The nucleoporin Nup214 sequesters CRM1 at the nuclear rim and modulates NFκB activation in *Drosophila*

Nikos Xylourgidis*, Peggy Roth*, Nafiseh Sabri, Vasilios Tsarouhas and Christos Samakovlis‡

Department of Developmental Biology, Wenner-Gren Institute, Stockholm University, S-10691, Stockholm, Sweden

*These authors contributed equally to this work

‡Author for correspondence (e-mail: christos@devbio.su.se)

Accepted 4 August 2006
Journal of Cell Science 119, 4409-4419 Published by The Company of Biologists 2006
doi:10.1242/jcs.03201

Summary

CRM1-mediated protein export is an important determinant of the nuclear accumulation of many gene regulators. Here, we show that the NFκB transcription factor Dorsal is a substrate of CRM1 and requires the nucleoporin Nup214 for its nuclear translocation upon signaling. Nup214 bound to CRM1 directly and anchored it to the nuclear envelope. In *nup214* mutants CRM1 accumulated in the nucleus and NES-protein export was enhanced. Nup214 formed complexes with Nup88 and CRM1 in vivo and Nup214 protected Nup88 from degradation at the nuclear rim. In turn, Nup88 was sufficient for targeting the complex to the nuclear pores. Overexpression experiments indicated that Nup214 alone attracts a fraction of CRM1 to the nuclear envelope but does not interfere with NES-GFP export. By contrast, overexpression of the Nup214-Nup88 complex trapped CRM1 and Dorsal to cytoplasmic foci and inhibited protein export and immune response activation. We hypothesize that variation in levels of the Nup214-Nup88 complex at the pore changes the amount of NPC-bound CRM1 and influences the relative strength and duration of NFκB signaling responses.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/119/21/4409/DC1

Key words: *Drosophila*, Nucleoporins, Nup214, CRM1 export, NFκB signaling, Nuclear transport

Introduction

Nuclear pore complexes (NPCs) are protein channels spanning the nuclear envelope to conduct macromolecular transport between the nucleus and the cytoplasm (reviewed by Suntharalingam and Wente, 2003). Genetic and biochemical studies in yeast and mammalian cells have identified ~30 nuclear pore proteins, which form phylogenetically conserved sub-complexes with defined composition and localization along the nuclear pore (Cronshaw et al., 2002; Rout et al., 2000). Although carrier-independent transport has been described for several proteins, most of the protein transport in or out of the nucleus relies on a family of soluble factors known as karyopherins. In facilitated transport events, importins and exportins recognize discrete signals on their cargoes and mediate either nuclear import or export. The karyopherin-cargo complex then binds to nucleoporins with phenylanine-glycine (FG) repeats, which may collectively provide a favorable environment for the passage of the complex (Becskei and Mattaj, 2005). Directionality is orchestrated by Ran, a small GTPase that is highly enriched in its GTP-bound form in the nucleus, where it dissociates the importin-cargo complexes and promotes the formation of cargo complexes with exportins (Weis, 2003). The exportin CRM1, recognizes and transports proteins bearing a leucine-rich nuclear export signal (Fornerod et al., 1997a; Fukuda et al., 1997; Stade et al., 1997). CRM1 binds cooperatively with RanGTP to the cargo to form a trimeric complex NES-RanGTP-CRM1, which then translocates through the NPC. Release of the export substrates in the cytoplasmic face of the NPC is accomplished by the RanGTPase-activating protein (RanGAP) and RanBP1, which hydrolyze RanGTP and dissociate the complex (Fornerod and Ohno, 2002; Kuersten et al., 2002).

Many cell-signaling events culminate with the selective translocation of gene regulators in and out of the nucleus. Cytoplasmic anchoring and posttranslational modifications of either cargo molecules or transport mediators regulate the nuclear accumulation of signaling effectors leading to changes in gene expression (Kaffman and O'Shea, 1999; Xu and Massague, 2004). A less-explored mechanism involves the modification of individual nucleoporins and transport factors leading to alterations of transport rates (Kehlenbach and Gerace, 2000; Makhnevych et al., 2003). A paradigm for the 'cytoplasmic anchoring' mechanism derives from studies of NFκB activation in flies and vertebrate systems. In *Drosophila*, Toll signaling determines dorsal-ventral axis specification in embryos and the activation of the innate immune responses in larvae and adults (Brennan and Anderson, 2004). Toll signaling upon bacterial infection, leads to the degradation of the IκB homolog, Cactus, and the nuclear accumulation of the NFκB proteins Dorsal and Dif. In mutant larvae lacking the nucleoporin Nup88, Cactus becomes degraded upon bacterial challenge but Dorsal and Dif fail to accumulate in the nucleus. Nuclear import of other transcription factors or artificial reporters bearing nuclear localization signals is not affected in

the mutants (Uv et al., 2000). Surprisingly, Nup88 acts as an attenuator of CRM1-mediated protein export by anchoring a fraction of CRM1 on the nuclear envelope (Roth et al., 2003) suggesting an additional mechanism controlling the nuclear accumulation of NFκB proteins.

The nucleoporin CAN-Nup214 binds to Nup88 and was originally identified as a putative oncogene, disrupted by chromosomal translocations associated with myeloid leukemias (Snow et al., 1987; von Lindern et al., 1992). It is localized at the cytoplasmic side of the NPC (Kraemer et al., 1994; Pante et al., 1994) and forms complexes with at least three other nucleoporins, Nup88-Nup82p, RanBP2-Nup358 and Nup62-Nsp1p (Belgareh et al., 1998; Bernad et al., 2004; Fornerod et al., 1997b). The binding of Nup214 to importin β and CRM1, and the phenotypic analysis of cells derived from Nup214-deficient mice or *Xenopus* nuclei depleted of Nup214, suggested a facilitative role for Nup214 in nucleocytoplasmic transport of proteins (Fornerod et al., 1997a; Moroianu et al., 1997). More recently, depletion of Nup214 in HeLa cells revealed a requirement for the nucleoporin in the nuclear export of the 60S pre-ribosomal subunit but no major defects in the export of NES-GFP reporters (Bernad et al., 2006). The FG repeats of Nup214 have also been directly implicated in the shuttling of signaling effectors such as mitogen-activated protein kinase (ERK) and Smad2 without the need for transport receptors (Matsubayashi et al., 2001; Xu et al., 2002).

Here we explore the interdependence of Nup214 and its partner Nup88 at the NPC and the function of the complex in NES export and NFκB protein transport in *Drosophila.* We find that Nup214 maintains high levels of Nup88 at the NPC and Nup88 is required for targeting the complex to the pores. Nup214 can bind to CRM1 directly and is required for anchoring a portion of CRM1 to the NPC. In *nup214* mutants NES export is surprisingly enhanced and dosage-sensitive genetic interactions between *crm1* and *nup214* mutants suggest that Nup214 acts as CRM1 inhibitor. Dorsal and Dif translocation upon bacterial infection is impaired in *nup214* mutants. We identified CRM1-dependent NES sequences in Dorsal and found that it becomes mislocalized after overexpression of the two nucleoporins. We propose a mechanism, where the amounts of the Nup214-Nup88 complex on the NPCs determine the levels of CRM1 export attenuation and thereby the degree of activation of NFκB downstream genes.

Results

Nup214 forms a complex with Nup88 and CRM1 in Drosophila embryos

Nup88 is required for the localization of Nup214 and a portion of CRM1 at the nuclear rim (Roth et al., 2003). Using yeast two-hybrid and in vitro binding assays, we first showed that the FG-rich, C-terminal part of *Drosophila* Nup214 binds directly to CRM1 (supplementary material Fig. S1). To analyze the complex in fly embryos, we prepared protein extracts from three embryonic stages and examined the relative amounts of each component in the complex by immunoprecipitations with a Nup214 antiserum. Nup214, Nup88 and CRM1 were then detected with specific antibodies on western blots of the bound and unbound fractions (Fig. 1). All three proteins were found in the precipitate, and although most Nup88 was precipitated from the extract, only a subfraction of CRM1 was detected in the complex (Fig. 1). In addition, the relative amounts of CRM1 in the complex varied at different stages of embryonic development suggesting that the composition of the complex is dynamic. The variations in the relative amounts of CRM1 bound to the nucleoporin complex at different developmental stages suggest that the affinity of the export factor for the NPC may be modulated during embryogenesis.

Interdependence of Nup214 and Nup88 at the nuclear rim

To investigate the function of Nup214, we generated deletion mutants by P-element transposon excision and characterized their lesions. Homozygous animals from one of these strains (from now on referred to as *nup214* mutants) lacked a 631 bp fragment including parts of the second and third exons of the gene and die as early third instar larvae. The expression of the full-length *nup214* cDNA clone, in *nup214* larvae under the control of the inducible *hsp70* promoter partially rescues the lethality of the mutants and extends their life span to the late third larval stage. Attempts to detect clones of *nup214* cells, generated by mitotic recombination, in imaginal discs and adult tissues failed, suggesting an essential function of Nup214 in cell division or cell survival. The phenotypic analysis of *nup214* was therefore conducted in early third instar mutant larvae that lack zygotic Nup214 expression (Fig. 2A). *nup214* mutants develop normally up to this stage owing to a robust contribution of maternal RNA and protein and do not show any gross defects in nuclear envelope integrity revealed by

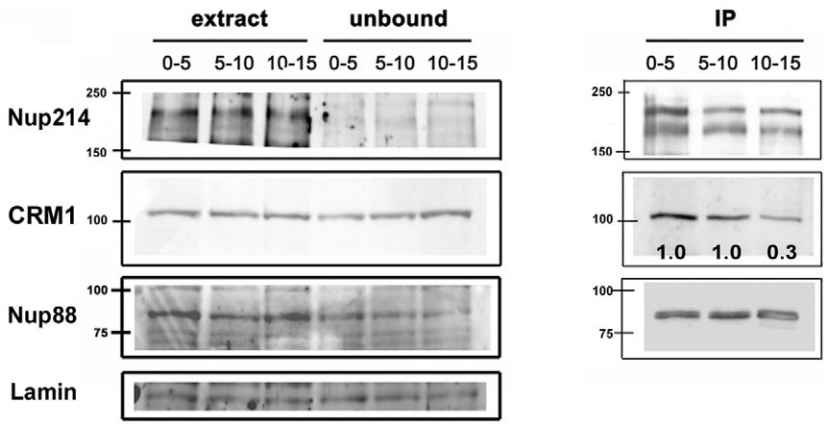

Fig. 1. *Drosophila* Nup214 is in complex with Nup88 and CRM1. The amounts of CRM1 bound to the Nup214-Nup88 complex vary during fly development. Nup214 antiserum was used to coimmunoprecipitate protein complexes of 0-5, 5-10 and 10-15 hour wild-type embryo extracts. Extracts, unbound fractions and precipitates (IP) were analyzed by labeling the western blots with Nup214, CRM1 and Nup88 antibodies. Lamin serves as a loading control. Total amounts of Nup214, Nup88 and CRM1 appear unchanged in the extracts from different embryonic stages. The amount of co-immunoprecipitated CRM1 relative to Nup214 does not change in 0-5 hour and 5-10 hour embryos whereas three times less CRM1 is brought down in 10-15 hour embryos.

stainings with the nucleoporin marker mAb414 (Davis and Blobel, 1986) and lamin (Stuurman et al., 1995) antibodies (Fig. 2A). To confirm that the lethality and the phenotypes in *nup214* mutants are due to a disruption of only the *nup214* gene, we also analyzed *nup214/Df(2R)3-70* mutant larvae. These animals revealed similar developmental and physiological phenotypes to the *nup214* homozygous mutants, indicating that the defects in *nup214* mutants are solely due to the lack of zygotic Nup214 protein.

Staining of wild-type larvae with anti-Nup88 antisera revealed a pronounced nuclear rim labeling, which appeared diffuse and drastically reduced in *nup214* mutants (Fig. 2A). Western blots of protein extracts from staged wild-type, *nup214* heterozygotes and *nup214* homozygous larvae showed that Nup88 was decreased in heterozygotes and was undetectable in the *nup214* homozygotes (Fig. 2B), whereas the *nup88* mRNA levels remained unchanged in *nup214* mutants compared to wild-type (Fig. 2C). This gradual reduction of Nup88 protein at decreasing concentrations of Nup214 suggests a role for Nup214 in titrating the amounts of Nup88 at the nuclear rim. Since the amount of the *nup88* transcript is not affected in *nup214* larvae, Nup214 might function in promoting the translation of Nup88 or protecting it from degradation.

To address the functional interplay between the two nucleoporins, we analyzed the Nup214-Nup88 complex in S2 cells. We used double-stranded RNA inhibition (RNAi) to reduce Nup214 and analyzed Nup88, Nup214 and β-Tubulin levels by western blotting (Fig. 3A). The Nup88 antibody recognizes two bands in S2 cell extracts and we performed a *nup88* RNAi experiment to identify the Nup88-specific band (Fig. 3A, right panel). In cells treated with *nup214* dsRNA for 3 days, Nup214 levels decreased by 50% and after 6 days of *nup214* RNAi they decreased by ~90%. Similarly to mutant larvae, the relative amount of Nup88 was also decreased proportionally to the decline of Nup214. To test if the reduction of Nup88 in the absence of Nup214 is due to protein degradation, we added the proteasome inhibitor epoxomicin (Meng et al., 1999) to cells treated with *nup214* dsRNA for three days in an attempt to restore the decrease of Nup88. Addition of the inhibitor compensated the reduction of Nup88 levels in cells lacking Nup214 (Fig. 3A) indicating that Nup214 protects Nup88 from proteasomal degradation at the nuclear pore.

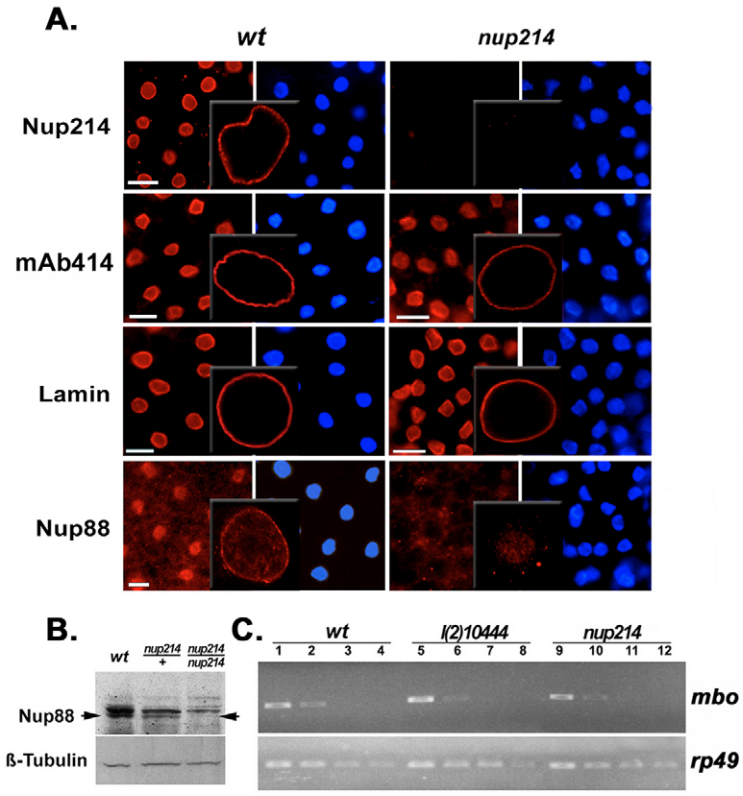

Fig. 2. Nup88 is degraded in *nup214* mutants. (A) Nup214, mAb414, lamin and Nup88 localization (red) in fat body cells of wild-type and *nup214* mutant larvae. Nuclei are visualized by DAPI in the adjacent panel. Confocal sections are shown in the insets. Bars, 35 μm. (B) Western blot of protein extract from wild-type, heterozygotes and *nup214* mutant larvae probed with Nup88 antibody. β-tubulin provides a loading control. Nup88 is reduced in heterozygote animals and totally abolished in the homozygous mutant. (C) RT-PCR for *mbo* on poly(A)$^+$-purified larval extracts of wild-type (lanes 1-4), P-element (*l(2)10444*) (lanes 5-8) and *nup214* mutants (lanes 9-12). (Fourfold serial dilutions of mRNA from each genotype were used in the RT-PCR step.) *rp49* provides a quantitative control. The mRNA levels of *mbo* remain the same in wild-type and mutant larvae.

Nup88 is required for the localization of Nup214 on the NPC because in *mbo* mutants Nup214 becomes nuclear (Roth et al., 2003). We further investigated the interdependence of the two components of the complex in vivo, by heat-shock-induced overexpression of each of the nucleoporins in the wild type and mutants lacking Nup88 or Nup214, followed by detection of both proteins with specific antibodies. Overexpression of Nup88 in wild-type larvae resulted in a punctuated cytoplasmic staining without affecting the localization of endogenous Nup214 (Fig. 3B). By contrast, Nup214 expressed under the control of the same promoter, was enriched only around the rim, suggesting that Nup88 at the NPC has the capacity to bind excessive Nup214 and anchor it to the pore (Fig. 3B). *hsp70*-driven Nup214 in *mbo* mutants accumulated in the nucleus, highlighting the anticipated role of Nup88 in anchoring Nup214 at the NPC (Fig. 3B). Nup214 was also nuclear when it was expressed in *nup214* mutants, which also lack Nup88 (Fig. 3B). By contrast, overexpression of Nup88 in *nup214* larvae resulted in a distinct nuclear rim staining indicating that Nup88 contains all the necessary sequences for targeting the NPC (Fig. 3B). The punctate Nup88 staining in these animals appeared much reduced in comparison with the levels of overexpressed Nup88 in the wild type, arguing again that Nup214 is required post translationally for the accumulation of high levels of Nup88. We also co-expressed Nup88 and Nup214 in *nup214* mutants. In these experiments, the relative amounts of the proteins varied in individual cells of the *nup214* larvae (supplementary material Fig. S2). In cells expressing high levels of Nup88 and low amounts of Nup214 after heat shock, the complex was strictly localized at the NPC. By contrast, in cells expressing relatively low levels of Nup88 and high amounts of Nup214, the complex was found in the

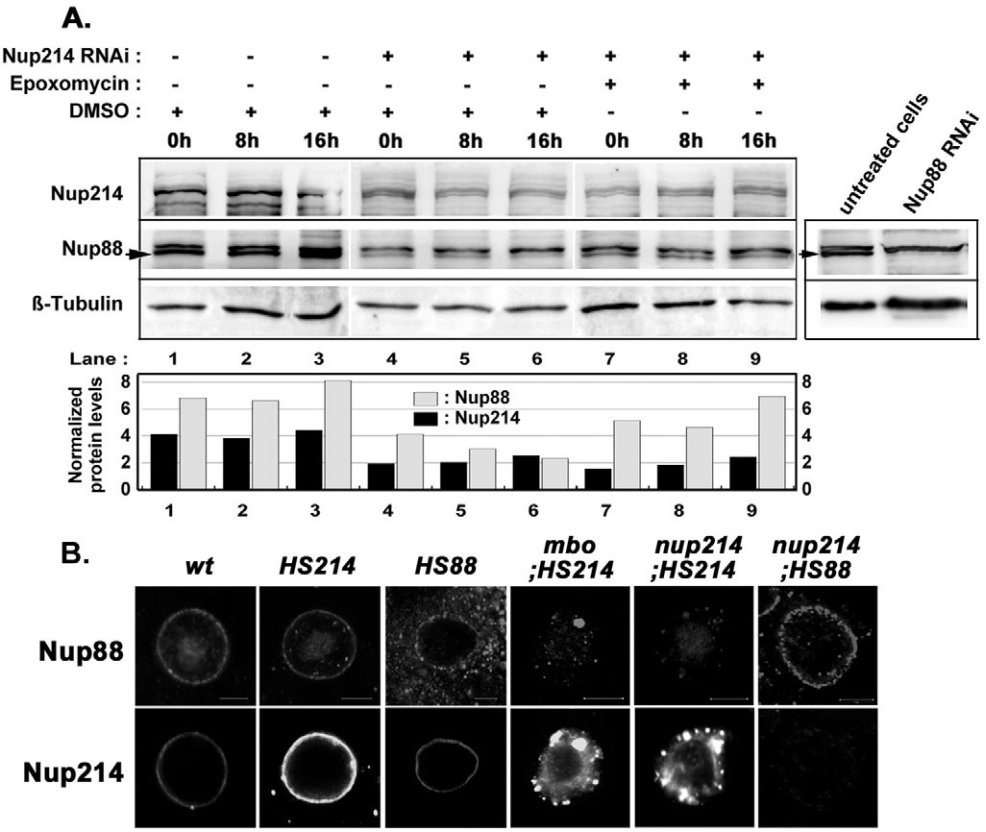

Fig. 3. Nup214 and Nup88 are interdependent. (A) Nup88 degradation in *nup214* RNAi cells is prevented by epoxomicin. *Drosophila* S2 cells were transfected with dsRNA against *nup214*. On day 3 post transfection, cells were treated with epoxomicin (or DMSO) for 0, 8 or 16 hours. Cell lysates were analyzed by western blot with Nup214 or Nup88 antibodies. β-tubulin served as a loading control. The arrow indicates the Nup88-specific band. The graph shows the levels of Nup88 (grey) and Nup214 (black) normalized against β-tubulin. The western blot to the right represents a control for the specificity of the Nup88 antibody. *Drosophila* S2 cells were exposed for 4 days to *nup88* RNAi. The Nup88-specific band (arrow) is absent in the RNAi cells. (B) Nup88 anchors Nup214 at the nuclear rim. Either Nup214 or Nup88 was expressed by the *hsp70* promoter in wild-type, *mbo* or *nup214* mutant larvae. The images show confocal sections of malpighian tube nuclei stained with anti-Nup214 and anti-Nup88. Bars, 5 μm.

nucleus. The overexpression analysis suggests that the stability and the localization of the complex may depend on the relative amounts of each constituent.

nup214 mutants show enhanced CRM1-mediated nuclear export

The cytoplasmic accumulation of a GFP-NES reporter is increased in *mbo* mutants and this phenotype could be reverted by LMB treatment of the animals, suggesting that the nucleoporin acts as an inhibitor of CRM1-mediated protein export (Roth et al., 2003). To investigate whether Nup214 has a similar function in protein export, we expressed GFP-NES under the control of the inducible *hsp70* promoter in wild-type, CRM1 (*emb*^{3}) (Roth et al., 2003) and *nup214* mutant larvae. GFP expression in wild-type and *nup214* larvae served as controls for the assay (supplementary material Fig. S3A). After a 1-hour induction and 3 hours recovery, we examined the levels and the localization of the reporter in larval gut and fat body tissues using a GFP antibody. GFP-NES fusions were detected in both the cytoplasm and nucleus of wild-type tissues (Fig. 4A). In *emb*^{3} (CRM1) mutants, the GFP-NES reporter accumulated inside the nucleus, indicating that it is recognized as a CRM1 substrate (Fig. 4A). In *nup214* mutants the distribution of GFP-NES was predominantly cytoplasmic, suggesting that CRM1-mediated protein export might be enhanced in the absence of Nup214 (Fig. 4A). To measure the increased cytoplasmic accumulation of GFP-NES in *nup214* mutants, we compared the ratios of nuclear versus cytoplasmic GFP-NES intensities in *nup214* and wild-type early third instar larvae. GFP-NES nuclear accumulation was decreased by 30% in *nup214*, whereas the nuclear accumulation of the same reporter was increased by 40% in *emb*^{3} mutants compared with the wild type (Fig. 4B).

To explore the mechanism underlying the inhibitory role of Nup214 on nucleocytoplasmic export, we analyzed the subcellular localization of CRM1 in the mutants. In several wild-type tissues, a substantial part of CRM1 staining was found along the nuclear rim and at lower levels in the nucleus (Fig. 4C). By contrast, in *nup214* mutants, most of the CRM1 staining at the nuclear rim was absent, and the protein appeared to be concentrated inside the nucleus (Fig. 4C). Western blots revealed that the total amount of CRM1 was similar in both wild-type and *nup214* extracts (Fig. 4D) suggesting a role of Nup214 in tethering CRM1 at the nuclear pore.

RanGAP together with RanBP1 promotes the hydrolysis of RanGTP and subsequent dissociation of the trimeric CRM1-NES-RanGTP export complex in the cytoplasm (Askjaer et al., 1999). It is localized at the cytoplasmic fibrils of the NPC through its association with RanBP2-Nup358 (Hopper et al., 1990; Matunis et al., 1996). Staining of wild-type tissues revealed a distinct accumulation of RanGAP at the nuclear rim, whereas in *nup214* mutants the staining appeared punctuated and reduced by ~30% (Fig. 4C,E). Thus, Nup214 is also required to maintain RanGAP on the cytoplasmic side of the NPC. Because the localization of Nup358 on the filaments depends on Nup214, the mislocalization of RanGAP in the mutants may be a secondary phenotype caused by the reduction of RanBP2-Nup358 at the NPC (Bernad et al., 2004). This function would also predict a facilitating role for Nup214 in the release of export substrates from CRM1. However, we did not detect any accumulation of GFP-NES either in the nucleus

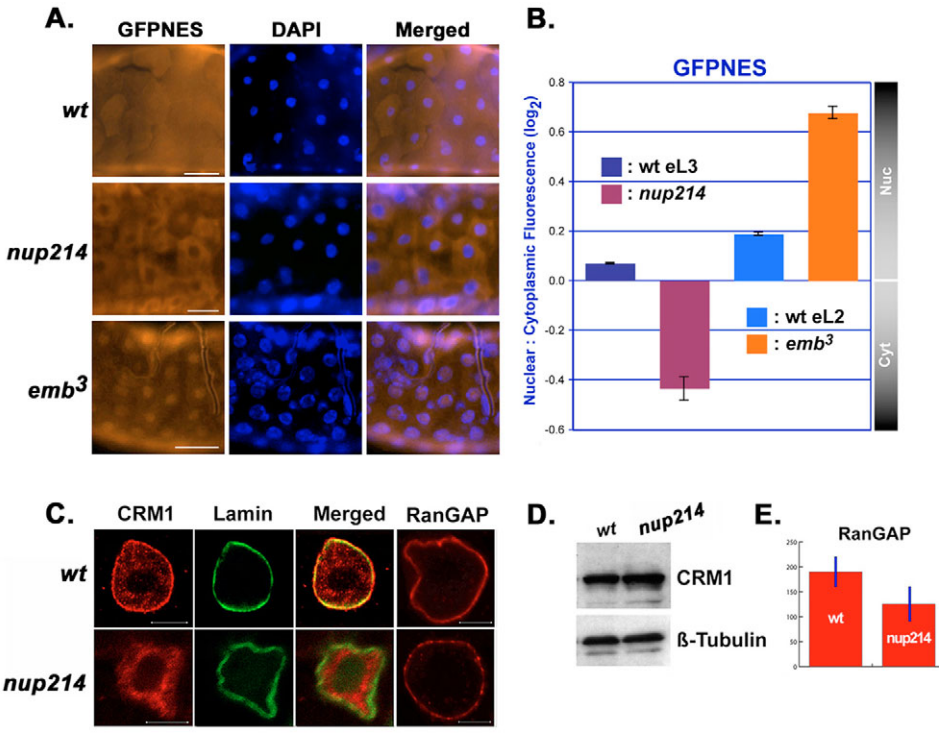

Fig. 4. *Drosophila* Nup214 attenuates CRM1-mediated protein export. (A) GFP-NES was expressed under the control of the *hsp70* promoter in wild-type, *emb*^{3} and *nup214* mutant larvae and detected with a GFP antibody (red). All panels show larval gut cells. Nuclei were visualized by DAPI staining (middle panels). Bar, 35 μm. (B) Nuclear export rates of GFP-NES are enhanced in *nup214* mutants. The ratios of nuclear:cytoplasmic GFP-NES intensities of early second (eL2) and early third (eL3) instar wild-type, *nup214* and *emb*^{3} larval gut cells are shown in a $\log_2$ graph. The nuclear accumulation of the GFP-NES reporter is decreased ~30% in *nup214* mutants, whereas in *emb*^{3} mutants the nuclear accumulation is increased by ~40% (P<0.0001 by pair-wise t-test). Error bars represent s.e.m. (C) Confocal sections of fat body cells from wild-type and *nup214* mutant larvae stained with anti-CRM1, anti-lamin and anti-RanGAP antibodies. Error bars, 5 μm. (D) Western blot of protein extract from wild-type and *nup214* mutant larvae probed with anti-CRM1. β-tubulin provides a loading control. (E) Quantification of RanGAP levels along the nuclear rim in wild-type and *nup214* mutants. The nuclear rim staining in the mutants is reduced by ~35%.

or at the nuclear rim in *nup214* larvae, suggesting that the release of CRM1 substrates is not significantly affected in these mutants. RanGAP is also required for NLS-nuclear import, because it facilitates the recycling of importin β by releasing it from RanGTP (Floer et al., 1997). We found that the nuclear accumulation of the β-Gal-NLS and NLS-GFP reporters is indeed reduced in *nup214* larvae compared with the wild type (supplementary material Fig. S3A,B). By contrast, the transcription factors Ultrabithorax (Ubx) and Grainyhead (Grh) entered the nucleus in wild-type and *nup214* mutants after induction, in all tissues examined (supplementary material Fig. S3A), arguing against a general role of Nup214 in nuclear protein import.

Nup214, like Nup88, binds directly to CRM1 and the analysis of GFP-NES localization suggests that they function to attenuate CRM1-mediated protein export. This inhibitory function of the two nucleoporins in protein export is surprising and we set to assess its significance in larval development and physiology. We performed dosage-sensitive genetic interaction experiments using null zygotic mutants of CRM1 (*emb*^{2}), which survive to larvae as a result of an abundant maternal CRM1 contribution (Collier et al., 2000; Roth et al., 2003), (Fig. 5). We reasoned that if the cytoplasmic accumulation of the GFP-NES reporter in *nup214* mutants also reflects an increase in the export of endogenous CRM1 targets, then decreasing the amount of Nup214 might ameliorate the *emb*

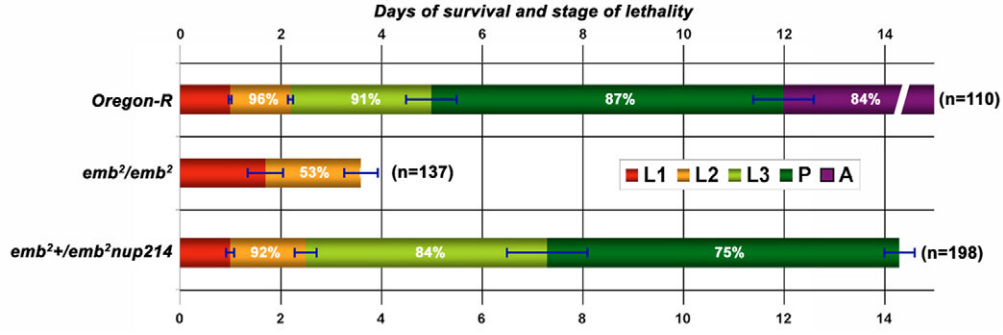

Fig. 5. Prolonged development of *emb*^{2} mutants lacking one copy of *nup214*. Bars represent survival (days) of the indicated genotypes. All values are significantly different (P<0.0001; pair-wise t-test) when compared with survival of the *emb*^{2}/*emb*^{2} mutants. Percentage values within bars represent the number of survivors through the developmental stages. A, adult stage; L1, first instar larvae; L2, second instar larvae; L3, third instar larvae; P, pupal stage.

mutant phenotypes. *emb* larvae die at the 2nd instar stage with underdeveloped anterior spiracles. Removal of one copy of *nup214* or the *mbo* gene from *emb* homozygous animals prolonged their life span drastically and also allowed the development and eversion of the anterior spiracles (Fig. 5). Thus, halving the amount of Nup214 can partially rescue all the defects caused by the reduction of CRM1 in the larva, offering genetic evidence that highlights the role of Nup214 as an antagonist of CRM1 in the animal.

Co-expression of Nup214 and Nup88 sequesters CRM1 in the cytoplasm and inhibits NES-mediated export

nup88 and *nup214* larvae show increased levels of NES-mediated protein export. Nup88 and Nup214 both bind to CRM1 and are required for anchoring a subpopulation of the exportin at the nuclear rim. We tested whether overexpression of the nucleoporins may be sufficient to capture CRM1 and interfere with its function in protein export. We used the *hsp70*-driven transgenes to express either Nup214 or Nup88 or both proteins together in third instar larvae, and analyzed the localization of the overexpressed proteins, CRM1, importin β, lamin and RanGAP by antibody staining. In a subset of these larvae treated in parallel, we also quantified the nuclear accumulation of GFP-NES and NLS-GFP to detect changes in nuclear protein export or import. Excess Nup88 alone did not affect the localization of CRM1, importin β, lamin and RanGAP (supplementary material Fig. S4A). Overexpression of Nup214 caused a slight increase in the accumulation of CRM1 on the nuclear rim, a minor mislocalization of importin β and no detectable change in lamin and RanGAP localization (supplementary material Fig. S4A). Neither of the single gene overexpression caused any notable defects in the ratios of nuclear to cytoplasmic accumulation of the GFP-NES and NLS-GFP cargoes ($P>0.05$ by pair-wise *t*-test, Fig. 6B and supplementary material Fig. 4B).

Simultaneous overexpression of Nup88 and Nup214 however, was lethal to the larvae and resulted in the pronounced reduction of the nuclear CRM1 pool and the mislocalization of the export factor in cytoplasmic complexes containing Nup88 and Nup214 (Fig. 6A). Consequently the GFP-NES reporter accumulated in the nucleus of these animals indicating a reduction of the level of protein export ($P<0.0001$ by pair-wise *t*-test, Fig. 6B). In animals treated in parallel, the distribution of RanGAP and lamin was indistinguishable from the wild type whereas importin β was slightly mislocalized (supplementary material Fig. S4A). Importantly, this defect in importin β localization was not accompanied by changes in the ratio of nuclear to cytoplasmic NLS-GFP signal which was indistinguishable from the wild type ($P>0.05$ by pair-wise *t*-test; supplementary material Fig. S4B). Thus, co-expression of Nup88 and Nup214 is sufficient to selectively attract CRM1 from the nucleus to the cytoplasm and to increase the nuclear accumulation of the GFP-NES reporter. This suggests that the two nucleoporins are functionally interdependent for the binding and anchoring of CRM1.

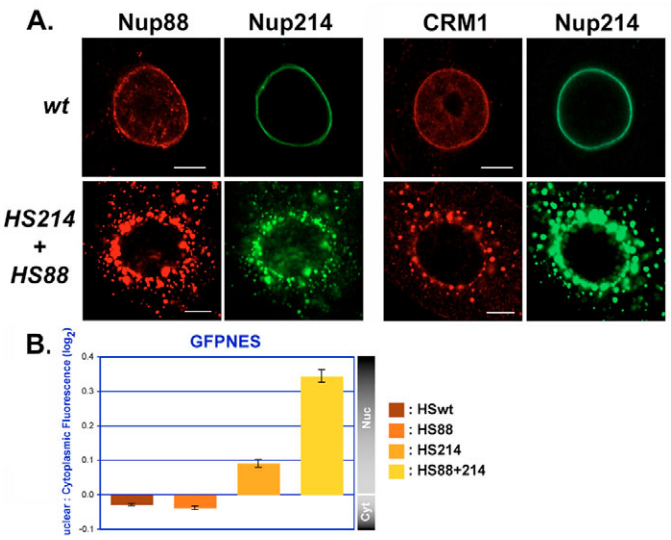

Fig. 6. Co-expression of Nup214 and Nup88 in the wild type causes formation of cytoplasmic foci and mislocalizes CRM1. (A) Nup214 and Nup88 (HS214+HS88) were expressed with the *hsp70* promoter in wild-type third-instar larvae. Heat-treated wild type (*wt*) served as a control. All panels show confocal sections of malphigian tube nuclei stained either with Nup88 and Nup214 or with Nup214 and CRM1 antisera. Bar, 5 μm. (B) Overexpression of both nucleoporins affects the localization of GFP-NES. GFP-NES was expressed together with Nup88, Nup214 or Nup214 and Nup88 by heat shock induction of third-instar larvae. The ratios of nuclear:cytoplasmic GFP-NES intensities are illustrated in a $\log_2$ graph. Nuclear accumulation of the GFP-NES is increased in cells co-expressing both Nup214 and Nup88 ($P<0.0001$ by pair-wise *t*-test). Error bars represent s.e.m.

NFκB factors require Nup214 for their nuclear accumulation upon signaling

The nuclear accumulation of Dorsal and Dif and the subsequent activation of an immune response are impaired in *mbo* larvae, suggesting that the activity of the two NFκB proteins depends on the function of Nup88 (Uv et al., 2000). Given the functional interdependence of Nup88 and Nup214, we first investigated the subcellular localization of Dorsal and Dif, in fat bodies of wild-type and homozygous *nup214* larvae before and after bacterial infection. In unchallenged larvae of both genotypes, Dorsal and Dif were detected in the nucleus and the cytoplasm (Fig. 7). After bacterial infection, both factors accumulated in the nucleus of wild-type larvae, but remained cytoplasmic in *nup214* mutants. This suggests that both members of the Nup214-Nup88 complex are necessary for the nuclear accumulation of Rel factors upon induction and the activation of the larval immune response (Fig. 7). In wild-type larvae, the nuclear translocation of NFκB proteins results in the rapid activation of genes encoding antimicrobial peptides (Lemaitre et al., 1997). We tested the activation of Rel targets in the mutants with two transcriptional reporter constructs, one containing the *cecropin A* promoter driving the expression of *lacZ* (*cecA1-lacZ*) (Petersen et al., 1995) and one expressing GFP under the control of the *drosomycin* promoter (Ferrandon et al., 1998). The expression of both reporters was strongly induced in wild-type larvae, whereas their expression after bacterial challenge was severely reduced in *nup214* mutants (Fig. 7). Thus, the inducible nuclear accumulation of two NFκB factors and the activation of their downstream genes upon bacterial infection are impaired in *nup214* mutants.

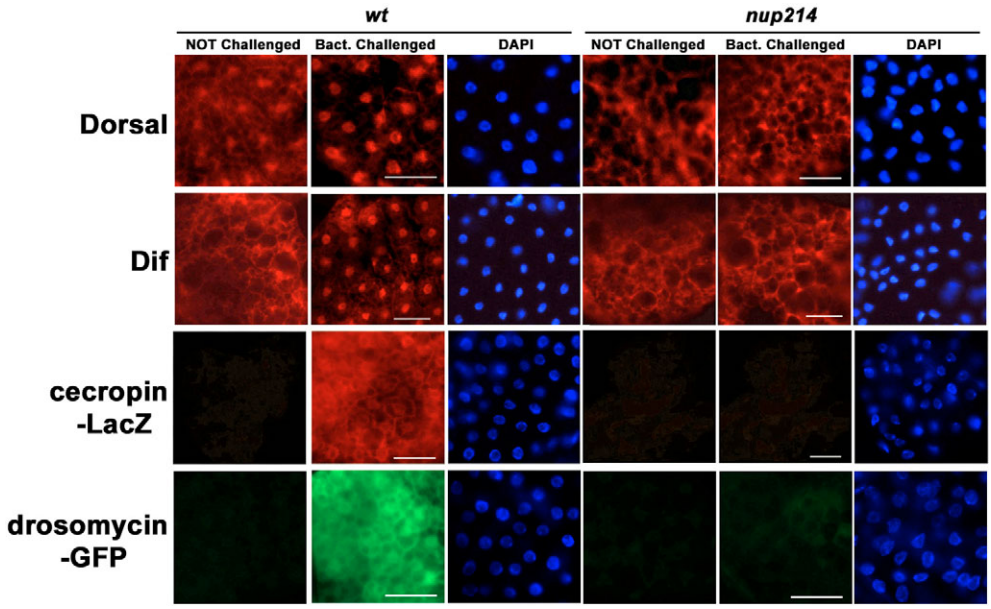

Fig. 7. The nuclear accumulation of Dorsal and Dif is impaired in *nup214* mutants. All panels show fat bodies of wild-type (wt) and *nup214* mutant larvae, before (not challenged) and after bacterial infection (bact. challenged) stained with specific antibodies for Dorsal, Dif, β-galactosidase for cecropin-lacZ or GFP for drosomycin-GFP expression. Nuclei are visualized with DAPI (blue). Bars, 35 μm.

To address the molecular defects underlying the phenotypes of *nup214* and *mbo* mutants we asked whether their endogenous targets might be direct substrates of CRM1-mediated nuclear export. Analysis of the Dorsal protein sequence revealed four putative leucine-rich nuclear export sequences (supplementary material Fig. S5A). To determine their relevance in Dorsal localization we fused these sequences to GFP and generated several Dorsal deletion and amino acid substitution constructs. We analyzed the subcellular distribution of these proteins before and after *crm1* RNAi or leptomycin B (LMB) treatment of transfected S2 cells (supplementary material Fig. S5B,C). The results indicate that Dorsal is a direct substrate of CRM1. The identified C-terminal NES4 is the major functional NES and is necessary for the cytoplasmic accumulation of Dorsal in the absence of signaling.

Overexpression of the Nup214-Nup88 complex interferes with Dorsal localization and the expression of downstream target genes

Dorsal requires CRM1 for its nuclear shuttling and the nuclear accumulation of the protein after signaling requires Nup214 and Nup88 function. In addition, overexpression of the nucleoporins traps CRM1 and interferes with GFP-NES export. We hypothesized that if the nuclear accumulation of Dorsal can be modulated by the amount of CRM1 captured by the Nup88-214 complex, we might be able to interfere with Dorsal localization and the activation of the immune response through overexpression of the nucleoporins. We first analyzed the expression levels of the inducible drosomycin-GFP reporter in wild-type larvae and animals overexpressing Nup214-Nup88 before and after bacterial infection. In unchallenged larvae of both genotypes, expression of the two reporters was weak or barely detectable (Fig. 8A). Upon bacterial infection, reporter-gene expression was strongly induced in wild-type larvae, but remained very low in larvae overexpressing Nup214 and Nup88 (Fig. 8A). We further examined whether the failure in reporter activation might be due to an aberrant localization of Dorsal after the overexpression of the nucleoporin complex. The distribution of Dorsal and Grainyhead (Grh), an unrelated transcription factor (Bray and Kafatos, 1991), was analyzed in untreated larvae and animals overexpressing Nup214 and Nup88. After heat shock induction of both Nups, Dorsal accumulated to cytoplasmic foci co-localizing with Nup88 and CRM1 (Fig. 8B), whereas the same treatment did not result in any defects in the localization of Grh (Fig. 8B). The results show that overexpression of the Nup214-Nup88 complex can selectively trap endogenous transcription factors and CRM1 and suggest that alterations in the levels of CRM1 bound to the complex may modulate the expression of Rel target genes upon activation of the immune response.

Discussion

The cytoplasmic filaments of the NPC extend ~40 nm from the nuclear envelope (Goldberg and Allen, 1993; Jarnik and Aebi, 1991). Functional analysis of their constituents suggests a role in the cytoplasmic release of nuclear export substrates and the assembly and docking of nuclear import complexes. Nup214 and Nup88 homologues form complexes at the cytoplasmic side of the pores in yeast, amphibians and mammals but their roles in animal development and physiology remain elusive. We have investigated the role of Nup214 in CRM1-mediated nuclear export in vivo and its functional relationship with its binding partner Nup88 in *Drosophila* larvae.

Interdependence of Nup214-Nup88 at the NPC

Nup214 does not play a key role in maintaining the NPC architecture. Staining with an antibody against several FG-repeat-containing nucleoporins did not reveal detectable abnormalities in their abundance or localization in *nup214* mutants. The major structural component of the cytoplasmic filaments is RanBP2-Nup358 (Delphin et al., 1997; Walther et al., 2002). Owing to the lack of a specific detection reagent for RanBP2-Nup358, we used antibodies against RanGAP, to indirectly assess the integrity of the cytoplasmic filaments in *nup214* larvae. RanGAP binds directly to RanBP2(Nup358)

and this binding is required for its accumulation at the nuclear rim. RanGAP was still localized along the nuclear envelope, but the staining appeared ~35% weaker and punctuated, suggesting that the Nup214-Nup88 complex does not play a major role in maintaining RanBP2(Nup358) on the cytoplasmic filaments.

The interdependence of Nup88 and Nup214 at the NPC however, has been consistently observed in all species in which it has been analyzed. Nup88 is undetectable in cells derived from Nup214-deficient embryos (Fornerod et al., 1997b) and RNAi inhibition of each of the genes in tissue culture results in reduced protein levels for the other (Bernad et al., 2004). In addition, yeast cells with a temperature-sensitive mutation of Nup82p, show a reduction of Nup159p from the nuclear rim (Belgareh et al., 1998). The molecular mechanisms underlying this interplay remain unknown. Nup88 is undetectable in *nup214* mutants whereas the levels of the *nup88* transcript remain constant. In tissue culture RNAi experiments, and in *nup214* heterozygotes and homozygous mutants, the reduction of Nup88 is proportional to the amount of Nup214. In addition, epoxomycin can inhibit the Nup88 reduction caused by the inactivation of Nup214. Since Nup214 and Nup88 bind to each other directly (Roth et al., 2003), the results suggest that Nup214 binding of Nup88 at the NPC protects it from proteasome degradation. This protection mechanism may involve interference with the degradation of Nup88 selectively at the pore, because Nup88 appears to be a stable protein in the cytoplasm when overexpressed.

In *mbo/nup88* mutants Nup214 detaches from the nuclear rim and localizes within the nucleus (Roth et al., 2003). Overexpression of Nup88 in *nup214* mutants, lacking endogenous Nup214 and Nup88, results in accumulation of the overexpressed protein at the nuclear rim indicating that Nup88 alone is sufficient to target the complex to the pore. The high levels of overexpressed Nup88 in *nup214* mutants might be explained by a protective function of minimal residual amounts of Nup214 or by the inability of the protein degradation machinery to cope with the overproduced Nup88. The analysis suggests an intriguing posttranslational mechanism for the interdependence of the two nucleoporins. Nup88 alone is sufficient to associate with the NPC and this association is a prerequisite for the localization of Nup214 to the nuclear membrane. In turn, the binding of Nup214 increases the stability of Nup88 proteins at the nuclear envelope and may thereby increase the potential of additional Nup88 molecules to associate with the NPC. In sporadic cells of *nup214* mutants that expressed high concentrations of Nup88 and low amounts of Nup214 after heat shock, the complex was localized along the nuclear envelope. By contrast, in cells expressing relatively low Nup88 and high Nup214 levels, localization of the complex became nuclear. This distinct distribution of the proteins correlating with the relative expression levels of the two Nups in different cells, suggests that localization of

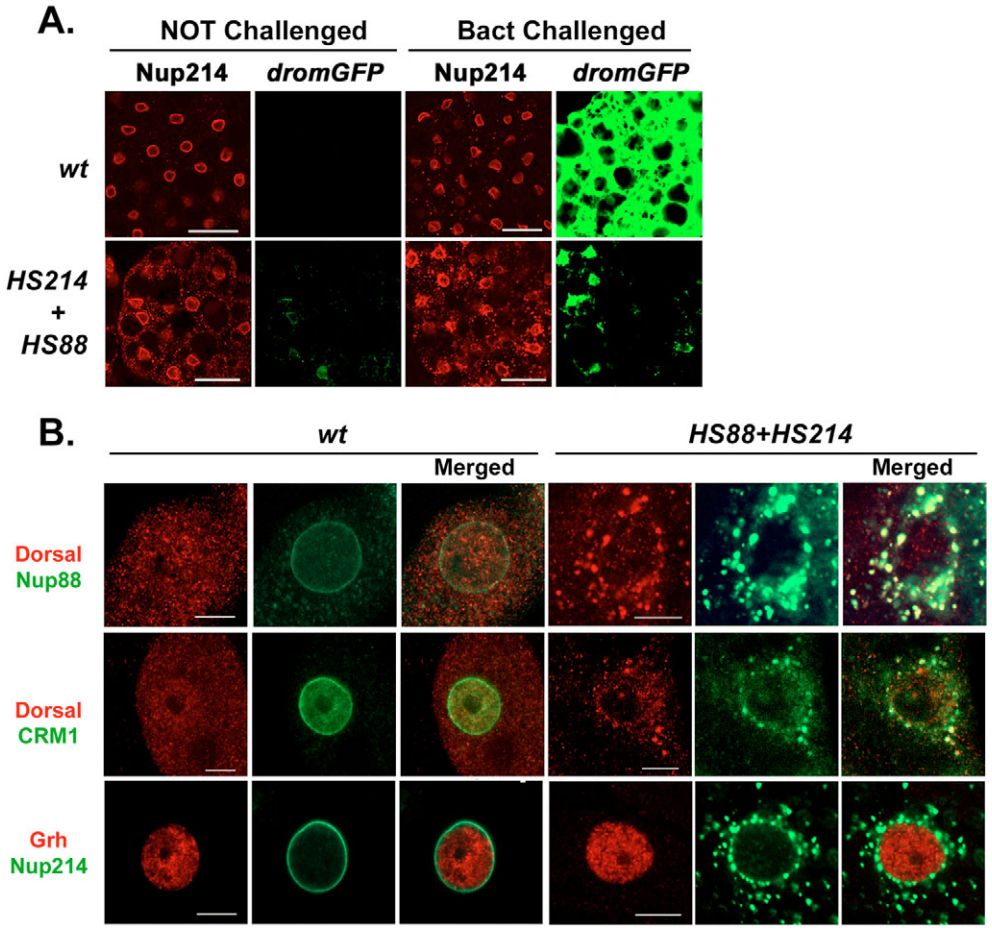

Fig. 8. Co-expression of Nup214 and Nup88 in the wild type is sufficient to mislocalize endogenous targets. Nup214 and Nup88 (HS214+HS88) expression was driven by the *hsp70* promoter in wild-type third instar larvae. Heat-treated wild-type (*wt*) served as a control. (A) Overexpression of Nup214 and Nup88 interferes with the expression of a drosomycin-GFP *(dromGFP)* reporter. All panels show fat bodies, before (not challenged) and after bacterial infection (bact. challenged) stained with GFP antibody. The images for HS214+HS88 unchallenged and infected larval fat bodies were acquired at three times lower intensity for Nup214 labeling compared with the wild type. (B) Confocal sections of malpighian tube nuclei stained with Nup88, Nup214, Dorsal, CRM1 or Grainyhead antiserum. Co-expression of Nup214 and Nup88 can mislocalize Dorsal but not Grainyhead. Bars, 35 μm (A); 5 μm (B).

the complex is dynamic and depends on the relative concentrations of the two nucleoporins.

The Nup214-Nup88 complex attenuates NES-protein export

Nup214 binds to CRM1 and the cytoplasmic accumulation of the GFP-NES reporter is increased in *nup214* mutants, whereas CRM1 is mislocalized from the NPC. These phenotypes suggest that Nup214 acts as an inhibitor of NES-mediated export. The physiological significance of the inhibitory function of Nup214 on CRM1 export is further emphasized by the phenotype caused by the reduction of Nup214 in *crm1* mutants. Removal of one chromosomal copy of *nup214* in emb^2 mutants, which die as second instar larvae, allows these animals to proceed into pupariation and to develop adult structures. This extension of the life span of emb^2 mutants suggests that anchoring of CRM1 to the NPC is a general mechanism that limits CRM1 activity. The endogenous substrates of CRM1 required for progression through the larval stages and pupariation remain to be identified.

The phenotypes of Nup214 mutants are identical to the previously described CRM1-export defects of mbo/nup88 larvae (Roth et al., 2003). However, overexpression of Nup88 alone in wild-type animals did not affect CRM1 localization and overexpression of Nup214 only caused a minor enrichment of CRM1 concentration on the nuclear rim and did not interfere with NES export. The co-expression of both nucleoporins under the control of the heat-shock promoter resulted in the gross mislocalization of CRM1 from the nuclear envelope and disrupted GFP-NES export. Thus, the Nup214-Nup88 complex is necessary and sufficient to tether a fraction of CRM1 and attenuate protein export.

Why would nuclear pore components negatively regulate CRM1 function? One possible explanation is that export complex formation depends on the levels of CRM1 in the nucleoplasm. The binding affinity of CRM1 to natural NESs varies, and cargoes with low affinity NESs may be exported less efficiently (Engelsma et al., 2004; Henderson and Eleftheriou, 2000; Kutay and Guttinger, 2005). The introduction of an artificial, high-affinity NES disrupts CRM1 export indicating that natural NESs are selected for their weaker affinity for the export factor (Engelsma et al., 2004). Removal of the Nup214-Nup88 sub-complex from the pore increases the nuclear concentration of CRM1 and it would also increase nuclear export of cargoes with low-affinity NESs. Tethering or releasing the NPC-bound fraction of the export factor may provide the means for controlling the nuclear concentration of proteins carrying low-affinity export signals.

Nup214-Nup88 functions in NFκB translocation and the activation of immune responses

Dorsal contains a functional NLS embedded in its Rel-homology domain. This sequence is sufficient to target a β-galactosidase reporter into the nucleus and is required for the nuclear accumulation of Dorsal during embryogenesis (Drier et al., 1999; Govind et al., 1996). Owing to the phenotypes of *nup214* larvae in the nuclear accumulation of import reporters we cannot exclude the fact that the defects of *nup214* mutants in the activation of immune responses may be partly due to a reduction in the nuclear import levels of Dorsal and Dif. We favor the hypothesis that the *nup214* phenotype in NFκB translocation is primarily due to increased levels of protein export. Mutations inactivating Nup88, the partner of Nup214, disrupt NFκB translocation and show concurrent enhanced levels of NES-mediated protein export but do not exhibit any detectable effects on the nuclear import of the same reporters (Roth et al., 2003). In addition, we identified a functional, CRM1-dependent NES, required for the cytoplasmic accumulation of Dorsal. This NES4 motif is deleted in hypomorphic *dorsal* alleles expressing truncated forms of the protein and causing an extended nuclear gradient in the embryo. These mutants still retain their Cactus-binding domain and their phenotypes become further enhanced by reduction of *cactus* activity, suggesting that CRM1 export is an additional novel determinant of Dorsal localization and activation in S2 cells and the embryo (Isoda et al., 1992; Rushlow et al., 1989). The requirement of the Nup214-Nup88 complex for the full activation of the immune response and its inhibitory function on CRM1 export in larvae suggest that the amplitude and duration of Toll signaling may be influenced by the export rates of Dorsal and Dif. The interference of the complex with NFκB localization and activity upon overexpression, further suggests that changes in the relative amounts of the nucleoporin complex and the fraction of CRM1 bound to it, may provide a regulatory node for the nuclear concentration of Dorsal and Dif. Variations in the NPC-bound CRM1 pool could be accomplished in two ways: First by modifications of Nup88 or/and Nup214, which could influence their binding capacity to CRM1. Variations in the affinity of the nucleoporin complex for CRM1 could explain the changes in the amounts of co-precipitated CRM1, whereas the amounts of Nup88, Nup214 and CRM1 remain constant in extracts from 5-10 and 10-15 hour *Drosophila* embryos. Nup88 phosphorylation has been detected in *Xenopus* oocytes (Bernad et al., 2004), and such modifications might influence the affinity of CRM1 for the complex. Alternatively, transcriptional control of Nup88 during fly development might also influence the levels of Nup214 and CRM1 in turn, at the NPC. The steady-state ratios of Nup88 to Nup214 have been determined by proteomic studies of yeast and rat liver NPCs and revealed a 2:1 and an 8:1 excess of Nup88, respectively (Cronshaw et al., 2002; Rout et al., 2000). Changes of the wild-type stoichiometry by overexpression of the two nucleoporins leads to lethality in *Drosophila* larvae and apoptosis and G0 arrest in human cells (Boer et al., 1998). The zygotic expression pattern of Nup88 in *Drosophila* is tissue- and stage-specific (Roth et al., 2003; Uv et al., 2000). The interdependence of Nup214 and Nup88 at the NPC may provide an elegant titration mechanism that continuously monitors the structure and function of the Nup214-Nup88 complex and the amount of CRM1 bound to it.

Materials and Methods

Drosophila strains

Nup214 in *Drosophila* is encoded by the CG3820 gene and the enhancer trap insertion l(2)10444 was identified as a P-element lethal mutation, where the transposon was inserted into the *nup214* gene at nucleotide position 122 after the translational initiation site of the predicted open reading frame (Flybase/BDGP). 100 excision strains deriving from l(2)10444 were generated as described (Robertson et al., 1988) and balanced over *CyO ftz-LacZ* and *CyO GFP*. The P-element excision generated several homozygous viable strains and the lethal *nup214*. This excision allele failed to complement *Df(2R)3-70* (Flybase/BDGP). The following fly strains were used: *mbo-1* (Uv et al., 2000), *hs-nup88* (Uv et al., 2000), *hs-grh* (Uv et al., 1994), *hs-ubx* (White and Wilcox, 1984), *hs-NLS-GFP* and *hs-GFP-NES* (Roth et al., 2003), *cecropin-lacZ* (Petersen et al., 1995) and *drosomycin-GFP* (Ferrandon et al., 1998). The *hs-nup214* transgenic fly strains were generated by P-element-mediated transformation (Spradling, 1986). *nup214* was

amplified from full-length cDNA using LA polymerase (TAKARA) for 12 cycles and cloned into the *Bgl*II and *Xba*I sites of pCaSpeR-hs.

Genetic interactions were assessed by examining the lethal phase of animals of the following genotypes: *Oregon-R, emb²/emb², nup214 emb²/emb²* and *emb²/emb²; Dfmbo* (*Dfmbo* corresponds to *Df(3R)Kar-Sz29f*, Flybase).

Sequencing and RT-PCR

For sequencing of the *nup214* excision allele, genomic DNA from wild-type and homozygous mutant larvae (Gloor et al., 1993) was amplified with the High Fidelity PCR system (Roche) using primer pair: 5′-TGCGATTGAATTCGAAGGAT-3′ and 3′-CAGCCTTGGGAGCATTTAGA-5′. PCR reactions were analyzed by electrophoresis in 1% agarose gel and purified using the QIAquick PCR Purification Kit (Qiagen). Sequencing reactions were performed with the same primer pair or with the internal primer: 5′-TCAAGCTAAGTTGGTTTCTTTT-3′ using BigDye Terminator v3.0 sequencing kit (Applied Biosystems).

Larval mRNA was extracted and annealed to magnetic oligo(dT) coupled beads (Dynabeads) according to the manufacturer's instructions. Reverse transcription was performed using SuperScriptII (Invitrogen). Serial dilutions were used as template for PCR amplification with specific primers for: C-terminal *mbo* (5′-GTTA-ACCAGCCCATCTTGGCGG-3′ and 3′-TTAGATGCCAACGATTTTATT-5′) (25 cycles) and *rp49* (5′-TGACCATCCGCCCAGCATACA-3′ and 3′-TCTCGCCGCA-GTAAAC-5′) (22 cycles). The products were analyzed by electrophoresis and visualized with ethidium bromide.

Binding assays

All constructs were generated by ten-cycle PCR amplification from cDNA clones. Fragments were cloned either into pGEX-5x (Amersham Bioscience) or in pRSET (Invitrogen). GST and His_6 fusion proteins were expressed in the bacterial strain BL21pLys. Bacteria were harvested and sonicated in a buffer containing 150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, 1% NP40 and 1 mM DTT. The bound proteins were analyzed on western blots using anti-RGS-His (Qiagen) diluted 1:2000 and anti-GST (1:1000; Santa Cruz Biotechnology). For the yeast two-hybrid system PCR fragments were cloned into either pAct2 (GAL4 DNA-binding domain vector; HA tagged; TRP selectable marker) or pAS1 (GAL4 activation domain vector; HA tagged; LEU2 selectable marker). The different constructs were pair-wise introduced in the yeast strain PJ69-4A (James et al., 1996) by LiAc transformation method (Gietz and Schiestl, 1995) and grown at 30°C for 3-4 days. Transformants were selected on SD-TRP^-LEU^- plates. Protein was extracted from positive clones as described in (Adams et al., 1997). Expression of the different fusion proteins was detected on western blots using an anti-hemagglutinin (HA) antibody (1:2000; Babco). Interaction was scored by growth on SD-$TRP^-LEU^-ADE^-$ plates and β-galactosidase assays were performed as described (Adams et al., 1997).

Cell culture and RNAi treatment

S2 cells were cultured at 25°C in Schneider's *Drosophila* medium (PAN) supplemented with 10% heat-inactivated FCS (Invitrogen), 50 μg/ml gentamycin, 50 U/ml penicillin, 50 μg/ml streptomycin (PAN) and 2 mM glutamine (Invitrogen). 700 bp fragments of *nup214*, *nup88* and *emb/crm1* were amplified by PCR using the nucleotides: 5′-TTAATACGACTCACTATAGGGAGAAAGCCTGATGACA-CTGAGC-3′ and 5′-TTAATACGACTCACTATAGGGAGAGGAGTCGGTCAAT-GTATCC-3′; 5′-TTAATACGACTCACTATAGGGAGAACTACAAGGATGGCA-AGCC-3′ and 5′-TTAATACGACTCACTATAGGGAGACAACACAGAGTCGT-CAACC-3′; TTAATACGACTCACTATAGGGAGATCGCAAATCTTGCGACGC-3′ and 5′-TTAATACGACTCACTATAGGGAGATGTCAATGATGAATGTCTCC-3′. These were then used as templates to produce dsNup214, dsNup88 and dsCRM1/emb RNA respectively with the MEGAscript RNAi kit (Ambion). The dsRNA treatment was performed as described (Clemens et al., 2000). For the Nup88 degradation assay, Nup214 RNAi cells at day 3 post treatment were incubated with 20 μM epoxomicin (Calbiochem).

Site-directed mutagenesis and live cell imaging

Mutations of the Dorsal NES3 and NES4 were created using the QuikChange mutagenesis kit (Stratagene). For both NESs the first two large hydrophobic residues were changed to alanines. NES3, $L^{553}SNL^{556}NNPFTM$, was changed to $A^{553}SNA^{556}NNPFTM$ and NES4, $DL^{669}QI^{671}SNLSIS$, was changed to $DA^{669}QA^{671}SNLSIS$. LMB was used at the final concentration of 10 ng/ml for 2 hours. For protein expression in *Drosophila* S2 cells the different constructs were cloned either in pAc5.1/V5-His or pMT/V5-His (Invitrogen). S2 cells were transfected with the TransFast™ Transfection Reagent according to the manufacturer's instructions (Promega). For staining of DNA in living cells, Hoechst 33342 (Sigma) was added to the culture medium at final concentration of 4 μM. Cells at day 4 and day 6 were viewed with a Leica DM IRB inverted Fluorescence microscope. Images were acquired using a Leica DC300F CCD camera and processed with Image Viewer 5.02 software (Kodak).

Immunostaining of larvae and *Drosophila* S2 cells

Larvae were heat induced for 1 hour at 37°C and analyzed after 2 or 3 hours. Antibody staining of larval tissues was performed as described (Patel, 1994). Dorsal and Dif translocation experiments were done as described (Uv et al., 2000). *Drosophila* S2 cells were directly fixed on a poly-L-lysine (Sigma)-coated coverslip in 4% freshly prepared paraformaldehyde in phosphate-buffered saline (PBS) for 30 minutes at room temperature. After fixing, the cells were washed in PBS and permeabilized in 0.1% Triton X-100 in PBS for 5 minutes. Cells were preincubated with PBS 0.1% Triton X-100 and 0.5% BSA (Albumin, Bovine, Sigma) for 30 minutes at room temperature followed by incubation with primary antibodies overnight at 4°C. After rinsing and preincubation with PBS 0.1% Triton X-100 and 0.5% BSA for 30 minutes, the cells were incubated with secondary antibody for 2 hours at room temperature, washed and incubated with DAPI (0.4 μg/ml) for 5 minutes. After rinsing, the cells were mounted in 70% glycerol containing 2.5% DABCO (1,4 diazabicyclo[2.2.2]octane; Sigma).

The following primary antibodies were used: anti-Nup214 (Roth et al., 2003) diluted 1:10,000, anti-CRM1 1:1000 (Roth et al., 2003); anti-GFP 1:1000 (Molecular Probes); anti-lamin Dm1 (ADL84) 1:500 (Stuurman et al., 1995); anti-Ubx 1:20 (White and Wilcox, 1984); anti-Grh 1:5 (Bray and Kafatos, 1991); anti-RanGAP 1:1000 (Merrill et al., 1999); anti-Nup88 1:100 (Uv et al., 2000), mAb414 1:5000 (Babco), anti-β-Gal 1:2000 (Promega), anti-Dorsal 1:1000 (Gillespie and Wasserman, 1994), anti-Dif 1:300 (Cantera et al., 1999), C-terminal anti-His 1:2000 (Invitrogen), anti-V5 1:2000 (Invitrogen) and anti-Ketel (Lippai et al., 2000) 1:1000. Secondary antibodies conjugated to Alexa Fluor 488 (Molecular Probes) or Cy3 (Jackson Laboratories) were used as recommended.

Confocal images were acquired with a LSM 510 laser-scanning microscope (Zeiss). Quantitative image analysis was performed with the LSM 5 ver3 software (Zeiss). Fluorescent images were recorded on a Zeiss fluorescence microscope equipped with (Axio Cam HRc) CCD camera. GFP intensities were measured from a selected area in the cytoplasm and an area of the same size in the nucleus (visualized by DAPI staining) by using the Volocity 2.0.1 software (Improvision). The ratio of nuclear/cytoplasmic fluorescent signal was determined from ⩾25 cells.

Immunoprecipitation and western blots

Immunoprecipitations from embryonic extracts were performed as described (Edwards et al., 1997) except for the lysis buffer (10 mM Tris-HCl pH 8.0, 140 mM NaCl, 1.5 mM $MgCl_2$, 1% NP-40, protease inhibitor cocktail (Boehringer), 5 mM pyrophosphate, 10 mM NaF, 10 mM β-glycerol phosphate, 5 mM sodium vanadate). The following antibody dilutions were used in western blots: anti-β-tubulin (Amersham) (1:1000), anti-lamin Dm1 (ADL84) 1:500 (Stuurman et al., 1995), anti-Nup214 (Roth et al., 2003) 1:1000 and anti-Nup88 (Uv et al., 2000) 1:1000 and anti-CRM1 (Roth et al., 2003) 1:1000. Signals on western blots were quantified with the Fuji Image Gauge V3.45 software.

We thank J. Szabad (University of Szeged, Hungary), P. A. Fisher (Stony Brook University, Stony Brook, NY), B. Ganetzky (University of Wisconsin, Madison, WI) for reagents and K. Senti and N. Visa for helpful comments on the manuscript. This work was supported by grants from the Cancerfonden and Wallenberg Consortium North to C.S.

References

Adams, A., Gottschling, D. E., Kaiser, C. A. and Stearns, T. (1997). *Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual*. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Askjaer, P., Bachi, A., Wilm, M., Bischoff, F. R., Weeks, D. L., Ogniewski, V., Ohno, M., Niehrs, C., Kjems, J., Mattaj, I. W. et al. (1999). RanGTP-regulated interactions of CRM1 with nucleoporins and a shuttling DEAD-box helicase. *Mol. Cell. Biol.* **19**, 6276-6285.

Becskei, A. and Mattaj, I. W. (2005). Quantitative models of nuclear transport. *Curr. Opin. Cell Biol.* **17**, 27-34.

Belgareh, N., Snay-Hodge, C., Pasteau, F., Dagher, S., Cole, C. N. and Doye, V. (1998). Functional characterization of a Nup159p-containing nuclear pore subcomplex. *Mol. Biol. Cell* **9**, 3475-3492.

Bernad, R., van der Velde, H., Fornerod, M. and Pickersgill, H. (2004). Nup358/RanBP2 attaches to the nuclear pore complex via association with Nup88 and Nup214/CAN and plays a supporting role in CRM1-mediated nuclear protein export. *Mol. Cell. Biol.* **24**, 2373-2384.

Bernad, R., Engelsma, D., Sanderson, H., Pickersgill, H. and Fornerod, M. (2006). The Nup214/Nup88 nucleoporin subcomplex is required for CRM1 mediated 60S preribosomal nuclear export. *J. Biol. Chem.* **281**, 19378-19386.

Boer, J., Bonten-Surtel, J. and Grosveld, G. (1998). Overexpression of the nucleoporin CAN/NUP214 induces growth arrest, nucleocytoplasmic transport defects, and apoptosis. *Mol. Cell. Biol.* **18**, 1236-1247.

Bray, S. J. and Kafatos, F. C. (1991). Developmental function of Elf-1: an essential transcription factor during embryogenesis in Drosophila. *Genes Dev.* **5**, 1672-1683.

Brennan, C. A. and Anderson, K. V. (2004). Drosophila: the genetics of innate immune recognition and response. *Annu. Rev. Immunol.* **22**, 457-483.

Cantera, R., Roos, E. and Engstrom, Y. (1999). Dif and cactus are colocalized in the larval nervous system of Drosophila melanogaster. *J. Neurobiol.* **38**, 16-26.

Clemens, J. C., Worby, C. A., Simonson-Leff, N., Muda, M., Maehama, T., Hemmings, B. A. and Dixon, J. E. (2000). Use of double-stranded RNA interference in Drosophila cell lines to dissect signal transduction pathways. *Proc. Natl. Acad. Sci. USA* **97**, 6499-6503.

Collier, S., Chan, H. Y., Toda, T., McKimmie, C., Johnson, G., Adler, P. N., O'Kane, C. and Ashburner, M. (2000). The Drosophila embargoed gene is required for larval progression and encodes the functional homolog of schizosaccharomyces Crm1. *Genetics* **155**, 1799-1807.

Cronshaw, J. M., Krutchinsky, A. N., Zhang, W., Chait, B. T. and Matunis, M. J. (2002). Proteomic analysis of the mammalian nuclear pore complex. *J. Cell Biol.* **158**, 915-927.

Davis, L. I. and Blobel, G. (1986). Identification and characterization of a nuclear pore complex protein. *Cell* **45**, 699-709.

Delphin, C., Guan, T., Melchior, F. and Gerace, L. (1997). RanGTP targets p97 to RanBP2, a filamentous protein localized at the cytoplasmic periphery of the nuclear pore complex. *Mol. Biol. Cell* **8**, 2379-2390.

Drier, E. A., Huang, L. H. and Steward, R. (1999). Nuclear import of the Drosophila Rel protein Dorsal is regulated by phosphorylation. *Genes Dev.* **13**, 556-568.

Edwards, D. N., Towb, P. and Wasserman, S. A. (1997). An activity-dependent network of interactions links the Rel protein Dorsal with its cytoplasmic regulators. *Development* **124**, 3855-3864.

Engelsma, D., Bernad, R., Calafat, J. and Fornerod, M. (2004). Supraphysiological nuclear export signals bind CRM1 independently of RanGTP and arrest at Nup358. *EMBO J.* **23**, 3643-3652.

Ferrandon, D., Jung, A. C., Criqui, M., Lemaitre, B., Uttenweiler-Joseph, S., Michaut, L., Reichhart, J. and Hoffmann, J. A. (1998). A drosomycin-GFP reporter transgene reveals a local immune response in Drosophila that is not dependent on the Toll pathway. *EMBO J.* **17**, 1217-1227.

Floer, M., Blobel, G. and Rexach, M. (1997). Disassembly of RanGTP-karyopherin beta complex, an intermediate in nuclear protein import. *J. Biol. Chem.* **272**, 19538-19546.

Fornerod, M. and Ohno, M. (2002). Exportin-mediated nuclear export of proteins and ribonucleoproteins. *Results Probl. Cell Differ.* **35**, 67-91.

Fornerod, M., Ohno, M., Yoshida, M. and Mattaj, I. W. (1997a). CRM1 is an export receptor for leucine-rich nuclear export signals. *Cell* **90**, 1051-1060.

Fornerod, M., van Deursen, J., van Baal, S., Reynolds, A., Davis, D., Murti, K. G., Fransen, J. and Grosveld, G. (1997b). The human homologue of yeast CRM1 is in a dynamic subcomplex with CAN/Nup214 and a novel nuclear pore component Nup88. *EMBO J.* **16**, 807-816.

Fukuda, M., Asano, S., Nakamura, T., Adachi, M., Yoshida, M., Yanagida, M. and Nishida, E. (1997). CRM1 is responsible for intracellular transport mediated by the nuclear export signal. *Nature* **390**, 308-311.

Gietz, R. D. and Schiestl, R. H. (1995). Transforming yeast with DNA. *Methods Mol. Cell. Biol.* **5**, 255-269.

Gillespie, S. K. and Wasserman, S. A. (1994). Dorsal, a Drosophila Rel-like protein, is phosphorylated upon activation of the transmembrane protein Toll. *Mol. Cell. Biol.* **14**, 3559-3568.

Gloor, G. B., Preston, C. R., Johnson-Schlitz, D. M., Nassif, N. A., Phillis, R. W., Benz, W. K., Robertson, H. M. and Engels, W. R. (1993). Type I repressors of P element mobility. *Genetics* **135**, 81-95.

Goldberg, M. W. and Allen, T. D. (1993). The nuclear pore complex: three-dimensional surface structure revealed by field emission, in-lens scanning electron microscopy, with underlying structure uncovered by proteolysis. *J. Cell Sci.* **106**, 261-274.

Govind, S., Drier, E., Huang, L. H. and Steward, R. (1996). Regulated nuclear import of the Drosophila rel protein dorsal: structure-function analysis. *Mol. Cell. Biol.* **16**, 1103-1114.

Henderson, B. R. and Eleftheriou, A. (2000). A comparison of the activity, sequence specificity, and CRM1-dependence of different nuclear export signals. *Exp. Cell Res.* **256**, 213-224.

Hopper, A. K., Traglia, H. M. and Dunst, R. W. (1990). The yeast RNA1 gene product necessary for RNA processing is located in the cytosol and apparently excluded from the nucleus. *J. Cell Biol.* **111**, 309-321.

Isoda, K., Roth, S. and Nusslein-Volhard, C. (1992). The functional domains of the Drosophila morphogen dorsal: evidence from the analysis of mutants. *Genes Dev.* **6**, 619-630.

James, P., Halladay, J. and Craig, E. A. (1996). Genomic libraries and a host strain designed for highly efficient two- hybrid selection in yeast. *Genetics* **144**, 1425-1436.

Jarnik, M. and Aebi, U. (1991). Toward a more complete 3-D structure of the nuclear pore complex. *J. Struct. Biol.* **107**, 291-308.

Kaffman, A. and O'Shea, E. K. (1999). Regulation of nuclear localization: a key to a door. *Annu. Rev. Cell Dev. Biol.* **15**, 291-339.

Kehlenbach, R. H. and Gerace, L. (2000). Phosphorylation of the nuclear transport machinery down-regulates nuclear protein import in vitro. *J. Biol. Chem.* **275**, 17848-17856.

Kraemer, D., Wozniak, R. W., Blobel, G. and Radu, A. (1994). The human CAN protein, a putative oncogene product associated with myeloid leukemogenesis, is a nuclear pore complex protein that faces the cytoplasm. *Proc. Natl. Acad. Sci. USA* **91**, 1519-1523.

Kuersten, S., Arts, G. J., Walther, T. C., Englmeier, L. and Mattaj, I. W. (2002). Steady-state nuclear localization of exportin-t involves RanGTP binding and two distinct nuclear pore complex interaction domains. *Mol. Cell. Biol.* **22**, 5708-5720.

Kutay, U. and Guttinger, S. (2005). Leucine-rich nuclear-export signals: born to be weak. *Trends Cell Biol.* **15**, 121-124.

Lemaitre, B., Reichhart, J. M. and Hoffmann, J. A. (1997). Drosophila host defense: differential induction of antimicrobial peptide genes after infection by various classes of microorganisms. *Proc. Natl. Acad. Sci. USA* **94**, 14614-14619.

Lippai, M., Tirian, L., Boros, I., Mihaly, J., Erdelyi, M., Belecz, I., Mathe, E., Posfai, J., Nagy, A., Udvardy, A. et al. (2000). The Ketel gene encodes a Drosophila homologue of importin-beta. *Genetics* **156**, 1889-1900.

Makhnevych, T., Lusk, C. P., Anderson, A. M., Aitchison, J. D. and Wozniak, R. W. (2003). Cell cycle regulated transport controlled by alterations in the nuclear pore complex. *Cell* **115**, 813-823.

Matsubayashi, Y., Fukuda, M. and Nishida, E. (2001). Evidence for existence of a nuclear pore complex-mediated, cytosol-independent pathway of nuclear translocation of ERK MAP kinase in permeabilized cells. *J. Biol. Chem.* **276**, 41755-41760.

Matunis, M. J., Coutavas, E. and Blobel, G. (1996). A novel ubiquitin-like modification modulates the partitioning of the Ran-GTPase-activating protein RanGAP1 between the cytosol and the nuclear pore complex. *J. Cell Biol.* **135**, 1457-1470.

Meng, L., Mohan, R., Kwok, B. H., Elofsson, M., Sin, N. and Crews, C. M. (1999). Epoxomicin, a potent and selective proteasome inhibitor, exhibits in vivo antiinflammatory activity. *Proc. Natl. Acad. Sci. USA* **96**, 10403-10408.

Merrill, C., Bayraktaroglu, L., Kusano, A. and Ganetzky, B. (1999). Truncated RanGAP encoded by the Segregation Distorter locus of Drosophila. *Science* **283**, 1742-1745.

Moroianu, J., Blobel, G. and Radu, A. (1997). RanGTP-mediated nuclear export of karyopherin alpha involves its interaction with the nucleoporin Nup153. *Proc. Natl. Acad. Sci. USA* **94**, 9699-9704.

Pante, N., Bastos, R., McMorrow, I., Burke, B. and Aebi, U. (1994). Interactions and three-dimensional localization of a group of nuclear pore complex proteins. *J. Cell Biol.* **126**, 603-617.

Patel, N. H. (1994). Imaging neuronal subsets and other cell types in whole-mount *Drosophila* embryos and larvae using antibody probes. In *Drosophila melanogaster: Practical uses in Cell and Molecular Biology* (ed S. B. Goldstein and E. A. Fyrberg), pp. 45-487. Cambridge, MA: Academic Press.

Petersen, U. M., Bjorklund, G., Ip, Y. T. and Engstrom, Y. (1995). The dorsal-related immunity factor, Dif, is a sequence-specific trans-activator of Drosophila Cecropin gene expression. *EMBO J.* **14**, 3146-3158.

Robertson, H. M., Preston, C. R., Phillis, R. W., Johnson-Schlitz, D. M., Benz, W. K. and Engels, W. R. (1988). A stable genomic source of P element transposase in Drosophila melanogaster. *Genetics* **118**, 461-470.

Roth, P., Xylourgidis, N., Sabri, N., Uv, A., Fornerod, M. and Samakovlis, C. (2003). The Drosophila nucleoporin DNup88 localizes DNup214 and CRM1 on the nuclear envelope and attenuates NES-mediated nuclear export. *J. Cell Biol.* **163**, 701-706.

Rout, M. P., Aitchison, J. D., Suprapto, A., Hjertaas, K., Zhao, Y. and Chait, B. T. (2000). The yeast nuclear pore complex: composition, architecture, and transport mechanism. *J. Cell Biol.* **148**, 635-651.

Rushlow, C. A., Han, K., Manley, J. L. and Levine, M. (1989). The graded distribution of the dorsal morphogen is initiated by selective nuclear transport in Drosophila. *Cell* **59**, 1165-1177.

Snow, C. M., Senior, A. and Gerace, L. (1987). Monoclonal antibodies identify a group of nuclear pore complex glycoproteins. *J. Cell Biol.* **104**, 1143-1156.

Spradling, A. C. (1986). P element-mediated transformation. In *Drosophila: A practical Approach* (ed. D. B. Roberts). Oxford: Oxford University Press.

Stade, K., Ford, C. S., Guthrie, C. and Weis, K. (1997). Exportin 1 (Crm1p) is an essential nuclear export factor. *Cell* **90**, 1041-1050.

Stuurman, N., Maus, N. and Fisher, P. A. (1995). Interphase phosphorylation of the Drosophila nuclear lamin: site- mapping using a monoclonal antibody. *J. Cell Sci.* **108**, 3137-3144.

Suntharalingam, M. and Wente, S. R. (2003). Peering through the pore: nuclear pore complex structure, assembly, and function. *Dev. Cell* **4**, 775-789.

Uv, A. E., Thompson, C. R. and Bray, S. J. (1994). The Drosophila tissue-specific factor Grainyhead contains novel DNA-binding and dimerization domains which are conserved in the human protein CP2. *Mol. Cell. Biol.* **14**, 4020-4031.

Uv, A. E., Roth, P., Xylourgidis, N., Wickberg, A., Cantera, R. and Samakovlis, C. (2000). members only encodes a Drosophila nucleoporin required for rel protein import and immune response activation. *Genes Dev.* **14**, 1945-1957.

von Lindern, M., Fornerod, M., van Baal, S., Jaegle, M., de Wit, T., Buijs, A. and Grosveld, G. (1992). The translocation (6;9), associated with a specific subtype of acute myeloid leukemia, results in the fusion of two genes, dek and can, and the expression of a chimeric, leukemia-specific dek-can mRNA. *Mol. Cell. Biol.* **12**, 1687-1697.

Walther, T. C., Pickersgill, H. S., Cordes, V. C., Goldberg, M. W., Allen, T. D., Mattaj, I. W. and Fornerod, M. (2002). The cytoplasmic filaments of the nuclear pore complex are dispensable for selective nuclear protein import. *J. Cell Biol.* **158**, 63-77.

Weis, K. (2003). Regulating access to the genome: nucleocytoplasmic transport throughout the cell cycle. *Cell* **112**, 441-451.

White, R. A. and Wilcox, M. (1984). Protein products of the bithorax complex in Drosophila. *Cell* **39**, 163-171.

Xu, L. and Massague, J. (2004). Nucleocytoplasmic shuttling of signal transducers. *Nat. Rev. Mol. Cell Biol.* **5**, 209-219.

Xu, L., Kang, Y., Col, S. and Massague, J. (2002). Smad2 nucleocytoplasmic shuttling by nucleoporins CAN/Nup214 and Nup153 feeds TGFbeta signaling complexes in the cytoplasm and nucleus. *Mol. Cell* **10**, 271-282.