in Fig. 2A. The growth curve shows that full length NUP214-ABL1 transformation ability is low in comparison to BCR-ABL1 (Fig 4). Neither the NUP214-coils-ABL1 (Fig 4) nor any of the other deletion proteins (data not shown) were capable to induce IL3-independent growth. This result suggests that neither simple dimerization of Nup214 via the coiled-coil region or insertion into the NPC are unlikely to activate Abl1 and that multiple domains of Nup214 contribute to its transforming capacity.

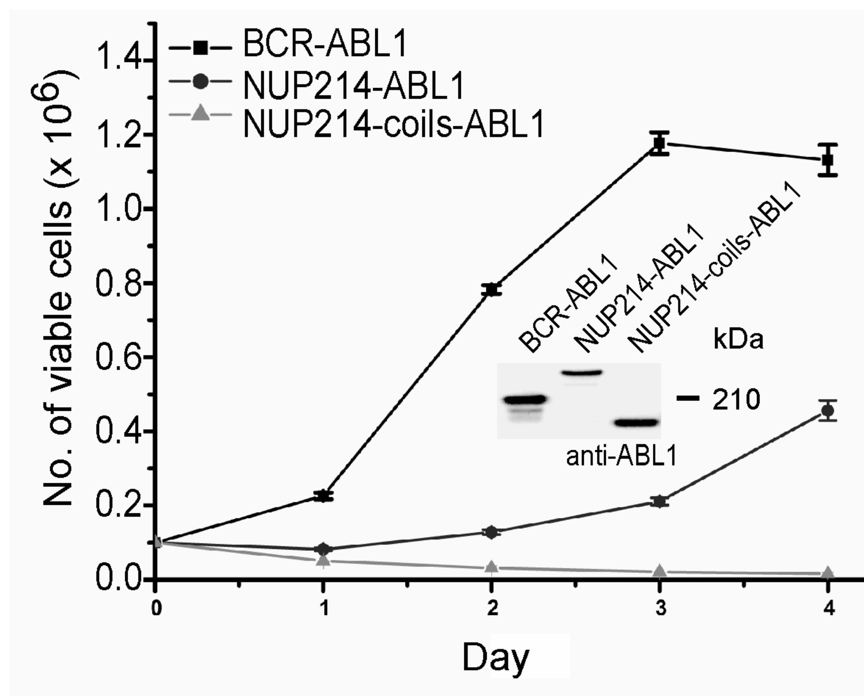
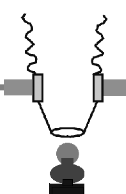


Figure 4. Il3-independent growth of Ba/F3 cells expressing BCR-ABL1, Nup214-ABL1 and Nup214-coils-ABL1. Western blot shows expression of the proteins, detected with anti ABL1.

Deletions of NUP214-ABL1 alter phosphorylation targets. Constitutively activated kinase activity of ABL1 plays a central role in leukemogenesis (Pui et al., 2004). We have studied ABL1 kinase activity on cells expressing NUP214-ABL1 constructs. We only detected phosphorylation on ABL1 tyrosine 245 when full length NUP214-ABL1 was expressed (Fig 5A, upper panel). This phosphorylation was inhibited by Imatinib indicating that is dependent on Abl1 kinase activity. Interestingly, antibodies against tyrosine 245 phosphorylated ABL cross-react to a protein running at ~350 kDa that co-migrates with full length Nup214-ABL1 when ABL1 proteins constructs containing Nup214 coiled coils were expressed (Fig. 5A upper panel, asterisks). This band was found to correspond to Nup358 (not shown) which may react to the anti-P-Abl1 antibody due to a region of similarity around tyrosine 785 of Nup358 (Fig. 5B). This suggests that ABL1 preserves kinase activity when incorporated into the NPC.



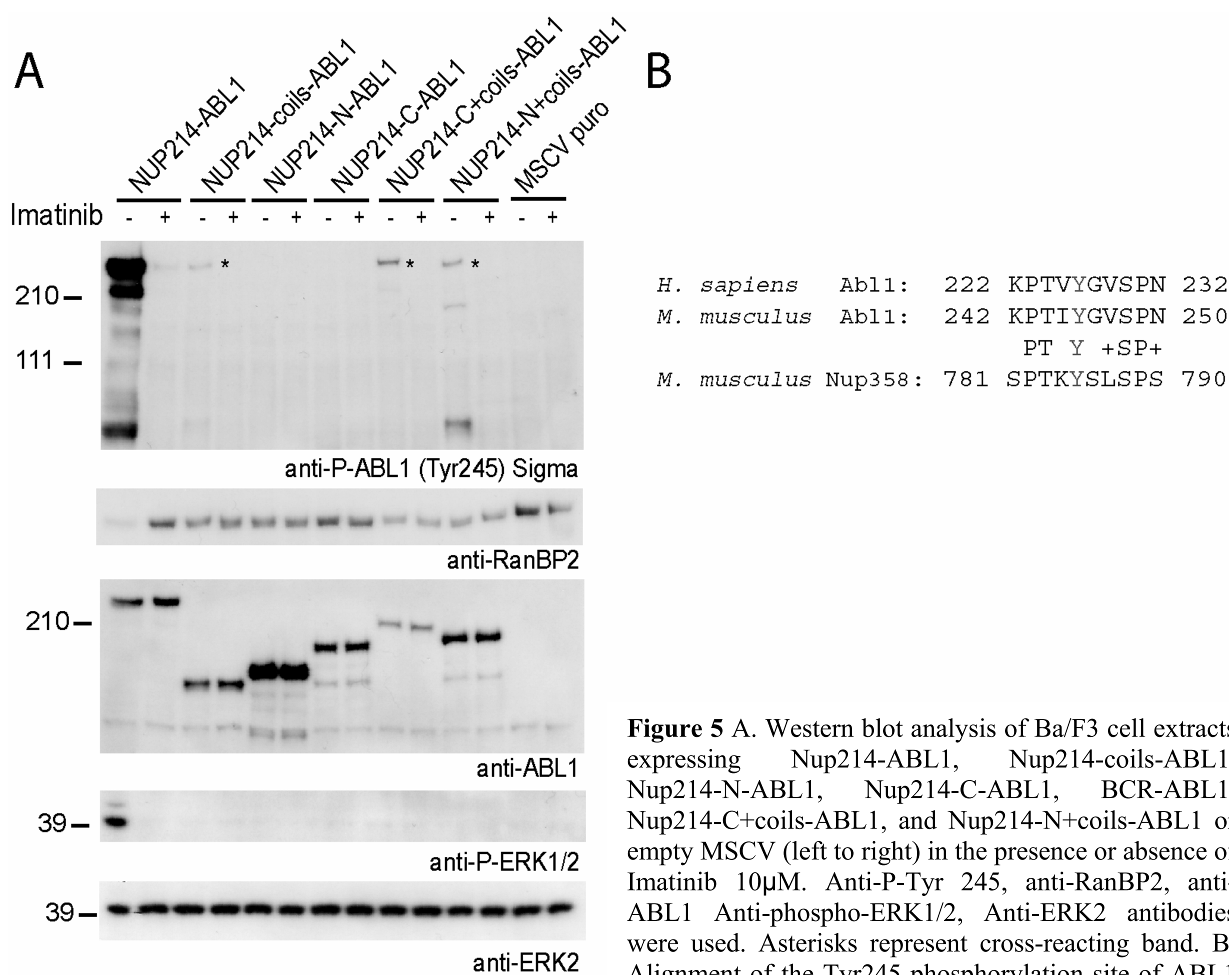


Figure 5 A. Western blot analysis of Ba/F3 cell extracts expressing Nup214-ABL1, Nup214-coils-ABL1, Nup214-N-ABL1, Nup214-C-ABL1, BCR-ABL1, Nup214-C+coils-ABL1, and Nup214-N+coils-ABL1 or empty MSCV (left to right) in the presence or absence of Imatinib 10μM. Anti-P-Tyr 245, anti-RanBP2, anti-ABL1 Anti-phospho-ERK1/2, Anti-ERK2 antibodies were used. Asterisks represent cross-reacting band. B. Alignment of the Tyr245 phosphorylation site of ABL1 and a region of similarity in Nup358.

However, only expression of full length NUP214-ABL1 could induce phosphorylation of the ABL downstream target ERK2 (Towatari et al., 1997).This finding indicates that hyperactivity of the Abl1 kinase is defined by multiple Nup214 domains including those required for NPC localization. One possibility is that only the full length Nup214-ABL1 as incorporated in the NPC brings the ABL1 kinase domains in sufficient proximity for autophosphorylation and subsequent hyperactivity to occur. We have previously shown that the Nup214 coiled coils domain by itself can incorporate into the NPC efficiently (Chapter 5, Fig 5). We predict that overexpression of these coils will be able to compete NUP214-ABL1 out of the NPC. Analysing transformation potential and NUP214-ABL1 under these conditions may reveal if NPC targeting is required for NUP214-ABL1 oncogenic transformation.

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Nup358 localisation is not perturbed on NUP214-ABL1 positive cell lines. Nup358 is hyperphosphorylated in mitosis (Favreau et al., 1996). This process is thought to be related with NPC disassembly. We reasoned that abnormal phosphorylation on Nup358 tyrosine 785 may provoke aberrant Nup358 disassembly. We studied the localisation of Nup358 on the

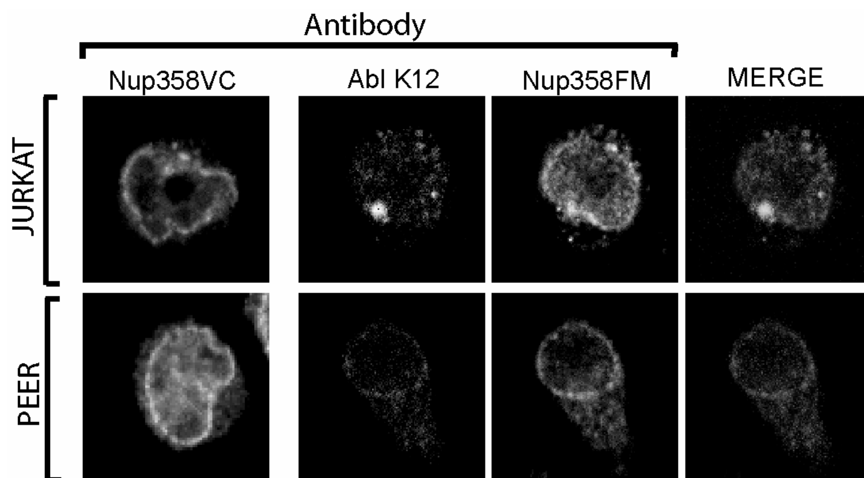
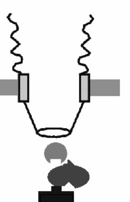


Figure 6 Immunofluorescence of ALL cell lines JURKAT and PEER. Note that JURKAT cell line is NUP214-ABL negative. Cells were fluorescently double-labeled with anti-Abl K12 in combination with anti-Nup358FM; or anti-Nup358VC.

NUP214-ABL1 positive PEER cell lines. Immunofluorescence images show no major change in Nup358 localisation on these cell lines suggesting that Nup358 tyrosine 785 phosphorylation does not provoke its disassembly from the NPC (Figure 6). We suggest that Nup358 phosphorylation is a consequence of the proximity to NUP214-ABL1.

In conclusion, we have shown that NUP214-ABL1 is targeted to the NPC in a manner analogous to Nup214. Furthermore, our data indicate that activation of Nup214-ABL1 is not mediated by simple dimerization, but that multiple domains of Nup214 are required. Resistance to treatment with Imatinib mesylate in chronic myeloid leukemia (CML) and some *NUP214-ABL* positive cell lines was reported (Graux et al., 2004; Shah, 2005). Alternative therapies for treatment are then of great interest. Taking advantage of our current knowledge about the dependency on protein stability of the two components of the Nup88-Nup214 subcomplex (Chapter 3) and the toxic effects of the FG-domain of Nup214 (Boer et al., 1998), we will perform Nup88 knockdowns and Nup214ΔFG or Nup214 coiled coils overexpression on *NUP214-ABL* positive cells lines to study possible changes on *NUP214-ABL* stability and content that could lead to a consequent growth delay and a possible therapeutical strategy.



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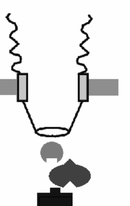
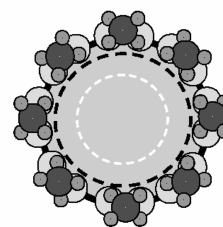


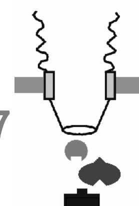
"They say I won't make it to the top of the hill
Won't take the fruit off the tree"
[...]

ACCEPT
"I'm a rebel"



CHAPTER 7

DISCUSSION

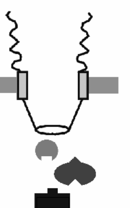


Establishment of communication between the nuclear and cytoplasmic compartments is crucial for eukaryotic life. The nuclear pore complex (NPC) is a multiproteinic structure adapted to elicit nucleocytoplasmic shuttling of a broad variety of molecules ranging from ions to complex ribonuclear proteins. It is of great interest for the understanding of cell biology to elucidate how this is accomplished. This thesis contains intellectual and experimental work that improves the current knowledge concerning nuclear transport.

1. How are the cytoplasmic Nups organized?

The NPC is composed of several copies of each of the ~20 different components, denominated nucleoporins or Nups (Cronshaw et al., 2002; Rout et al., 2000). We studied the localization of these individual components to elucidate their neighbor counterparts and function. We have used electron microscopy techniques on *Xenopus* egg extracts to localize Nup88 (Chapter 3, Fig. 1) and map it in relation to Nup214 and Nup358, the other cytoplasmic oriented Nups (Walther et al., 2002). We show that Nup88 is in close proximity to both Nups. It has been shown before that Nup214 and Nup88 form a stable association (Bastos et al., 1997; Fornerod et al., 1997; Matsuoka et al., 1999). This fact prompted us to further investigate the physical interactions between Nup88 and Nup358. We found that, as with Nup214, Nup88 and Nup358 interact (Chapter 3, Fig. 2). Interestingly, the interaction between Nup88 and Nup214 was detected as well and we could not exclude other Nups mediating interaction with Nup358. This suggests that Nup358 interacts with Nup88 when associated with Nup214 in a subcomplex. Further evidence of this fact was found when we studied protein stability and localization of Nup88, Nup214 and Nup358 in *HeLa* cells under normal conditions or after RNA interference-mediated depletion of each individual component. We found that Nup358 could no longer incorporate to the nuclear envelope (NE) when the Nup214/Nup88 subcomplex was absent (Chapter 3, Fig. 3). This finding indicates that the Nup214/Nup88 subcomplex is the anchoring site of the cytoplasmic filaments to the NPC and establishes the hierarchy of interactions for the cytoplasmic Nups.

NPCs undergo organised rounds of disassembly and re-assembly every cell cycle and Nups show a dynamic behaviour during interphase (Doye and Hurt, 1997; Rabut et al., 2004; Ryan and Wente, 2000). These facts indicate that Nups need to be tightly regulated. Nups associate



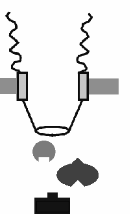
in subcomplexes prior to incorporation to the NPCs. We studied the fate of the components of the Nup214/Nup88 subcomplex when their expression was inhibited through RNAi. We showed a co-dependence of these Nups in both protein stability and NPC incorporation (Chapter 3, Fig. 3 and 4) which is in turn required for Nup358 NPC incorporation. Intriguingly, microarray based expression profiles showed that Nup88 and Nup214 had a tendency for co-regulation (Perou et al., 1999; Ross et al., 2000), indicating that transcription of these two Nups is tightly coordinated. The mechanism of these networks is not clear but it is likely to serve as a system to maintain the correct stoichiometry for these NPC components. Nup88 function is largely unknown. Proteomic analysis of the NPC indicate that it is 4-fold more abundant than Nup214 at the NPC (Cronshaw et al., 2002). The reason for this higher abundance of Nup88 may be related to its capacity to interact with other NPC components (Griffis et al., 2002) and surplus Nup88 explains why *in vitro* reconstituted nuclei from Nup214 depleted *Xenopus* still retain Nup358 (Walther et al., 2002).

Electron microscopy techniques have provided extensive information of the NPC structure (Akey, 1989; Beck et al., 2004; Goldberg and Allen, 1993; Stoffler et al., 2003). In contrast, very little is known about how this macromolecular complex is constructed at atomic resolution. The main reason for this is the unstructured nature of many Nups provided by the presence of repeated phenylalanine and glycine motifs (FG-repeats) (Denning et al., 2003), which impedes the crystallization procedure. Solving Nup214/Nup88 subcomplex structure at the molecular level may provide very important information towards understanding how NPC subcomplexes assemble. We have shown that Nup214 and Nup88 stability is maintained upon co-expression (Chapter 3, Fig. 4) and we have mapped the Nup214 domain that mediates Nup88 stabilization to a central region containing two coiled coils (Chapter 5, Fig. 5 and (Fornerod et al., 1996). We propose to co-express this domain with Nup88 in an attempt to obtain a stable and structured complex for crystallization.

2. What is the role of Nup358 in nuclear transport?

Transport receptors and the Ran system co-operate with the NPC to achieve nucleocytoplasmic transport (Allen et al., 2000; Bayliss et al., 2000; Görlich and Kutay, 1999; Hetzer et al., 2005; Mattaj and Englmeier, 1998; Weis, 2003; Wente, 2000). Transport receptors can interact with

FG-domain containing Nups and promote translocation of their cargoes (Allen et al., 2001; Bayliss et al., 2002; Görlich et al., 1997). Interestingly, differential binding affinities exist between transport receptors and specific FG-Nups (Iovine et al., 1995; Strawn et al., 2001) suggesting that these interactions may be relevant for nuclear transport. It has even been proposed that an affinity gradient for transport receptors mediates nuclear translocation (Ben-Efraim and Gerace, 2001). However, recent evidence shows that most FG-repeats, especially those of asymmetric Nups, are dispensable for NPC viability, exclusion diameter and transport in yeast (Strawn et al., 2004; Zeitler and Weis, 2004). In a typical round of CRM1-mediated export (Chapter 2, Fig. 3), this transport receptor binds a NES-containing cargo in the presence of RanGTP and releases it upon RanGTP hydrolysis in the cytoplasm. Nup358 contains four RanBP1-like RanGTP binding domains that act as RanGTPase coactivators (Beddow et al., 1995; Bischoff et al., 1995; Richards et al., 1995; Villa Braslavsky et al., 2000), and two RanGDP binding Zinc-finger domains (Yaseen and Blobel, 1999). Nup214 contains, in addition to the two central coiled coils domains that associate with Nup88 and target them to the NE, a carboxy-terminal FG-repeat domain that strongly binds the export receptor CRM1 (Fornerod et al., 1996; Fornerod et al., 1997). None of the cytoplasmic Nup214 nor Nup358 were required to mediate import of proteins on *in vitro* assembled NPCs (Walther et al., 2002) indicating that they may play a role in export. For that reason our work focused on the function of the cytoplasmic Nups in export pathways. First, we have found that Nup358 depletion provoked a defect on export when we used a sensitive Rev-GFP export assay (Chapter 3, Fig. 6). Furthermore, we show that this Nup can hold empty CRM1 at the cytoplasmic side of the NPC (Chapter 3, Fig. 5). These results suggest that Nup358 may have a role in CRM1-mediated export as a supporting element providing the platform where the export complexes are disassembled. Studies performed on supraphysiological nuclear export signals (NES) further strengthen this hypothesis (Chapter 4). These superNESs bound CRM1 strongly independently of RanGTP. Export complexes containing a superNES accumulated at Nup358 after NPC translocation (Chapter 4, Fig 4 and 5), suggesting that cargo release is less efficient and that export complex disassembly occurs at Nup358. The fact that freely diffusing cytoplasmic RanBP1 is capable to perform the same reaction (Beddow et al., 1995; Bischoff et al., 1995; Richards et al., 1995; Villa Braslavsky et al., 2000) explains why, in contrast to the results found in *Drosophila* that lacks RanBP1 homologue (Samaklovis, personal



communication), many depleted cells are still capable of exporting substrates even in a sensitive export assay. In addition, it further suggests that Nup358 is the immediate location where export complexes can disassemble upon translocation and release cargo while retaining emptied CRM1 for a rapid recycling to the nucleus (Chapter 4, Fig 7). A similar model of empty receptor retention has been proposed for the NFX1-p15 heterodimer mRNA export receptor, which was not present at the NPC upon depletion of Nup358 in *Drosophila* (Forler et al., 2004). All these facts indicate that Nup358 presence at the NPC increases the efficiency of export.

3. Why are nuclear export signals born to be weak?

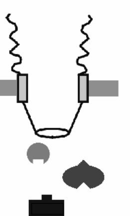
We have selected NESs with strong, RanGTP independent, CRM1 affinities and shown that they are not optimal for export. In contrast to other export receptors CAS, exportin-t and exportin-4 (Kutay et al., 1997; Kutay et al., 1998; Lipowsky et al., 2000), the affinity of CRM1-RanGTP complex for endogenous NESs is weak (Askjaer et al., 1999; Paraskeva et al., 1999). Irrespective of their weakness, NESs are diverse and they provide with different export efficiencies to the proteins that contain them (Henderson and Eleftheriou, 2000). Our results show that weak affinities are not trivial but crucial for efficient export (Chapter 4). Furthermore and since supraphysiological NESs arrest only after nuclear translocation, they suggest that, under physiological conditions, the affinities of NESs for CRM1 contribute to export efficiency by determining the rate of export complex assembly and disassembly, but not by altering the translocation process itself.

But, are supraphysiological signals functioning in nature? We have shown that they may provoke toxic effects at high levels (Chapter 4, Fig 5), suggesting that they may play a role in the pathology of infection. It has been shown that Nup358 is required for kinetocore assembly, implicating CRM1 pathway in mitosis (Salina et al., 2003). SuperNES may have a function different to export after nuclear envelope breakdown by regulating CRM1 in mitosis. Recycling of the small nuclear ribonucleoprotein import receptor snurportin is mediated by high affinity interaction with CRM1 indicating that strong interactions can occur in cells with no deleterious effect (Paraskeva et al., 1999). This can be possible at low concentrations or in the presence of alternative release mechanisms for these specific complexes. The proposed

ribosomal export adaptor NMD3 (Thomas and Kutay, 2003; Trotta et al., 2003) contains a targeting signal that resembles the strong NES consensus (Kutay and Guttinger, 2005) and shows under certain conditions, (when tagged with protA), very strong CRM1 affinity (Chapter 4, Fig 3 and 6). Although this effect can be considered as an artifact provoked by the alteration of the sequences around the NES, its presence in preribosomal export complexes is intriguing. A possible reason for the requirement of strong NESs would be that the residence time at the nuclear side of the NPC would have to be longer for certain type of particles. Strong NESs would be required for sufficiently long interaction between the NPC and the export complex. As simple supraphysiological NES cargoes accumulate at the NPC, the preribosomal particle (as well as snurportin) should have a specific release mechanism to prevent this. Further investigation should be performed to address this possibility.

4. What is the role of the high affinity interaction between Nup214 and CRM1?

When we depleted Nup214 by RNAi, we found only moderate defects in export of three different reporters: a PKI NES-bearing GFP molecule and two Rev-GFP reporters containing Rev-NES and the supraphysiological NESs S1 (Chapter 5, Figs 1 and 2), These defects are comparable to an absence of Nup358 at the NPC, known to be dependent on the Nup214/Nup88 subcomplex (Chapter 3, Fig 3), indicating that these Nups are not essential for CRM1-mediated export and suggesting that the *in vitro* high affinity of CRM1 for the Nup214 FG-domain may not reflect any function of Nup214 in CRM1-mediated transport. This result is in agreement with the results found on yeast strains devoid of FG-Nups (Strawn et al., 2004; Zeitler and Weis, 2004). Furthermore, Zeitler and co-workers fused the Nup159 (Nup214 yeast homologue) high affinity Xpo1 (CRM1) binding domain to the nuclear Nup1 and showed that NES mediated export was not compromised. All these data clearly argue against a gradient affinity model for nuclear transport (Ben-Efraim and Gerace, 2001), that consider asymmetric FG-domains essential. We propose that the FG-domain of Nup214 may participate redundantly on the formation of the NPC kinetic/hydrophobic barrier. As is the case for Nup358, Nup214 may play a role in mitosis regulating CRM1 activity and/or localization (Salina et al., 2003). As an alternative of the affinity gradient model, it has been proposed that the Nup153 and



Nup214 domains that contain high affinity binding sites for transport receptors are able to cross the NPC barrier and escort the transport complex through the pore (Fahrenkrog et al., 2002; Paulillo et al., 2005). Our *in vivo* studies on cytoplasmic-Nups depleted cells exclude this possibility for CRM1 export. Furthermore, we show that Nup214 can not access the nuclear compartment *in vivo* (Chapter 5, Fig. 3), indicating that this model is not applicable to Nup214. We consider that the reason for this discrepancy is that sub-optimal antibody specificity and sample processing for electron microscopy led Paulillo and co-workers to a incorrect interpretation of the data. Nevertheless, the fact that the high affinity interaction between Nup214 and CRM1 is conserved indicates that it has a function, possibly implicated in the retention and recycling of empty CRM1, as the domain swap experiment suggests (Zeitler and Weis, 2004).

5. Is there a specific role for Nup214/Nup88 subcomplex in nuclear export?

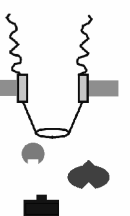
Our studies on NLS-GFP-NES and Rev-GFP assays showed that depletion of Nup214/Nup88 subcomplex provoked a moderate export defect which can be attributable to the consequent lack of Nup358 at the NPC (Chapter 3, Fig. 3). However, we found a dramatic export defect of the ribosomal export adaptor NMD3. Since NMD3 mediates export of the 60S preribosomal subunit (Ho et al., 2000; Thomas and Kutay, 2003; Trotta et al., 2003; Warner, 2001), a consequent delay in preribosome export and maturation occurred when Nup214 was reduced (Chapter 5, Fig 2). This indicates that Nup214 is implicated in this specific transport process. Also, it shows that diverse cargos utilizing the same export receptor can trigger different transport mechanisms and raises the possibility that transport mechanisms are defined by the characteristics of the cargo or the cargo-transport receptor interaction. Intriguingly, a minimum Nup214 derivative containing the central coiled coil domains were able to restore NMD3 export capacity (Chapter 5, Fig. 5). This domain is required for Nup88 interaction and NPC incorporation, probably through Nup62 binding (Chapter 5, Fig. 5 and (Belgareh et al., 1998; Fornerod et al., 1996). Considering the size of the central Nup214 coiled coil domain and that Nup214 localises to the cytoplasmic side of the NPC (Walther et al., 2002), we can exclude any direct interaction between the preribosomal export complex and Nup214 as a requirement for export. This indicates that Nup214 plays a dual role in NPC function mediated by different

domains: participation in the barrier through its FG-rich domain and 60S export through association with Nup88 and Nup62.

More extensive research is required to further determine whether all large complexes require a specific transport mechanism for translocation irrespective of their transport receptor. Messenger RNPs are large complexes. They are exported by specific export receptors and require the action of specific RNA helicases and adaptor proteins (Huang et al., 2003; Izaurralde, 2004; Reed and Hurt, 2002). Furthermore, the mRNA export receptor affinities are regulated by phosphorylation suggesting the existence of an alternative release mechanism for mRNAs (Gilbert and Guthrie, 2004). There is a close relationship between NPC function and transcription activation indicating that transcription and mRNA export are coupled processes (Aguilera, 2005; Schmid et al., 2006). Interestingly, genetic depletion of Nup214 in mice provoked nuclear poly(A)$^{+}$ RNA accumulation (van-Deursen et al., 1996). The yeast counterpart Nup159 is also required for poly(A)$^{+}$ RNA export (Belgareh et al., 1998). Forler and co-workers found in *Drosophila* that dsRNA mediated depletion of either Nup358 or Nup214 provoked accumulation of poly(A)$^{+}$ RNA suggesting that mRNA export is blocked in the absence of these Nups. *hsp70* mRNA accumulated in the nucleus as well on Nup358 dsRNA treated cells after heat shock. But a moderate reduction of cytoplasmic mRNAs was found upon Nup358 depletion. It is not clear whether this reflects a failure in NPC translocation or an indirect cellular response as a consequence of a defective NPC. The NE localization of NFX1 and the delayed mRNA export found on a stress response suggests that Nup358 plays a supporting role by favoring recycling of receptors. Based on our finding concerning the different behavior of large complexes on transport, we predict that the size of mRNPs may influence export dynamics and propose that a detailed study on the role of Nup214 on export mRNA should be performed taking these factors into consideration.

6. Is the Nup214/Nup88 interaction at the NPC required for gating?

Mathematical modeling of nuclear translocation predicts three major classes of transport: partitioning, enhanced diffusion and NPC gating (Becskei and Mattaj, 2005). The partitioning model is related to the traditional well-supported hydrophobic exclusion and virtual gating models (Jaggi et al., 2003; Ribbeck and Görlich, 2002; Rout et al., 2003). Enhanced diffusion



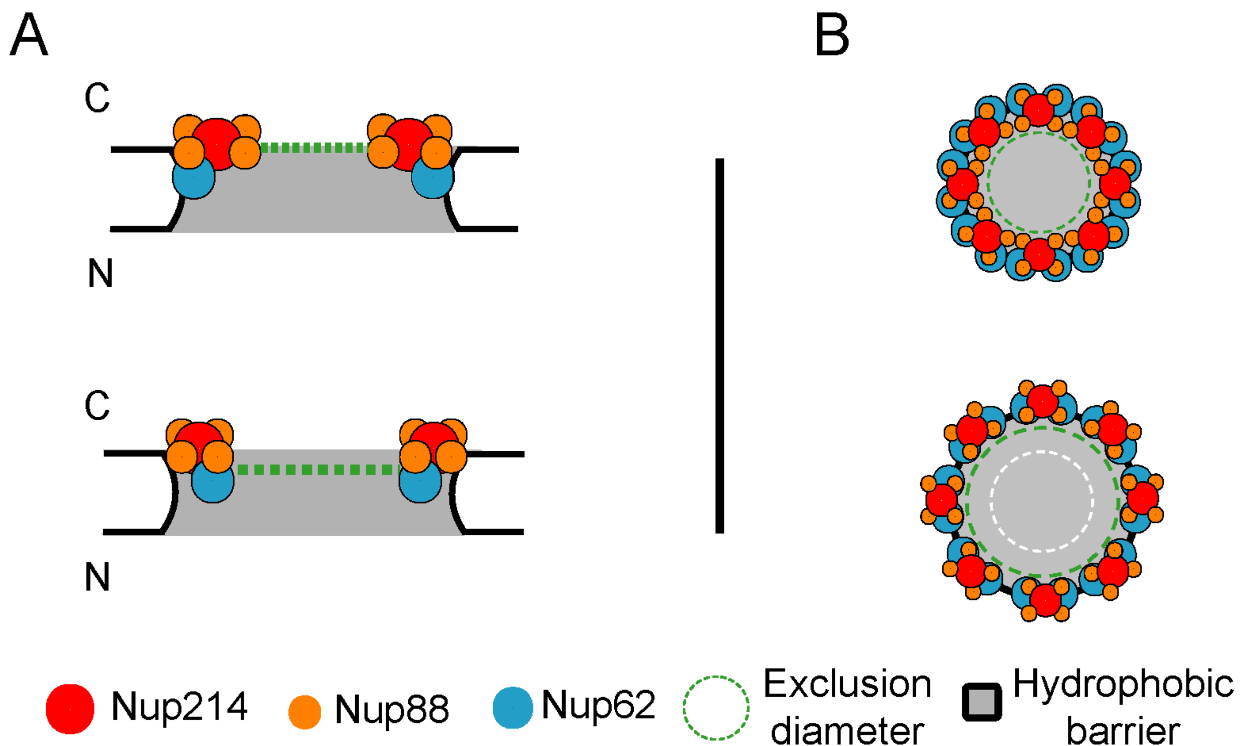


Figure 1. NPC gating model. Side (A) and top (B) view representation of the NPC showing two conformations: closed (top) and gated (bottom). Nup stoichometry is based on (Cronshaw et al., 2002). Note the actual exclusion diameter (black) is increased upon gating (Full-colour image in cover).

and NPC gating are less consolidated and postulate that, in order to accomplish otherwise unlikely transport reactions, the NPC changes its properties favoring transport complex translocation. While enhanced diffusion does it by modulating the domain interactions within the meshwork, NPC gating requires a conformational change. No experimental evidence supports the enhanced diffusion model. The size of the 60S preribosomal subunit complex is close to the exclusion size limit of the NPC (Feldherr and Akin, 1990), suggesting that a major change of NPC characteristics may be required to accommodate translocation of large cargoes. We have shown that, while small GFP reporter derivatives are not affected, a 100-fold larger cargo fails to export in the absence of Nup214/Nup88 subcomplex (Chapter 4). Furthermore we revealed that just the small Nup214 domain that interacts with its neighbor Nups is sufficient to mediate 60S export suggesting that this can not occur by altering the characteristics of the NPC at a molecular level but at a supramolecular level. We propose that a NPC gating mechanism is required for export of preribosomes and that Nup214 plays an important role in this mechanism by acting as a structural scaffold between its counterparts and

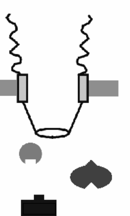
mediating, in a hinge like manner, transition between NPC conformational states (Figure 1). Our data represent evidence supporting this model and several microscopy studies have shown structural changes that alter the permeability of the NPC (Beck et al., 2004; Jaggi et al., 2003; Kiseleva et al., 1998; Stoffler et al., 2003).

Several lines of evidence suggest that NPC gating may be dependent on Ca^{2+} levels (Stoffler et al., 1999) and a study on *Saccharomyces cerevisiae* revealed changes in cargo exclusion size upon treatment of cells with aliphatic alcohols which alter NPC properties (Shulga and Goldfarb, 2003). Based on this, we predict that intracellular calcium depletion would prevent NPC gating. Investigation on the gating mechanism is necessary to further determine if it is required for all large cargoes, if it is directional, if it requires energy, which other Nups are required and, if so, which are able to initiate it.

7. What are the roles of Nup214 and Nup88 in cancer?

Although higher content of Nups can be related to an increased cell metabolism and proliferation (Feldherr and Akin, 1993), the finding that Nup88 levels are high in relation to other Nups in more aggressive tumors is intriguing (Agudo et al., 2004). Nup88 and Nup214 are closely co-regulated (Chapter 2 and (Perou et al., 1999; Ross et al., 2000), suggesting that both components are required for tumor development. Nup214 is frequently found in leukemia associated chromosomal translocations (Fornerod et al., 1995; Kraemer et al., 1994; von Lindern et al., 1992; von Lindern et al., 1990) and both components of the Nup214/Nup88 subcomplex are highly expressed in leukemias (Perou et al., 1999; Ross et al., 2000).

Recently, an episomal aberration was found in Acute Lymphoblastic Leukemia (ALL) that expresses a fusion between NUP214 and ABL1 and, as a consequence, a constitutively activated tyrosine kinase NUP214-ABL1 is overexpressed (Graux et al., 2004). In contrast to other Nup214 and Nup translocations, NUP214-ABL1 product lacks most of the FG-repeats indicating that the mechanism of oncogenesis may differ to that previously suggested (Kasper et al., 1999). *Abl* autophosphorylation is a prerequisite for activation (Brasher and Van Etten, 2000) and it has been shown that oligomerization of fusion kinases can induce constitutive kinase activity (Golub et al., 1996). This suggests that Nup214 coiled coils may allow NUP214-ABL to oligomerize and provide capacity to autophosphorylate. We analyzed the



localization and function of this product as well as deletion derivatives containing either the amino-terminal, central or carboxy-terminal domains of Nup214. Irrespective of the presence of the coiled coils, none of the deleted constructs were capable to induce transformation of Ba/F3 cells (Chapter 6, Fig. 4), indicating that, in contrast to TEL-ABL1 fusion (Golub et al., 1996), coiled coils mediated oligomerization is not sufficient for transformation. Intriguingly, immunofluorescence results clearly show that, as it was for all NUP214-ABL1 positive cell lines, all constructs that contained the coiled coils could incorporate to the NPC (Chapter 6, Fig 1 and 2). In contrast, only full-length NUP214-ABL1 was autophosphorylated and could phosphorylate the downstream *Abl* target ERK2 (Chapter 6, Fig. 5). Surprisingly another phosphorylated target was detected when constructs contained Nup214 coiled coils: Nup358 (Chapter 6, Fig 4 and not shown). This result suggests that derivatives containing the coiled coils have kinase activity, as they phosphorylate Nup358, but fail to target themselves and downstream oncogenic targets. We propose that, instead of oligomerization, the coiled coils domain of Nup214 provides a platform that permits, within the symmetry of the NPC, proximity between neighbor *Abl1* molecules and activation. Supporting this fact is the capacity of all coiled coils containing constructs to co-precipitate with all Nup214 neighbor Nups (Chapter 6, Fig 3 and not shown). This hypothesis would reason that deleted constructs are not close enough for autophosphorylation and oncogenic activation. We propose to study this hypothesis at the molecular level through rapamycin-induced heterodimerization of Nup214 with free *Abl1* and/or FRET (Fluorescence Resonance Energy Transfer) and proximity techniques. Phosphorylation on Nup358 tyrosine was never reported. It is unknown whether this has any effect on Nup358 function and oncogenesis. We found no change in the localization of Nup358 in NUP214-ABL1 positive cell lines suggesting that phosphorylation does not prevent its incorporation to the NPC (Chapter 6, Fig 6). Further research is required to determine if Nup358 is implicated in the oncogenesis of NUP214-ABL.

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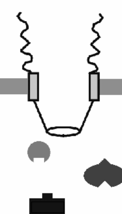

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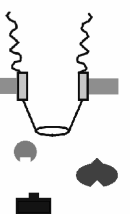

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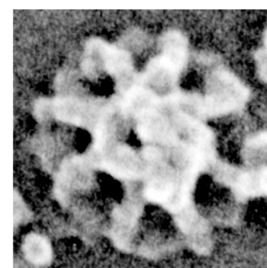
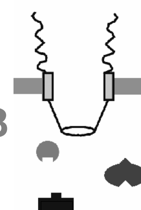
"De pequeño me impusieron las costumbres
me educaron para hombre adinerado
pero ahora prefiero ser un indio
que un importante abogado"

Extremoduro
"Ama, ama y ensancha el alma"



CHAPTER 8

Resumen en castellano para no científicos



Resumen en castellano para no científicos

La célula es la unidad vital porque es la estructura mínima capaz de realizar las funciones biológicas tanto en organismos unicelulares como pluricelulares. Cada célula esta aislada del exterior por una membrana celular. En su interior contiene su información genética en su Ácido DesoxirriboNucleico (ADN) y distintos tipos de orgánulos que realizan diversas funciones, como, por ejemplo, el metabolismo energético. Las proteínas son el resultado de la expresión del ADN y cumplen las funciones necesarias para mantener la célula viva construyendo la estructura de los orgánulos celulares y catalizando reacciones bioquímicas. A su vez, construyen la maquinaria necesaria para que el propio ADN se exprese y se duplique en cada división celular. La síntesis de proteínas requiere dos procesos principales: la transcripción y la traducción. La transcripción es el proceso por el cual, a partir del ADN, se sintetiza una molécula de Acido RiboNucleico (ARN), denominada ARN mensajero, que es el encargado de transmitir la información que se encuentra codificada en los genes. Esta información será convertida a proteína mediante la traducción del código genético presente en el ARN mensajero. Este proceso se lleva a cabo en los ribosomas, factorías biológicas de proteínas.

Los seres vivos se clasifican, según su estructura celular, en procariotas y eucariotas. A diferencia de la célula procariota, la célula eucariota ha desarrollado un sistema de membranas y de compartimentalización celular que le ha permitido realizar procesos más complejos. Los orgánulos celulares están embebidos en un sistema de membranas en el citoplasma de la célula mientras que el ADN se encuentra en un núcleo separado del citoplasma por la envoltura nuclear. Se considera que esta adaptación evolutiva ha servido para proteger el cada vez más complejo material genético de agentes que lo dañen, como virus o agentes mutágenos. Pero esta adaptación conlleva otra serie de dificultades. Las células eucariotas han separado la transcripción, que ocurre en el núcleo, de la traducción, que ocurre en el citoplasma. Además, la expresión genética está regulada por señales recibidas del exterior de la célula. Para poder transmitir estas señales a su diana, el ADN, la célula provoca una cascada de reacciones que desemboca en la activación de factores de transcripción que promueven la expresión de genes determinados. Estos procesos requieren que el ADN sea accesible en determinadas circunstancias. Por otro lado, los ribosomas, encargados de producir proteínas en el citoplasma, están compuestos ellos mismos de proteínas y de ARN (ARN ribosómico) y su síntesis es un proceso complejo que se inicia en el núcleo y concluye en el citoplasma. Es evidente que, a pesar de aislar su ADN, la célula eucariota necesita mantener un sistema efectivo de comunicación entre el núcleo y el citoplasma. Si la envoltura nuclear supone una barrera que aísla el núcleo del citoplasma, ¿Cómo consigue mantener la célula esta comunicación? El complejo del poro nuclear lo permite reservando a su vez su derecho de admisión, ya que mantiene elementos indeseables excluidos del núcleo.

Los poros nucleares son complejos de múltiples proteínas que atraviesan la envoltura nuclear creando un canal entre el núcleo y el citoplasma (Ver representación en el Chapt. 2, Fig. 2, pág. 17). Los poros nucleares son uno de los complejos proteínicos mas grandes de la célula. Sus componentes se denominan nucleoporinas y en el caso de vertebrados, cada poro nuclear contiene unas 35 nucleoporinas (Ves lista de nucleoporinas en el Chapt. 2, Tabla 1, pág. 18) presente en múltiples copias siguiendo dos simetrías respecto al plano de la envoltura nuclear,

una rotacional de base 8 en el plano perpendicular, y una horizontal en el plano paralelo (Ver imagen de microscopio electrónico en Chapt. 2 Fig. 1, pág. 16).
La envoltura nuclear y los poros nucleares se desmontan y se vuelven a formar cada vez que la célula se divide. Además, células con un alto metabolismo o actividad requieren un alto número de poros nucleares. Por ello y debido a su complejidad, la síntesis y el ensamblaje de esta estructura debe estar regulado de manera precisa. Las nucleoporinas se asocian en subcomplejos que actúan como bloques construyendo el complejo final. Unos de esos subcomplejos es el formado por las nucleoporinas Nup88 y Nup214. El gen NUP214 fue descubierto y caracterizado a partir de investigaciones en Leucemias (una forma de cáncer de glóbulos blancos) y fue denominado inicialmente Caín, debido a su proximidad al gen ABL (Abel). Por otro lado, se cree que la nucleoporina Nup88 también está relacionada con cáncer puesto que ha sido asociada a tumores agresivos.
El paso de una molécula o cargo por el complejo del poro nuclear se denomina transporte núcleo-citoplasmático y se clasifica en importación, cuando es desde el citoplasma al núcleo, y exportación, cuando es desde el núcleo al citoplasma (Ver representación en la Chapt 2, Fig. 3, pág. 21). Además de la estructura del complejo del poro nuclear, el transporte núcleo-citoplasmático requiere elementos adicionales. Entre ellos están los receptores de transporte, que son las únicas moléculas que son capaces de interaccionar con el poro nuclear y por ello mediar el transporte de ellas mismas y de los cargos a los que se asocian. Se dividen en importinas y exportinas según su función y reconocen y se unen a determinadas señales presentes en los cargos o elementos a transportar. Un ejemplo de exportina es CRM1, un receptor que es capaz de unirse a proteínas que contienen unas señales denominadas señales de exportación nuclear (NES), formando un complejo trimérico en presencia de RanGTP. Una vez transportado al citoplasma, este complejo se separa liberando el cargo en su destino (Ver animación junto al número de página). Ciertos virus son capaces de utilizar esta maquinaria para salir y entrar en el núcleo cuando lo requieren.
A pesar de todos los avances conseguidos en los últimos años, todavía quedan muchas preguntas sin responder en el campo de los poros nucleares y el transporte entre el núcleo y el citoplasma. Se desconoce cómo una sola estructura es capaz de transportar cargos de tan diferente naturaleza que van desde pequeños solutos hasta un ARN mensajero o un preribosoma, cuyo tamaño está al límite del tamaño de exclusión del poro nuclear. Además, todavía no se ha determinado cual es la función específica, si la hay, de cada uno de los componentes del complejo en el transporte de los distintos cargos. Aunque se cree que su superficie hidrofóbica permite la interacción con el poro, se desconoce también cómo los receptores de transporte son capaces de atravesar el poro nuclear. Aunque se han propuesto diversos teorías, todavía no se ha definido un modelo de transporte único capaz de abarcar todas las modalidades de transporte encontradas y se cree que distintos mecanismos de transporte co-existen en una misma estructura. Determinar cómo se regula la función del complejo del poro nuclear y cómo se realiza el transporte núcleo-citoplasmático es de gran importancia para conocer funciones básicas de la célula y entender su biología tanto en condiciones de salud como de enfermedad.
El trabajo presentado en este libro es el producto de un proyecto destinado al estudio de los poros nucleares y transporte núcleo-citoplasmático. Hemos estudiado tres nucleoporinas: Nup88, Nup214 y Nup358, y hemos completado el estudio de su localización exacta respecto del resto del poro utilizando técnicas de microscopia electrónica (Chapter 3). Nup358 es el principal componente de los filamentos citoplasmáticos del poro y descansa sobre el complejo Nup88-Nup214 que se encuentra mas próximo al canal central. Para estudiar estas

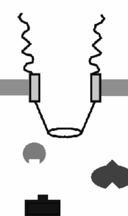
nucleoporinas en detalle, hemos utilizado una técnica denominada interferencia de RNA, que promueve la destrucción de un RNA mensajero específico y con ello reduce el contenido celular de la proteína que codifica. Hemos sido capaces de reducir dramáticamente los niveles endógenos de cada una de estas nucleoporinas o Nups. Hemos estudiado entonces qué sucede a la célula y a las otras Nups con técnicas de microscopía confocal que permiten estudiar estructuras intactas en tres dimensiones en células incluso vivas. Hemos descubierto que Nup358 necesita la presencia del complejo Nup88/Nup214 para incorporarse al poro nuclear y que estas dos Nups, Nup88 y Nup214, presentan co-dependencia, tanto para mantener su estabilidad como para incorporarse al poro nuclear. La localización de estas tres Nups sugiere que desempeñan una función en el mecanismo de importación. Sin embrago, ciertas investigaciones excluyen esta posibilidad. Hemos analizado si Nup88, Nup214 y Nup358 están implicadas en el mecanismo de exportación y hemos encontrado que así es. Nup358 tiene una función de apoyo en el mecanismo de exportación mediado por la exportina CRM1. Gracias a Nup358, el complejo de exportación, formado por CRM1, RanGTP y un cargo, se separa. Este proceso permite la liberación del cargo y ayuda a reciclar CRM1 al núcleo para otra ronda de exportación, consiguiendo un transporte más eficiente.

Hemos estudiado la naturaleza de las señales de exportación (Chapter 4) por técnicas de bioquímica y biología molecular y celular. Hemos creado señales artificiales que presentan alta afinidad por CRM1 (suprafisiológicas) y hemos concluido que la afinidad de las señales de exportación endógenas por CRM1 debe ser baja en condiciones normales de lo contrario el complejo de exportación es desensamblado menos eficientemente. Este estudio ha servido para definir más claramente la función de Nup358 en exportación nuclear mediada por CRM1.

El receptor de exportación CRM1 es capaz de interaccionar con nucleoporinas del poro nuclear. De todas las Nups, Nup214 es con la que interacciona con mayor afinidad. Debido a esto, hemos estudiado en más detalle la función de Nup214 en la exportación mediada por CRM1. Hemos analizado la capacidad de exportación de una serie de cargos en células a las que se ha reducido el contenido de Nup214 por técnicas de RNAi (Chapter 5). Sorprendentemente, no hemos encontrado ningún defecto en exportación de GFP o Rev-GFP, dos cargos de pequeño tamaño. Sin embrago, el defecto en exportación es evidente cuando estudiamos la exportación del preribosoma, un cargo de gran tamaño. Nuestro estudio muestra que los mecanismos de transporte son diferentes dependiendo del cargo a pesar de utilizar el mismo receptor de exportación. También muestra que Nup214 juega un papel importante en la exportación de este cargo específico. Creemos que este tipo de exportación es ejecutado para cargos de gran tamaño y que requiere un proceso que ha sido denominado apertura, que supone un cambio estructural en el poro nuclear para acomodar el paso de grandes cargos (Ver representación en Chapt. 7, Fig.1, pág 116 y en portada).

Muy recientemente, una mutación que implica los genes ABL y Nup214 ha sido definida en un subtipo de Leucemias. Actualmente y en un intento de mejorar las terapias para este tipo de enfermedad, estamos investigando el mecanismo de transformación tumoral provocado por esta mutación (Chapter 6).

En conclusión, el trabajo presentado en esta tesis ha contribuido en un mejor conocimiento del complejo del poro nuclear y, en definitiva, de la biología de la célula y es de potencial interés por su aportación a la sociedad en general y al mundo de la biomedicina en particular.



List of abbreviations

NPC	Nuclear Pore Complex
NE	Nuclear Envelope
NLS	Nuclear Localization Signal
NES	Nuclear Export Signal
GTP	Guanosine TriPhosphate
GDP	Guanosine DiPhosphate
FG	Phenylalanine Glycine
RanBP	Ran Binding Protein
RNAi	Ribonucleic Acid Interference
tRNA	Transfer Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
mRNA	Messenger Ribonucleic Acid
snoRNA	Small Nucleolar Ribonucleic Acid
EM	Electronic Microscopy
TEM	Transmission Electronic Microscopy
FESEM	Field Emission Scanning Electronic Microscopy
ProtA	Protein A
GFP	Green Fluorescent Protein
FRAP	Fluorescence Recovery After Photobleaching
FRET	Fluorescence Resonance Energy Transfer
LMB	Leptomycin B
CML	Chronic Myeloid Leukemia
AML	Acute Myeloid Leukemia
ALL	Acute Lymphoblastic Leukemia

Curriculum Vitae

Rafael Bernad was born in the city of Zaragoza, Spain the 20th October of 1975. He attended successfully primary studies in the public school Gascón y Marín. Noticeable achievements during this period were to interpret the angel Gabriel in a Christmas performance and to lead a rebellion against too many homework exercises at the age of 12. From 1989 to 1994, he completed secondary studies at Miguel Servet High School, where he raised his interest for sciences. After passing University selection exams in June 1994, he moved to Oviedo in Asturias, Spain, where he did university studies in Biology. His passion for molecular biology led him to gain a collaboration grant and join the group of Prof. Francisco Parra in 1997 at the Department of Biochemistry and Molecular Biology, where he was initiated in laboratory investigation for research on the Rabbit Haemorrhagic Disease Virus. An interesting opportunity rose when Dr. J.J. Blankert visited Oviedo University in June 1999 to present an International MSc in Biotechnology, hosted by the Monfort University in UK and Hogeschool Brabant in The Netherlands. Rafael Bernad joined this Masters study after working during the summer as environmental inspector for the construction of France-Valencia highway. As MSc student, he joined Genencor International B.V. in Leiden, The Netherlands, for a three months project in 2000. After that, he stayed in Amsterdam and joined The Netherlands Cancer Institute from November 2000. First, in a six months project based on LPA receptors at the Cellular Biochemistry Department under the supervision of Dr. Ben Giepmans, and then as a PhD student at the Tumor Biology Department and under the supervision of Dr. Maarten Fornerod, where he performed the work presented in this book.

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