Chapter 1

General Introduction
1. Nuclear transport

The word eukaryote derives from the Greek words εὖ (good/true) and κάρυον (nut, nucleus), indicating that a eukaryotic cell contains a “real” nucleus, opposed to prokaryotes that do not have a nucleus. The nucleus separates the genomic DNA from the cytoplasm by a double membrane, termed the nuclear envelope (NE), while prokaryotes have their DNA freely floating in the cytoplasm. One of the functions of the NE may be to form an additional protective layer to shield the precious genomic information from environmental damage. Furthermore, the NE spatially separates DNA transcription in the nucleus from protein translation in the cytoplasm and as a consequence these two processes can be better regulated independently.

To allow macromolecular communication between both compartments, the NE is penetrated by large protein complexes that form aqueous channels, termed nuclear pore complexes (NPCs). These pores allow diffusion of small molecules as well as facilitated transport of proteins, RNAs and ribonucleoproteins (RNPs). This facilitated transport is achieved by soluble transport receptors, termed karyopherins that shuttle between both compartments, picking up cargo at one side of the NE and releasing it at the other side. These transport receptors are divided into importins, importing cargo into the nucleus and exportins that transport cargo out of the nucleus (reviewed in (Weis, 2003)) (Figure 1). Nuclear transport is an extremely quick and efficient process. Each NPC has been calculated to transport up to one thousand molecules per second (Ribbeck and Gorlich, 2001; Smith et al., 2002).

Proteins are translated in the cytoplasm and as a consequence proteins that function in the nucleus need to transverse the NE. In contrast, nuclear export is needed for the regulation of nuclear proteins, like transcription factors, or for recycling of import adaptors (see Figure 1). However, the largest group of molecules that have to be exported from the nucleus are RNA molecules and protein/RNA complexes, termed ribonucleoproteins (RNP). Some karyopherins transport a plethora of cargos, while others are specialized in the transport of one specific molecule or group of molecules (reviewed in Gorlich, 1999 #174)). In brief, the direction of transport is governed by the small Ras-like GTPase Ran, of which the concentration of the GTP-bound form is high in the nucleus and low in the cytoplasm. This will be further discussed in 1.2. Importins bind their cargo only in the presence of RanGTP and therefore only in the nucleus, whereas importins bind their cargo in the absence of RanGTP and release their cargo upon RanGTP binding. After dissociation from the cargo in the opposite compartment, the empty karyopherin recycles back to the original compartment (Figure 1). Both import and export pathways have one very general transport receptor that transports a large number of cargo. The importin responsible for this general import is Importin β (also termed karyopherin β) and the exportin is termed CRM1 or exportin 1 (see 1.3.3). Most of their cargo is recognized by the exposure of short signals, termed nuclear localization signals (NLS) and nuclear export signal (NES), respectively (reviewed in (Weis, 2003)).

1.2 Ran system

Ran is a member of the small Ras-like GTPases. Like all GTPases, Ran cycles between an ‘active’ GTP-bound form and an ‘inactive’ GDP-bound form and undergoes significant conformational changes upon hydrolysis (Scheffzek et al., 1995). Only in its active GTP-bound form, can it bind transport receptors (Figure 2). RCC1 (regulator of chromosome condensation 1) is Ran’s guanine nucleotide exchange factor (GEF), exchanging RanGDP to RanGTP. Ran possesses very weak GTPase activity, but this is stimulated by several orders of magnitude by its specific GTPase activating protein, RanGAP1 (Klebe et al., 1995). Ran binding protein 1 (RanBP1) further boosts the hydrolysis of RanGTP. By binding RanGTP, RanBP1 stimulates a brief dissociation of GTP, facilitating hydrolysis by RanGAP. Perhaps more importantly, RanBP1 is essential for dissociating RanGTP from nuclear transport receptors (Bischoff and Gorlich, 1997)(Figure 2). In interphase cells, RanGTP is present in a steep gradient over the nuclear envelope, being at a high concentration in the nucleus and low in the cytoplasm (Kalab et al., 2002). This gradient is maintained by a separated localization of its cofactors. RCC1 is mainly nuclear, as it is bound to chromatin, whereas the RanGTP hydrolyzing factors RanGAP1 and RanBP1 are restricted to the cytoplasm (Itzaurralde et al., 1997). Moreover, SUMOylated RanGAP is attached to the cytoplasmic side of the NPC, which also has RanBP1-like domains (Wu et al., 1995). This will lead to an immediate GTP hydrolysis upon arrival of export complexes at the cytoplasmic face of the NPC. RanGDP in turn is recycled back into the nucleus by the import receptor NTF2 (nuclear transport factor 2) (Figure 2) (Ribbeck et al., 1998).

But how does the RanGTP gradient regulate the direction of transport? This is achieved by the Ran-binding properties of karyopherins. Importins bind their cargo in the cytoplasm and are translocated to the nucleus where RanGTP binding to the Ran binding domain of importins will lead to dissociation of cargo (Figure 1). The opposite holds true for exportins, as the exportin-cargo interaction is rather weak and needs to be stimulated by RanGTP.
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binding. Upon arrival in the cytoplasm, Ran will either be dissociated at the NPC or, as a backup mechanism, by soluble RanBP1 and RanGAP in the cytoplasm. Because RanGTP is high in the nucleus and low in the cytoplasm, import complexes can only form in the cytoplasm and export complexes only in the nucleus (reviewed in (Fornerod and Ohno, 2002)).

Besides Ran’s regulatory function in nuclear transport, it has also been implicated at several stages of mitosis. Ran is important for entry into mitosis (reviewed by (Sazer and Dasso, 2000)). It regulates the spindle formation and it is required for proper functioning of the spindle checkpoint (Arnaoutov et al., 2005). This checkpoint ascertains whether chromosomes in metaphase are properly aligned before onset of anaphase. Finally, RanGTP regulates NPC assembly by recruiting nucleoporins to chromatin, inserting them into membranes and by stimulating the assembly of NPC subcomplexes (Walther et al., 2003).

1.3 Transport receptors

Most nuclear transport receptors fall into the class of karyopherin β proteins, also termed importin β-like proteins as importin β was the first identified nuclear transport factor. Fourteen different karyopherins have been identified in yeast and at least twenty in humans (Mosammaparast et al., 2001). Some karyopherins transport a whole range of proteins, whereas others are specialized in the transport of only one molecule, or a specific subset of molecules. For example Importin α/β imports a wide range of proteins, importin 4, 5 and 9 import ribosomal proteins and histones, transportin 1 imports RNA binding protein and histones, transportin 2 imports HuR. CRM1 is a very generic receptor, exporting a wide range of proteins, while exportin-t exports only t-RNA, and exportin 6 is the receptor for profilin and actin. Two karyopherins function in two directions: vertebrate importin 13 functions as an importin for Ubc9, RBM8 and Pax6, but also as an exportin for elf-1A (Mosammaparast et al., 2001). Yeast Msn5 also mediates bidirectional transport (Yoshida and Blobel, 2001).

Karyopherins range between 95 and 145 kDa and possess a rather weak sequence homology (15-20% identical), being most identical in the N-terminal Ran binding or “CRIME” domain (for CRM1, Importin β, etc) (Fornerod et al., 1997a; Gorlich et al., 1997). Structurally, karyopherins are almost entirely composed of 19-20 HEAT (named after the proteins where they were first identified in, Huntington, Elongation Factor A and TOR) repeats, presumably rendering the protein the plasticity to bind different cargos (Cook et al., 2007). Most kary-
Karyopherins can bind cargo directly, although in some cases an adaptor is used (Fig. 1) providing an additional level of specificity and regulation. Another common feature of karyopherins is their ability to interact with the NPC, in particular through hydrophobic interactions with FG-repeats. Because of these abundant but rather weak interactions karyopherins can translocate rapidly through the NPC, both in the absence and presence of cargo, exceeding the size limit of diffusion (Bayliss et al., 2000; Cook et al., 2007).

1.3.1 Importin α/β

Importin α (karyopherin α)-mediated import is defined as the “classical” import pathway that transports proteins containing nuclear localization signals (NLS) to the nucleus. The NLS is a short monotripe or bipartite sequence of basic residues, mainly consisting of lysines and arginines. The first NLS identified was a monotripe sequence (PKKKRKV) in the simian virus 40 (SV0) large T antigen and is still the most widely used signal for heterologous nuclear targeting (Kalderon/Roberts 1984). NLS-containing cargo does not bind directly to importin β, but is recognized by an adapter protein, importin α (also termed karyopherin α) (see Fig. 1). Importin α interacts with importin β via the amino-terminal importin β binding domain (IBB) and this interaction stabilizes the importin α/cargo interaction (Efthymiadis et al., 1997; Rexach and Blobel, 1995). Importin α does not contain a HEAT-repeat domain, but is composed of ten armadillo (ARM) repeats that are related to HEAT repeats. Importin β can also bind its cargo directly via a nonclassical NLS (Moore et al., 1999; Palmeri and Malim, 1999; Truant et al., 1999). This could be explained by the similarity between the structure of importin α IBB domain in complex with importin β and the IBB domain in complex with an NLS (Conti et al., 1998). However, two different cargoes that bind importin β directly have been characterized, PTHrP and SREBP-2 (Chubb et al., 2002; Lee et al., 2003) and although SREBP-2 binds at the same site as IBB, PTHrP binds importin β at a different site. These different interactions underscore the flexibility of transport receptors. The release of cargo occurs by binding RanGTP to the Ran binding domain of importin β, followed by a conformational change and subsequent destabilization of the import complex. Empty importin β recycles back to the cytoplasm for another round of import. Importin α in turn, requires the binding of a specialized exportin, termed CAS (cellular apoptosis susceptibility) that is solely responsible for the export of importin α (see Fig. 1) (Kutay et al., 1997). Importin α/β are also responsible for the import of integral membrane proteins into the inner nuclear membrane via a signal resembling the classical import signal (Kiseleva et al., 1998). Another important function of importin α/β is the regulation of many processes during mitosis and NE assembly (Weis, 2003).

1.3.2 Transportin

Transportin 1, also termed karyopherin β2, shares ~24 % sequence homology with Importin β and was first identified as the import receptor for hnRNP A1 by recognizing a 39 amino acid motif termed the M9 signal (Pollard et al., 1996; Siomi and Dreyfuss, 1995). Subsequently, transportin 1 has been shown to import several mRNA binding proteins, e.g. hnRNP D, TAP, JKBTP, HIV Rev, and c-FOS (Imasaki et al., 2007). Unlike classical NLS sequences, the import signals recognized by transportin 1 are rather long and have only low sequence similarity. Recent mutational analysis has pinned down the importance of the PY motif, present in many of the transportin 1 cargoes (Iijima et al., 2006; Suzuki et al., 2005).

1.3.3 CRM1

CRM1 (chromosome region maintenance-1) was originally identified in Schizosaccharomyces pombe, where cold-sensitive mutants led to deformed chromosome
domains (Adachi and Yanagida, 1989). Later, simultaneously with CAS, it was the first identified export receptor that required RanGTP binding to stimulate binding of cargo (Fornerod et al., 1997a; Kutay et al., 1997). Hence it was renamed exportin 1 (Xpo1 in yeast (Stade et al., 1997)). Whereas CAS is a highly specific exportin for importin α (Figure 1), CRM1 appeared to be a general exportin that recognizes proteins bearing a short hydrophobic sequence of ~10-12 amino acids, initially termed leucine-rich nuclear export signals (NES). The number of CRM1-mediated cargoes is still growing, including both shuttling transcription factors, small diffusible proteins and translation factors that have to be excluded from the nucleus, import adaptors, and also several RNA molecules. Besides an important role in interphase cells, CRM1 also has an important function in mitosis (see 1.3.3.3)(Arnaoutov et al., 2005).

1.3.3.1 CRM1 structure

CRM1 is a 115 kDa HEAT-repeat protein. Only the carboxy-terminal third (HEAT repeat 14-19) has been crystallized and together with electron microscopy analysis and homology modeling, we now have a better understanding of CRM1 at the structural level (Petosa et al., 2004). CRM1 is a superhelical structure composed of 19 HEAT repeat motifs of which the first three comprise the CRIME domain. This domain, together with an acidic loop in HEAT repeat 8-11, is responsible for RanGTP binding (Fornerod et al., 1997a). A central conserved region encompassing HEAT repeat 8-11 is responsible for NES binding. Interestingly, residues involved in NES binding (Leu525, Lys568 and Phe572) are located at the outer surface, which are also thought to be required for interactions with the NPC. The large acidic loop in HEAT repeat 8-11 has strong homology with transportin and has been proposed to alter its conformation upon RanGTP hydrolysis, resulting in displacement of cargo (Petosa et al., 2004).

1.3.3.2 RanBP3

Without RanGTP the affinity of the CRM1-cargo interaction is very weak, in the mM range. RanGTP greatly stimulates this interaction, though the Kd is often not lower than 0.5 μM (Askjaer et al., 1999). Because of this weak interaction, complex formation is thought to be the rate-limiting step in CRM1-mediated export. To aid CRM1 NES formation another Ran binding protein, termed RanBP3 stimulates these interactions in two ways. First, RanBP3 tethers CRM1 to RCC1 to ensure that RanGTP loading occurs in proximity of CRM1 molecules. Simultaneously, RanBP3 also stimulates RCC1’s exchange activity. Second, RanBP3 enhances the affinity of CRM1 for cargo by binding directly to CRM1 (Engelmeier et al., 2001; Lindsay et al., 2001). Evidence from the modeled structure of CRM1 suggest that RanBP3 interacts with the acidic loop and it has been suggested that RanBP3 stabilizes the loop in an "open" conformation to allow both cargo and RanGTP to bind simultaneously (Petosa et al., 2004).

1.3.3.3 CRM1 in mitosis

Besides its role in transport, CRM1 may also be used as a platform to attach NES-containing proteins to certain subcellular structures. For example, CRM1 attaches nucleo phosphosmin to centrosomes and this interaction is important for proper centrosome duplication (Wang et al., 2005). In certain cell types, CRM1 regulates proper chromosome segregation by being localized to kinetochores from the end of prophase till anaphase or telophase. The localization of CRM1 to kinetochores is required for attachment of Nup358 and RanGAP to these structures. This interaction can be abolished by leptomycin B (LMB), a specific CRM1 inhibitor (see below), suggesting that CRM1 binds in a RanGTP- and cargo-dependent manner (Arnaoutov et al., 2005; Joseph et al., 2004).

1.3.4 Transport inhibition

So far, CRM1 is the only karyopherin for which a highly specific inhibitor has been identified. This inhibitor, termed leptomycin B (LMB) is a small molecule, which was identified in Streptomyces and forms a covalent bond with cysteine 528 in human CRM1 or cysteine 529 in S. pombe. This cysteine is lacking in S. cerevisiae and is therefore completely resistant to LMB, emphasizing the specificity for CRM1. LMB irreversibly and immediately inhibits CRM1 and is active in a low nM range (Fornerod et al., 1997a; Wolff et al., 1997).

In addition to LMB other CRM1 inhibitors have been identified. Ratjadone A resembles LMB, binding CRM1 at cysteine 528 and is equally potent and specific (Koster et al., 2003). More interesting is the small compound PKF050-638, which is thought to interact with the same cysteine residue. However, unlike LMB and Ratjadone A, the inhibition by PKF050-638 is reversible after removal of the drug (Daelemans et al., 2002).

1.4 Nuclear export signals

In 1995 two studies simultaneously reported the existence of a leucine-rich nuclear export signal. One was identified in Human Immunodeficiency Virus type 1 (HIV-1) Rev, the export adaptor for unspliced viral mRNA and the other in the protein kinase A inhibitor (PKI) (Fischer et al., 1995; Wen et al., 1995). The export signals were termed leucine-rich nuclear export signals, because of the presence of four leucines that were regularly spaced by other residues, following the consensus sequence L-X$_1$-$X_2$-L-X$_3$-$X_4$-L-X-L. Subsequent descriptions of NESs revealed that leucines could be replaced by any other hydrophobic residue, although the two carboxy-terminal hydrophobic residues, which form the most important part of the NES, are often leucines or isoleucines (la Cour et al., 2003). Mutation of one of these core residues abolishes export activity completely (Wen et al., 1995). The residues in between the hydrophobic residues are frequently either
charged or small. Even though more than one hundred NESs have been identified, a more defined consensus sequence has not yet been described. In addition, several NESs have been described that do not follow the general consensus sequence, though they all expose several hydrophobic residues. Based on this loose consensus, most proteins are predicted to contain one or more NESs. However, the majority will not be functional, either because they are not exposed or simply because they do not bind CRM1. Ideally, to prove the export function of any NES the following experiments should be performed. First, the localization of the protein should be studied after LMB treatment or after mutagenesis of the NES. This however, does not prove a direct interaction with CRM1. One should be extra cautious if the structure of the protein is unknown, because the predicted NES could be part of an internal hydrophobic interaction that may be disrupted upon mutation. This could lead to protein misfolding and a subsequent altered localization. Second, to demonstrate a direct interaction, in vitro binding assays should be performed. The interaction should be RanGTP dependent and should be abolished upon LMB addition or by mutation of one or more hydrophobic residues. Third, the in vitro and in vivo experiments should also be carried out with the isolated NES.

1.4.1 Assays to study NESs

NESs have different affinities for CRM1, which results in different export capacities. The affinity of NESs can be measured in vivo with an export assay based on the shuttling HIV-1 Rev protein, which is fused to a fluorescent reporter protein, like GFP. The Rev NES is mutated, but its NLS is intact, resulting in nuclear localization. Insertion of an active NES, strong enough to overrule the NLS, will shift the reporter protein to the cytoplasm (Hendersom and Eleftheriou, 2000). A widely used quantitative in vitro assay is the CRM1 RanGAP assay. Strong NESs require lower CRM1 and RanGTP concentrations to form stable complexes. This assay measures the hydrolysis of RanGTP when it is not incorporated into export complexes. (Askjaer et al., 1999; Bischoff and Gorlich, 1997). These assays have proven very useful for the identification of true NESs and are frequently used for the studies described in this thesis (see Chapter 2, 5 and 6).

1.4.2 Structure

Mutational analyses combined with the putative CRM1 structure have identified three important residues for NES interaction; two hydrophobic residues, LeuS25 and PheS72 and one positively charged residue, LysS68. Besides a charged moiety, lysines also have a long flexible aliphatic chain that may be used to interact with hydrophobic residues of NESs. Crystal structures of five known NES-containing proteins computationally fitted onto CRM1 revealed that the NESs lie (partly) within an α helix and have a strong structural similarity (Rittinger et al., 1999). However, some doubt has been cast on at least two of these NESs (found in β-actin and IκBα). At least three out of four hydrophobic residues in the NES were shown to contact the proposed involved residues in CRM1 (Petosa et al., 2004). However the structure of an NES complexed with CRM1 awaits crystallization.

1.4.3 NES regulation

NESs can be regulated by several mechanisms. A frequently adopted mechanism is NES inactivation by phosphorylation in or near the NES sequence, directly lowering the affinity of the NES (Ikuta et al., 2004; Zhang and Xiong, 2001). Phosphorylation can also lead to activation of the NES as seen for the transcription factor Nuclear Factor of Activated T-cells (NFAT). Ca2+ influx leads to activation of the phosphatase calcineurin, which in turn dephosphorylates thirteen phosphoserine residues that are required to mask the NES. As a consequence the NLS is exposed and NFAT will be imported into the nucleus where it can perform its function as a transcription factor (Okamura et al., 2000). Third, inter- or intramolecular interactions can result in NES masking upon phosphorylation or other post-translational modifications of the protein. An example of intramolecular masking is shown for INI1 (Integrase interactor 1), a member of SWI/SNF chromatin remodeling complex, where the C-terminal 66 amino acids prevent CRM1 from binding the NES. This masking can be released upon a signal-induced conformational change or degradation of the C-terminus (Craig et al., 2002). An example of intermolecular masking will be given in Chapter 5. Finally, NES activity can also be influenced by retention either in the nucleus, compromising the export capacity of NESs, or in the cytoplasm, driving the direction of transport towards the cytoplasmic compartment. An example of nuclear retention is shown for the glucocorticoid receptor (GR), which contains a nuclear retention signal in the hinge region that actively impedes its export (Carvalho et al., 2001). Finally, an interesting example of NES regulation is seen in yeast. The yeast AP-1-like transcription factor ( Yap1p) is responsible for the upregulation of genes in response to oxidative stress. In an oxidative environment, the NES is no longer recognized by CRM1, because of intramolecular disulfide bond formation, resulting in nuclear Yap1p (Kuge et al., 2001; Yan et al., 1998).

1.5 NPC

1.5.1 Composition

The NE of a human cell contains between 500-5000 NPCs, depending on the metabolic state of the cell. The yeast NPC is ~50 MDa and the human NPC is ~120 MDa (Reichelt et al., 1990). Strikingly, both NPCs are composed of a similar set of only 30 different proteins, termed nucleoporins (or Nups). Despite its bulky mass, the composition of the NPC is simplified by an eight-fold rotational symmetry. Recent modelling suggests that the core-NPC consists of four spokes, which in turn consist of two highly
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homologous columns (Alber et al., 2007). The NPC has both vertical and horizontal symmetry, being only asymmetrical in its nuclear and cytoplasmic extremities (Figure 3). The metazoan NPC has a diameter of ~ 145 nm and is ~ 80 nm in length. The inner diameter has been estimated to be ~ 44 nm (Akey and Radermacher, 1993), which is in accordance with the maximum cargo size of 25-39 nm, based on transport studies with coated gold particles (Dworetzky and Feldherr, 1988; Pante and Kann, 2002).

As seen with scanning electron microscopy, the NPC has a basket-like structure on the nuclear side and eight protruding filaments on the cytoplasmic side (Goldberg and Allen, 1992). Some nucleoporins are highly dynamic and have short residency time at the NPC (minutes), whereas membrane-bound and more internally located nucleoporins are very stably localized (reviewed in (Rabut et al., 2004; Tran and Wente, 2006). Recently, yeast nucleoporins have been classified into three groups, based on their structural domains. The first group consists of membrane-bound nucleoporins, containing transmembrane α-helices. This group is thought to form the outermost layer of the NPC, responsible for anchoring the NPC into the NE. The innermost layer consists of nucleoporins containing phenylalanine-glycine (FG) repeat domains (Figure 3) (Devos et al., 2006). These domains are very abundant, highly unstructured and unfolded and fill most of the NPC channel (Ribbeck and Gorlich, 2001; Rout et al., 2000) and references therein). Most of these nucleoporins contain coiled coiled with which they are thought to interact with other NPC components. The last group, consisting of approximately half of the nucleoporins has an α-solenoid, a β-propeller or a combination of both domains. These nucleoporins form a structural scaffold between the membrane-bound nucleoporins and the FG-repeat-containing nucleoporins (Figure 3) (Devos et al., 2006).

1.5.2 Models of translocation through the NPC

How the FG-repeats selectively allow transport of substrates is still a matter of debate. One model, termed “virtual gating” predicts that the unstructured filamentous FG-repeats fill the central channel, restricting molecules larger than ~ 30-40 kDa from passing through. Receptor/cargo complexes however, by virtue of interactions with the FG-repeats can translocate through the NPC channel (Rout et al., 2000). This hypothesis is supported by a recent study where the composition of the NPC was constructed based on computational modeling of biochemical interaction data. The inner layer of the NPC reveals an FG-repeat-containing cloud that thins towards the interior of the central channel, leaving an ~ 10 nm channel in the middle, which is consistent with the maximal diffusible size (Alber et al., 2007). The second model, termed “selective phase” predicts that FG-repeats do interact with each other (Ribbeck and Gorlich, 2002). Evidence for this model comes from a study in yeast where it was shown that most FG-repeat containing nucleoporins present at the core of the NPC possess a weak affinity for each other. The FG-repeats of the asymmetrically located nucleoporins do not interact with other FG-repeats, suggesting that these nucleoporins either form repulsive bristles or stimulate more specific nuclear transport receptor interactions (Patel et al., 2007). In other studies it has been shown that certain FG nucleoporins form a saturated hydrogel in vitro (Frey and Gorlich, 2007; Frey et al., 2006). The interacting FG-filaments form a meshwork that functions as a sieve, permitting only small molecules to pass through. In this model receptor/cargo complexes will dissolve the meshwork locally, allowing the complex to slide through (Frey and Gorlich, 2007; Ribbeck and Gorlich, 2001; Weis, 2007). A third model predicts that affinities of karyopherins for FG nucleoporins increase towards the final compartment. This implies that direction of nuclear transport is not solely governed by the Ran system, but is aided by interactions at the NPC (Ben-Efraim and Gerace, 2001). However, experiments in yeast, where asymmetrically located nucleoporins were either deleted or swapped, showed no defects in nuclear transport (Strawn et al., 2004; Zeitler and Weis, 2004). Moreover, permeabilized cell assays revealed that the transport direction of receptor/cargo complexes can be reversed by switching the RanGTP gradient, which also strongly argues against the affinity gradient model (Na-chury and Weis, 1999).

1.5.3 Flexibility of the NPC

The centre of the NPC is filled with an electron-dense structure as seen with electron microscopy, which is thought to be at least partly composed of transiting cargo. Cryoelectron tomography in the slime mould Dictostelium analyzed this central plug in NPCCs incubated with gold-labeled import complexes and in principle two different states could be identified, which are thought to represent different steps in transport. One state shows an NPC with a more internally located plug and cytoplasmic filaments that are unordered and variably protrude from the cytoplasmic side of the NPC core. In the second state, the central plug is located in the plane of the cytoplasmic filaments. The filaments are bent towards the central plug and are thought to be interacting with cargo at this state. These results also demonstrate a more general conformational change in the two states (Beck et al., 2004; Beck et al., 2007). This flexibility of the NPC has also been shown for the diameter of the central channel (Akey, 1989; Beck et al., 2004; Kiseleva et al., 1998). Recently, structural evidences for this flexibility has been obtained by analyzing two internally located nucleoporins, Nup58 and Nup45, whose α-helices interact with each other. These dimers form tetramers through hydrophilic interactions. Interestingly, these latter interactions are variable, leading to sliding along the dimer surface, resulting in a shift of ~ 11 Å and an increase of the entire NPC channel of ~ 30 Å. This sliding of nucleoporin may facilitate the passage the large cargo (Melcak et al., 2007).
The diameter of the pore is also thought to be influenced by changes in Ca²⁺ influx. Several studies have shown either a structural change of the NPC, in particular of the nuclear basket, or a functional change, leading to changes in permeability and nuclear transport (Erickson et al., 2006; Thorogate and Torok, 2007) and references therein.

The dynamics of the NPC can also take place at the level of NPC density in the NE. For example, quickly dividing cells require a fast incorporation of NPCs in the NE. Alternatively, incorporation of specific nucleoporins may alter the function of the NPC. A nucleoporin storage site could be useful to accomplish this rapid increase in NPC density. In the cytoplasm, stacks composed of double membranes are present, which are perforated by NPCs in both orientations. These organelles are termed annulate lamellae (AL) and have been proposed to serve as a storage site for nucleoporins. Interestingly, besides the standard subset of nucleoporins to build up an NPC, transport factors associated with the NPC are also present at AL (Kessel, 1992).

1.6 Nucleoporins

The structural components that form the NPC are termed nucleoporins or nups and they are often named after their molecular weights. Even though the protein sequences of yeast and vertebrate nucleoporins are poorly conserved, more than eighty percent of the vertebrate nucleoporins have a structural counterpart in yeast. The vertebrate NPC differs however by having a subset of nucleoporins with more complex domains or modifications that are not present in yeast. For example, general structures present in multiple vertebrate nucleoporins are the zinc-fingers, implicated in Ran binding (Nakielny et al., 1999; Wang et al., 2003) and in coordinating NE breakdown (Higa et al., 2007; Nakielny and Dreyfuss, 1999; Prunuske et al., 2006). Tryptophan-alanine (WD) repeat-containing nucleoporins are more abundant in vertebrates and are thought to mediate the assembly of protein complexes (Cronshaw et al., 2002). Moreover, serines or threonines in vertebrate nucleoporins can be phosphorylated in a cell-cycle dependent manner or O-linked glycosylated, though little is understood about the precise role of these modifications (Miller et al., 1999).

The eminent nuclear basket structure at the nuclear side of the NPC is mainly composed of Nup153 and TPR (translocated promoter region), of which the latter has been mapped more exteriorly and is attached to the pore via Nup153 (Figure 3) (Hase and Cordes, 2003; Krull et al., 2004). At the other side of the vertebrate NPC, three exclusively cytoplasmic nucleoporins are located: Nup358, Nup214 and Nup88. These nucleoporin will be discussed independently in the next section. Nup214 forms a dynamic subcomplex with Nup88 and their stability is directly dependent on their interaction (Bastos et al., 1997; Bernad et al., 2004; Fornerod et al., 1997b; Xy-lourigidis et al., 2006). Nup88, but not Nup214, interacts with Nup358 as shown by immunoprecipitation studies. Depletion of either Nup88 or Nup214, results in a strong reduction of Nup358 from the NE. These results suggest that Nup88 forms a platform for attaching Nup358 to the NPC (Bernad et al., 2004).

1.6.1 Nup358

Nup358 is one of the few nucleoporins that is specific for metazoans. It was initially termed Ran binding protein 2 (RanBP2) when identified in a yeast two-hybrid screen for Ran binding proteins (Yokoyama et al., 1995). Nup358 is the largest nucleoporin, ~35 nm in length, and is present at the cytoplasmic side of the NPC, where it is the main constituent of the cytoplasmic fibrils (Delphin et al., 1997; Walther et al., 2002; Wilken et al., 1995; Wu et al., 1995; Yokoyama et al., 1995). It has multiple domains for which increasing number of interacting proteins and functions have been identified. These domains are illustrated in Figure 7 of Chapter 2. The amino-terminus has been proposed to be the site of attachment to the NPC core, possibly via the predicted leucine zipper and a coiled-coil. Nup358 has four Ran binding domains (Wu et al., 1995) and a zinc-finger domain, which has been shown to coordinate NE breakdown (Prunuske et al., 2006). The internal repeat domain (IR1) attaches RanGAP (see 1.4) to the NPC via a SUMO moiety and also interacts with the SUMO E2 conjugase, Ubc9. Interestingly, Nup358 itself has SUMO E3 ligase activity and together with Ubc9 it can SUMOylate proteins like p53 and IkBα. RanGAP however is not SUMOylated by Nup358 (Reverter and Lima, 2005). At the carboxy-terminus of Nup358 is a domain with homology to cyclophilin A, which has been shown to play a role in the ubiquitin-proteasome system (Yi et al., 2007). Nup358, together with other NPC components and transport factors (see 1.5.3.3) also plays a role during mitosis as the Nup358-RanGAP complex, when SUMOylated, is directly required for the attachment of microtubules to kinetochores (Joseph et al., 2004). Nup358 does not seem to be required for general import or export via the importin α/β or CRM1 pathway, as depletion of Nup358 has no or only little effect on these pathways (Bernad et al., 2004). Finally, Drosophila Nup358 has been shown to play a role in mRNA export. Depletion of Nup358 results in an mRNA export defect and in the cytoplasmic accumulation of the mRNA export factor NXF1/p15. Binding of the mRNA export factor NXF1/p15 to Nup358 inhibits leakage of the export factor to the cytoplasm, with the resultant increased concentration of nuclear NXF1/p15 making mRNA export more efficient. However, Drosophila lacks RanBP1, so depletion of Nup358 here depletes the cell of all RanBP1-like activity, likely having general effects on many transport processes.

As mentioned before, Nup358 does not have a counterpart in yeast. However, a recent study by Stelter et al. described the presence of dynein light chain (Dyn2) at
the yeast NPC. Five copies of Dyn2 have been shown to bind the dynein light chain interacting domain (DID) of Nup159, the yeast counterpart of Nup214. This array of Dyn2 interacting with Nup159 forms a rod-like structure of ~ 20 nm. Because the yeast NPC lacks Nup358, Dyn2 has been hypothesized to be the vertebrate counterpart of the cytoplasmic filaments and may play a role in organizing the FG-repeats of Nup159 (Stelter et al., 2007).

1.6.2 Nup214

Nup214/CAN was first identified as an oncogenic fusion product causing acute myeloid leukemia (AML) when fused to the protein tyrosine kinase c-Abl. Because of this fusion with Abl, the protein was termed Cain (Can) (von Lindern et al., 1990). Shortly after, Nup214/Can was also found in translocations with the chromatin protein DEK and with the PP2A inhibitor SET, causing AML and acute undifferentiated leukemia (AUL), respectively (von Lindern et al., 1992a; von Lindern et al., 1992b). Nup98, a nucleoporin located at the nuclear side of the NPC, has also been implicated in multiple forms of leukemia, by being fused to homeodomain transcription factors or histone methyl transferases. Interestingly, all translocations retain the FG repeats of Nup214 or Nup98, suggesting this domain is important for the oncogenic properties of the fusion proteins (reviewed by Kalverda, 2007 #1000) (Kasper et al., 1999). Depletion of Nup214 also results in cell cycle arrest, reduction in protein import and mRNA export defects (Boer et al., 1998). However, it may be hard to distinguish the functions of Nup88 and Nup214 as the stability of both proteins depend on each other (Bernad et al., 2004). Nup214/CAN also has an amino-terminal β-propeller structure and two central coiled-coils through which it interacts with Nup88 (Nehrbass and Blobel, 1996). Nup214 plays a role in mRNA export by attaching the RNA helicase Dpb5 (DEAD box protein 5) and the mRNA export factor GLE1 (Kohler and Hurt, 2007).

The high-affinity interaction of Nup214’s FG-repeat region with the export receptor CRM1 has been studied extensively, as demonstrated both in vitro and in vivo (Fornerod et al., 1996; Fornerod et al., 1997b; Xylourgidis et al., 2006). Because of this strong interaction, Nup214 has been speculated to play a role in preventing the reimport of export complexes (Askjaer et al., 1999), or to be the site of complex dissociation (Askjaer et al., 1999; Hutten and Kehlenbach, 2006; Kehlenbach et al., 1999). The role of Nup214 in export however, remains controversial. Hutten and Kehlenbach demonstrated a strong CRM1 export defect upon knockdown of Nup214 by RNAi (Hutten and Kehlenbach, 2006), whereas a study by Bernad et al. showed only a minor export defect (Bernad et al., 2004), similar to the defect observed in Nup358 knockdown. In Drosophila S2 cells, CRM1-mediated export is not compromised in Nup214 mutants that are unable
to bind CRM1. Instead Nup214 mutant larvae show an increase in CRM1-mediated export, suggesting that the CRM1-Nup214 interaction is actually inhibiting the CRM export pathway (Sabri et al., 2007). These discrepancies will be discussed in Chapter 7.

1.6.3 Nup88

Nup88 does not have an FG-rich repeat, but its yeast counterpart is predicted to have an amino-terminal β-propeller and a carboxy-terminally placed coiled coil (Alber et al., 2007). Whereas Nup214 and Nup358 have only eight copies at a each NPC, Nup88 is predicted to have 32 copies (Cronshaw and Matunis, 2004). Nup88 is also abundant in the cytoplasm, although the purpose of this excess has not yet been determined. Nup88 has been shown to be upregulated in many cancers and pre-malignant lesions, however in these studies Nup88 was predominantly detected in the nuclei of cells (Martinez et al., 1999), which contrasts with its proposed regular cytoplasmic localization. This overexpression is correlated with tumorigenesis and aggressiveness of colorectal cancers (Enterling et al., 2003) and also with the metastatic potential of melanomas (Zhang et al., 2002). Whether Nup88 functions as an oncogenic protein or only follows an increased demand for nuclear transport is not understood at present.

1.7 RNA export

Export of RNA molecules is required for two reasons. First, RNA molecules are transcribed in the nucleus, but most of them exert their function, or need to be translated, in the cytoplasm. Second, for nuclear RNAs like small nucleolar RNAs, certain maturation steps take place in the cytoplasm. The karyopherins responsible for this transport are sometimes very specialized, e.g. exportin-t only exports tRNAs, whereas other RNAs, like rRNAs, snRNAs and some mRNAs, are exported by the generic export receptor CRM1. The export of the majority of messenger RNAs (mRNAs) is mechanistically different, as the export factors involved do not resemble karyopherins (Kohler and Hurt, 2007).

1.7.1 tRNA and miRNA

tRNAs are exported by exportin-t that, instead of recognizing linear sequences like classical transport signals, interacts with secondary and tertiary structural elements in the tRNA (Arts et al., 1998; Lipowsky et al., 1999). Exportin-5 is also able to export tRNA, and this receptor is also responsible for miRNA export (Lund et al., 2004). miRNAs are transcribed either from the intron of a coding gene or from a separate miRNA coding gene. The miRNA precursors form hairpin structures that are exported in a RanGTP-dependent manner and are further processed in the cytoplasm to single stranded RNA molecules. Subsequently, these RNAs are incorporated into the RISC complex and basepair with their target mRNAs, leading to translational inhibition (reviewed in (Kohler and Hurt, 2007)).

1.7.2 rRNA

rRNAs are ribosomal RNA molecules that are incorporated into large protein complexes together constructing the ribosomal subunits. Each ribosome is composed of a 40S and 60S subunit that together contain four rRNA molecules (28S/5S rRNA, 5.8S rRNA, 5S rRNA and 18S rRNA). Ribosomal biogenesis is rather complex and involves processing of rRNAs and association with ribosomal proteins to form ribonucleoproteins (RNP). These processes take place in the nucleus as well as in the cytoplasm. The final maturation of the 40S and 60S subunits takes place in the nucleus and their subsequent export into the cytoplasm depends on CRM1. For both subunits the CRM1 interaction is thought to be bridged by export adaptors (Zemp and Kutay, 2007). The export of the 40S subunit is not completely understood, although the involvement of CRM1 is evident (Gleizes et al., 2001; Moy and Silver, 2002). Several other factors have been implicated although it is unclear at present whether these are true ribosomal export adaptors (Oeffinger et al., 2004). CRM1 mediates the export of the 60S ribosomal subunit and this interaction is bridged by the export adaptor NMD3 (non-sense mediated mRNA decay protein 3) that bears a classical NES required for the CRM1 interaction (Thomas and Kutay, 2003). In the cytoplasm, CRM1 is released from NMD3 by RanGTP hydrolysis. Subsequently, the GTPase Lsg1 dissociates NMD3 from the 60S subunit (Hedges et al., 2005; West et al., 2005). In Chapter 6 a specific role for Nup214 in 60S ribosomal export will be described.

1.7.3 snRNA

Small nuclear RNAs (snRNAs) are spliceosomal RNA that are incorporated in the spliceosome and are required for proper splicing of RNAs. This splicing occurs in the nucleus, but the assembly of the snRNAs U1, U2, U4, U5 and U6 with a group of seven proteins known as Sm ribonucleoproteins, occurs in the cytoplasm, at least in higher eukaryotes. snRNAs are exported by CRM1, but similar to rRNA, adaptor proteins are required to bridge this interaction. Before being exported, snRNAs acquire a 5' cap structure (Hamam et al., 1990), which is recognized by a protein termed CBC (cap binding complex) (Izaurralde et al., 1995). Subsequently phosphorylated PHAX (phosphorylated adaptor for RNA export) will bind the complex and recruit CRM1 via its NES. The PHAX/CRM1/RanGTP complex by itself is rather weak, but upon CBC binding this interaction is greatly enhanced. In the cytoplasm,
dephosphorylation of PHAX, together with RanGTP hydrolysis, results in dissociation of the complex (Ohno et al., 2000). In the cytoplasm snRNAs are assembled into mature snRNP and imported back into the nucleus by importin β. In this case, instead of importin α, a highly specialized adaptor, termed snurportin 1, is used (Huber et al., 1998; Palacios et al., 1997). Interestingly, CRM1 has a dual function in snRNP maturation. Besides being directly involved in the export of snRNAs, CRM1 also recycles snurportin 1 back to the cytoplasm. Snurportin 1 however, lacks a classical NES, but binds CRM1 through large domains, involving residues located throughout the protein. This finding is supported by mutation analysis of CRM1, in which none of the single, double or triple mutations abolished Snurportin binding completely. This result showed that unlike the classical NES binding site, snurportin 1 binding requires multiple residues spread out over CRM1 (Petosa et al., 2004). Another characteristic is the unusually high affinity for CRM1 (KD ≈ 10 nM), which may be explained by the increase in avidity due to the multiple interactions. The affinity of small classical NESs is approximately 100-fold lower (Paraskeva et al., 1999).

1.7.4 mRNA export

Export of general mRNA molecules is perhaps the most complex export pathway and involves a wide range of proteins and processes before an export-competent mRNP is formed. The major processes before mRNA export are 5' capping, splicing of the introns, cleavage and polyadenylation of the 3' end. During these processes, mRNA is assembled into an mRNP particle, which will be bound by proteins that serve as export adaptors, like YRA1 or ALY/REF (Lei et al., 2001; Zenklusen and Stutz, 2001; Zhou et al., 2000). The generic export receptor TAP-p15 (also termed NXF1-p15 and Mex67/Mtr2 in yeast) is recruited to the mRNP via these adaptors (Kohler and Hurt, 2007). TAP-p15 does not belong to the karyopherin family and has no RanGTP binding domain, although it does interact with FG-rich nucleoporins. The lack of RanGTP binding predicts the existence of a different dissociation mechanism at the cytoplasmic site of the NPC (Kohler and Hurt, 2007). Remodelling of the mRNP particle could form an irreversible action that prevents mRNPs from re-import. Studies in yeast suggest that this remodelling is established by the RNA helicase Dpb5, which forms a key player in mRNA export. It unwinds mRNA in an ATP-driven fashion and is attached at the cytoplasmically oriented nucleoporin Nup159. Dpb5 is a shuttling protein that is also present in the nucleus where it is recruited to nascent mRNPs during transcription (Hodge et al., 1999; Schmitt et al., 1999; Zhao et al., 2002). However, Dpb5 will not be active in the nucleus as it has very low intrinsic helicase activity. At the NPC, this activity is enhanced ~600-fold by the combined action of Gle1 and InsP6, both present at the cytoplasmic side of the NPC (Alcazar-Roman et al., 2006; Weirich et al., 2006). The unwinding of the mRNA may lead to dissociation of the mRNA-associated proteins, including its export factors (Cole and Scarcelli, 2006). Dpb5 has also been proposed to function as a ratchet wheel, pulling the mRNP complexes out of the nucleus (Stewart, 2007). Although CRM1 is not the major mRNA export factor (discussed in (Fornerod and Ohno, 2002)), it is responsible for export of a specific subset of mRNAs. For example, export of AU-rich elements-containing mRNAs present in short-lived mRNAs, like mRNAs coding for interferons, cytokines, proto-oncogenes and growth factors is mediated by CRM1. These AU-rich elements are recognized by the RNA-binding protein HuR, a protein involved in mRNA stabilization, and subsequent binding of the NES-containing proteins APRIL and pp32 recruits CRM1 to the complex (Gallouzi et al., 2001). Other examples of ARE-containing mRNAs exported via the CRM1 pathway are c-FOS, COX-2 and IFN-α (reviewed in (Hutten and Kehlenbach, 2007)).

1.8 Viruses and nuclear transport

Viruses often hijack the host transport machinery to promote their replication and infectivity (reviewed in (Fontoura et al., 2005)). Although some viruses replicate in the cytoplasm, viruses that replicate in the nucleus require the host’s import machinery to transport the viral genome into the nucleus and allow replication and gene expression. Adenovirus, for example, disassembles at Nup214 at the NPC and ‘injects’ its DNA into the nucleus, a process aided by host cell factors (Trotman et al., 2001).

Besides import, viruses often use the host cell’s nuclear export machinery to export their mRNAs and/or genomes. A well-studied example is the export of the intron-containing transcripts of the retrovirus HIV-1. HIV-1 has one pro-viral transcript that encodes the information for nine proteins that are expressed from different mRNA molecules formed by alternative splicing. The mRNAs for the regulatory proteins Tat, Rev and Nef are fully spliced and exported via the canonical mRNA export pathway. The other six transcripts are either unspliced or partially spliced. These transcripts would normally be retained in the nucleus, but the virus encodes a non-structural protein named Rev that allows nuclear export of these transcripts. Rev is a shuttling protein that specifically recognizes a tertiary structural element in these transcripts, termed the Rev response element (RRE). Rev mediates the export of these mRNAs via its leucine-rich NES, which was one of the first NESs identified (Fischer et al., 1995). At steady state, Rev predominantly localizes to nucleoli by interacting with the nucleolar protein B23, where it is thought to inhibit cell growth by inhibiting transport of (pre-) ribosomal proteins (Miyazaki et al., 1996).

Whereas the HIV-1 genome harbours the information for Rev, which recognizes viral transcripts, simian type D
retroviruses, like the Mazon-Pfizer monkey virus, require a host factor for the binding of their transcripts. Like in HIV-1, the export of unspliced mRNA molecules is mediated by a structural element, termed the constitutive transport element (CTE). The mRNA export factor TAP is responsible for recognition and export of CTE-containing transcripts (Grüter et al., 1998). Interestingly, the intron-containing TAP mRNA also contains a CTE and is transported by the TAP protein itself. Therefore, it is thought that TAP plays a more general role in the export of intron-containing mRNAs (Li et al., 2006).

Viruses can also inhibit the host’s transport pathways in order to cause cytotoxicity to the host and/or to promote their own transport. An example of this is inhibition of host mRNA export by the Vesicular Stomatitis Virus (VSV) Matrix protein (M protein). M protein exerts this function by inhibiting Rae1, a mRNA export factor that is attached at the nuclear located nucleoporin Nup98. M protein binding is thought to interfere with mRNA export by competing for the Rae1-Nup98 interaction (Faria et al., 2005; von Kobbe et al., 2000). In Chapter 4, I will describe the inhibition of the host mRNA export pathway by the parvovirus NS2 protein of MVM (see 1.8.1).

Viruses may also adopt the host’s nuclear transport system to export themselves out of the nucleus. Although most nuclear assembled viruses are too big to pass the NPC, one exception is the small parvovirus with a diameter of only 25 nm, which equals the size of a ribosome and is therefore small enough to translocate through NPCs. Larger viruses escape the nucleus either by cell lysis, or by using alternative ways to penetrate the NE. One elegant example is the nuclear egress of the large enveloped herpesvirus. Studies in cytomegalovirus, a member of the β-herpesvirus subfamily, revealed that two viral proteins, M53/p38 and M50/p35 that together form a docking site for the virus, govern the first step in nuclear egress. M50/p35 recruits protein kinase C, which phosphorylates the nuclear lamina causing its dissolution (Muranyi et al., 2002). Subsequently, the herpesvirus translocates through the NE by an initial envelopment at the inner nuclear membrane, followed by a re-envelopment upon fusion with the outer nuclear membrane. Upon arrival in the cytoplasm, the capsid acquires its final envelope by fusing with vesicles derived from the Golgi network (reviewed in (Mettenleiter et al., 2006)).

1.8.1 Minute Virus of Mice

Minute Virus of Mice (MVM) is the mouse species of the parvovirus genus. It is a small single stranded DNA virus, composed of an icosahedral capsid, entirely made up of the two viral proteins, VP1 and VP2, that surround the 5 kb single-stranded DNA molecule. Natural infections are asymptomatic (Kimsey et al., 1986; Rubio et al., 2005), but an immunosuppressive variant of MVM, termed MVMi, causes death in newborns and leukopenia in SCID mice (Ramirez et al., 1996; Segovia et al., 1999). Interestingly, cells transformed by various mechanisms have an increased susceptibility for cytotoxicity caused by MVM (Cornelis et al., 1988a; Cornelis et al., 1988b; Mousset et al., 1986).

The MVM life cycle starts with uptake of the virus at the cell membrane through receptor-mediated endocytosis. As nuclear assembly and DNA replication take place in the nucleus, MVM should insert its DNA in the nucleus or alternatively, the complete virus should enter the nucleus. For MVM, evidence for both mechanisms have been demonstrated. In lysosomes, the low pH causes structural rearrangements of the capsid, resulting in the exposure of an N-terminal NLS in VP1, which is required for MVM import (Lombardo et al., 2002; Mani et al., 2006). Evidence for an alternative mechanism has been shown in Xenopus oocytes, where MVM injections led to small breaks in the NE through which MVM could enter the cell nucleus (Cohen and Pante, 2005). In the nucleus, the replication of the ssDNA requires DNA polymerase and as a consequence MVM can only infect dividing cells. The viral genome contains the information for two structural proteins VP1 and VP2 and at least two non-structural proteins NS1 and NS2 (Cotmore et al., 1983). VP1 and VP2 form heterotrimer and VP2-only heterotrimers in the cytoplasm and their import relies on the N-terminal NLS in VP1 and on a nuclear localization motif present in both VP1 and VP2. In the nucleus these trimers assemble into icosahedral capsids composed of twenty trimeric subunits (Lombardo et al., 2002; Riolobos et al., 2006). MVM spreads by cell lysis, but it is able to escape the nucleus prior to cell lysis (Miller and Pintel, 2002). The mechanism of the latter is not precisely known, although this viral egress is mediated by an N-terminal peptide of VP2, termed 2Nt. This signal is exposed upon insertion of the replicated ssDNA into newly assembled capsids (Mauroto et al., 2004).

The two non-structural proteins NS1 (83 kDa) and NS2 (25kDa) are expressed early during infection (Clemens and Pintel, 1988) and have a relatively short half-life of less than one hour (Schoborg and Pintel, 1991). Together they are responsible for managing all processes required for viral replication. NS1 is a nuclear phosphoprotein that is imported via a classical NLS, composed of at least two lysine-containing domains (Nuesch and Tattersall, 1993). It is required for viral DNA replication (Cotmore and Tattersall, 1987) and for causing cytotoxicity to the host cell (Caillet-Fauquet et al., 1990). NS2 has been shown to function in multiple processes like capsid assembly (Cotmore et al., 1997), viral messenger translation (Naeger et al., 1993) DNA replication (Choi et al., 2005), virus production (Naeger et al., 1990), cytotoxicity (Legrand et al., 1993) and capsid egress from the nucleus (Eichwald et al., 2002; Miller and Pintel, 2002) though its mode of action has not yet been unraveled. NS2 exists in a non-phosphorylated and a phosphorylated form, of which the latter is excluded from the nucleus (Cotmore and Tattersall, 1990). Thus far, only three interactors of NS2 are
known: Smn (Survival Motor Neuron), a protein mutated in the neurodegenerative disease spinal muscular atrophy, the phosphothreonine and phosphoserine-binding protein 14-3-3 and CRM1, which is its most studied interaction partner. Whereas 14-3-3 predominantly binds the phosphorylated form of NS2 in the cytoplasm (Bodendorf et al., 1999; Brockhaus et al., 1996), CRM1 interacts with the non-phosphorylated form in both compartments (Bodendorf et al., 1999). CRM1 exports NS2 out of the nucleus by binding the NS2 NES (82-MTKKFGTLT-91) (Askjaer et al., 1999; Eichwald et al., 2002). This NES has been used in many studies because of its potent binding to CRM1 (Askjaer et al., 1999; Petosa et al., 2004). The CRM1-NS2 interaction has been implicated in ssDNA replication (Choi et al., 2005) and viral egress from the nucleus (Eichwald et al., 2002; Miller and Pintel, 2002). Early on in infection, NS2 is mainly localized in the cytoplasm, while at later time points, NS2 accumulates in the nucleus. Furthermore, an enhancement of the CRM1-NS2 interaction has been shown to improve viral fitness pointing to a role for nuclear export pathways in MVM pathogenicity (Lopez Bueno et al., 2004). In Chapter 3 of this thesis I describe a study on the NES of MVM NS2 and show that this NES has all the features of a supraphysiological NES. We further showed that this NES is required for parvoviral egress from the nucleus. Chapter 4 is a short report on another yet unknown function of MVM NS2, the inhibition of cellular mRNA.

Thus, as in recent years much progress has been made in understanding the basics of nucleocytoplasmic transport, basic questions still need to be elucidated. Major issues are the precise requirements of nuclear transport signals, whether different classes of cargo require different types of signals and which interactions at the NPC are required for nucleocytoplasmic transport. In this thesis I address these questions using nuclear export signals and the transport receptor CRM1. I emphasize however, that the answers obtained may in general be applicable to many other nucleocytoplasmic transport pathways as well.

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General Introduction

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