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The *Drosophila* nucleoporin DNup88 localizes DNup214 and CRM1 on the nuclear envelope and attenuates NES-mediated nuclear export

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Many cellular responses rely on the control of nucleocytoplasmic transport of transcriptional regulators. The *Drosophila* nucleoporin Nup88 is selectively required for nuclear accumulation of Rel proteins and full activation of the innate immune response. Here, we investigate the mechanisms underlying its role in nucleocytoplasmic transport. Nuclear import of an nuclear localization signal-enhanced green fluorescent protein (NLS-EGFP) reporter is not affected in DNup88 (*members only; mbo*) mutants, whereas the level of CRM1-dependent EGFP-nuclear export signal (EGFP-NES) export is increased. We show that the nuclear accumulation of the *Drosophila* Rel protein Dorsal requires CRM1. DNup88 binds to DNup214 and DCRM1 in vitro, and both proteins become mislocalized from the nuclear rim into the nucleus of *mbo* mutants. Overexpression of DNup88 is sufficient to relocalize DNup214 and CRM1 on the nuclear envelope and revert the mutant phenotypes. We propose that a major function of DNup88 is to anchor DNup214 and CRM1 on the nuclear envelope and thereby attenuate NES-mediated nuclear export.

Introduction

Transport across the nuclear envelope occurs through nuclear pore complexes (NPCs) and determines the temporal changes in nuclear concentration of gene regulators during cell growth, differentiation, and responses to environmental signals (Komeili and O'Shea, 2001; Lei and Silver, 2002). The regulation of nucleocytoplasmic transport relies to a large extent on the recognition of the cargo by soluble transport receptors (importins and exportins), which translocate the protein through the pore to the appropriate compartment (Mattaj and Englmeier, 1998). Target protein binding and release by importins and exportins is controlled by the asymmetric distribution of the two nucleotide states of the small GTPase, Ran, across the nuclear envelope. The nuclear guanine nucleotide exchange factor, RanGEF, is keeping Ran in its GTP-bound form in the nucleus, whereas the GTPase-activating protein RanGAP hydrolyzes it to RanGDP in the presence of the Ran-binding proteins RanBP1 and RanBP2 in the cytoplasm. Both importins and exportins interact with RanGTP through a conserved NH_2-terminal domain. Proteins with a leucine-rich nuclear export signal (NES) bind to the export receptor CRM1 only at high concentrations of RanGTP in the nucleus, and the trimeric complex translocates through the pore. Once in the cytoplasm, the complex is dissociated by the hydrolysis of RanGTP (Fornerod et al., 1997a; Weis, 2003). In vertebrate tissue culture systems, CRM1 is found in a complex with the nucleoporins CAN/Nup214 and Nup88 at the cytoplasmic face of the NPC (Kraemer et al., 1994; Fornerod et al., 1997b). The potential function of this interaction in CRM1-mediated export events remains unknown.

The function of individual nucleoporins is often difficult to study in vivo, as their genetic ablations may result in the rapid accumulation of several indirect transport defects. *Drosophila* development may provide an advantageous system for the analysis of nuclear pore components. Females often deposit into the egg a sufficient amount of maternal gene product, which supports embryonic development and gradually decreases during larval life, enabling the phenotypic analysis of

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Abbreviations used in this paper: *emb*, *embargoed*; LMB, leptomycin B; *mbo*, *members only*; NES, nuclear export signal; NPC, nuclear pore complex.

transport events at different concentrations of the gene product in zygotic null mutants.

Mutants in *members only* (*mbo*), encoding the *Drosophila* nucleoporin DNup88, fail to accumulate the Rel proteins Dif and Dorsal in the nucleus after bacterial infection and also fail to fully activate their immune response (Uv et al., 2000). The nuclear translocation of several other proteins and RNA export are not affected in *mbo* mutants (Uv et al., 2000). Here, we investigate the mechanism of Nup88 function in nucleocytoplasmic transport.

Results and discussion

DNup88 acts as an inhibitor of CRM1-mediated protein export

We wanted to determine whether the defect in nuclear accumulation of *Drosophila* Rel proteins is caused by a failure in protein import or by another unknown mechanism that involves DNup88. We generated transgenic flies expressing either a native EGFP, an NLS-EGFP (nuclear localization signal of the SV40 large T antigen; Kalderon et al., 1984), or an EGFP-NES (leucine-rich NES of protein kinase A inhibitor; Wen et al., 1995) reporter construct under the control of the inducible *hsp70* promoter. These reporters were crossed into *mbo* mutants, and wild-type and *mbo* larvae heat-induced in parallel expressed similar levels of protein for each construct (Fig. 1 A). EGFP was homogenously distributed throughout the cell in wild-type larval tissues, presumably due to its small size and consequent diffusion through the NPC (Fig. 1 A). NLS-EGFP, on the other hand, appeared predominantly nuclear (Fig. 1 A), indicating that NLS-EGFP was recognized as a substrate for importin-mediated nuclear import. We did not detect any significant differences in the localization of EGFP or NLS-EGFP between *mbo* and wild-type larvae (Fig. 1 A; Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200304046/DC1), and together with the previous analysis of *mbo* mutants, this argued against a function of DNup88 in general protein import (Uv et al., 2000).

EGFP-NES protein was detected both in the cytoplasm and the nucleus of wild-type larvae, and in some tissues, like the gut and the fat body, it appeared more concentrated in the nucleus. In addition, the nuclear accumulation of EGFP-NES in a given tissue was dependent on the larval stage because it appeared more nuclear in wild-type 2nd instar compared with 3rd instar larvae (Fig. 1 A; Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200304046/DC1). To test if the localization of this reporter reflects CRM1 activity, we analyzed its subcellular distribution in two *embargoed* (*emb*) mutants, which are defective in the fly CRM1 gene and die as 2nd instar larvae (Collier et al., 2000). Sequence analysis of the two *emb* alleles showed that emb^2 contains a T–G transition, changing a conserved methionine at position 413 in the ORF into an arginine. emb^3 contains a TAG stop codon at aa position 488, leading to the absence of any detectable CRM1 in both mutants (see Fig. 4 C). The distribution of EGFP-NES in emb^2 and emb^3 mutants was clearly different from the wild type. In both mutants, EGFP-NES accumulated in the nucleus, confirming that EGFP-NES is a substrate for CRM1-mediated export. (Fig. 1 A; Fig. S1). To measure nuclear accumulation of EGFP-NES in *mbo* mutants, we compared the ratios of nuclear/cytoplasmic EGFP-NES intensity in *mbo* and wild-type 3rd instar larvae. Surprisingly, EGFP-NES nuclear accumulation was decreased by 38% in *mbo* mutants, whereas the nuclear accumulation of the same reporter was increased by 40% in emb^3 mutants compared with wild-type 2nd instar larvae. This observation suggests that CRM1-mediated export is enhanced in *mbo* mutants and that DNup88 may act as an inhibitor of CRM1-mediated protein export.

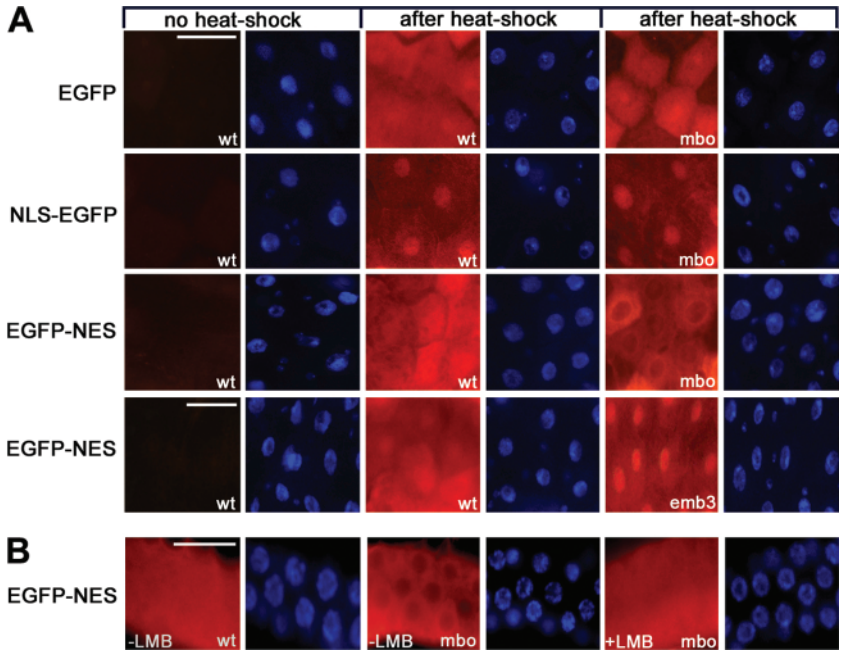

Figure 1. **DNup88 attenuates EGFP-NES export.** (A) EGFP, NLS-EGFP, and EGFP-NES in wild-type, *mbo*, and emb^3 larvae. All panels show gut cells stained for EGFP (red) or DAPI (blue). In each row, the left panel shows background levels in wild-type (wt) untreated animals. The middle panel shows EGFP reporters after heat shock in the indicated genotypes. Bar, 35 μm. (B) EGFP-NES localization in heat shock–induced salivary glands of wild-type (left) and *mbo* mutants (middle). Treatment with LMB (+LMB) can revert the EGFP-NES mislocalization in *mbo* mutants (right). Bar, 35 μm.

If the increased amount of cytoplasmic EGFP-NES observed in *mbo* mutants results from hyperactivated protein export, then treatment of the mutants with the CRM1 inhibitor leptomycin B (LMB) (Kudo et al., 1998) should revert this phenotype. In salivary glands of wild-type larvae, inducible EGFP-NES expression results in both nuclear and cytoplasmic localization of EGFP-NES (Fig. 1 B). Culture of wild-type glands with 10 nM LMB for 2 h after the induction of EGFP-NES expression did not have any detectable effect on EGFP amount or localization. However, in glands of *mbo* mutants, the same concentration of the drug was able to revert the decreased nuclear accumulation of the CRM1 cargo (Fig. 1 B). These results indicate that the exclusion of EGFP-NES from the nucleus in *mbo* mutants is due to an overactivation of the CRM1-mediated export pathway.

Dorsal is exported from the nucleus by CRM1

Is the defect in Rel protein nuclear accumulation and the lack of a fully active immune response in *mbo* mutants due to the increased levels of protein export? Previous extensive mutagenesis and functional analysis of Dorsal identified a short leucine-rich segment in the COOH end of the protein required for its cytoplasmic retention (Rushlow et al., 1989; Isoda et al., 1992; Bergmann et al., 1996). This segment is outside the Cactus-IκB–binding region of the protein and is strikingly similar to the CRM1-binding motif, suggesting that Dorsal nuclear concentration may be controlled by DCRM1. CRM1-mediated protein export has also been implicated in the control of Rel protein localization in mammalian cells (Carlotti et al., 2000). First, we tested whether the localization of the *Drosophila* Dorsal protein is sensitive to the CRM1 inhibitor LMB. *Drosophila* S2 cell lines expressing Dorsal fused to EGFP or an EGFP protein carrying the PKI export signal were generated, and both fusion proteins were found in the nucleus and the cytoplasm under normal culture conditions. LMB treatment of the cells resulted in stronger nuclear accumulation of both reporters, suggesting that their cytoplasmic localization requires CRM1 for export (Fig. 2 A).

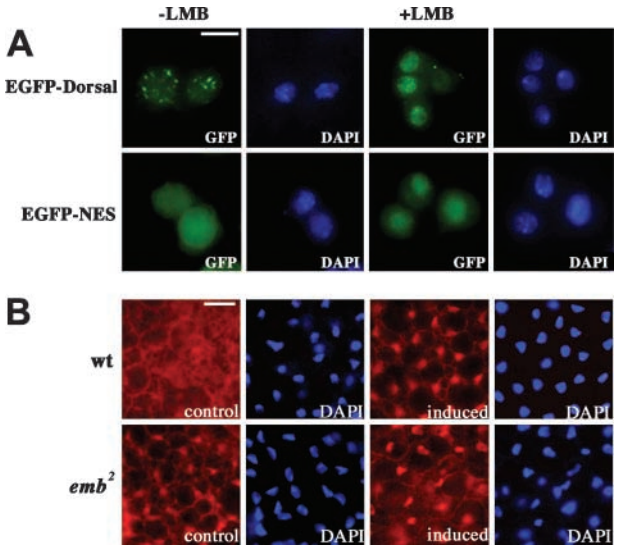

Figure 2. **Nuclear export of Dorsal is mediated by DCRM1.** (A) LMB inhibits nuclear export of EGFP-Dorsal and EGFP-NES in S2 cells. The fusion proteins are visualized by GFP, and nuclei are visualized by DAPI. LMB treatment enhanced the nuclear localization of both proteins. Bar, 2.5 μm. (B) Fat bodies from wild-type (wt) and *emb*^{2} mutant larvae stained for Dorsal (red) before (control) and after (induced) bacterial infection. Nuclei are shown by DAPI. Bar, 35 μm.

To explore whether mislocalization of Dorsal upon LMB treatment also reflects a requirement for CRM1 in the animal, we analyzed its subcellular distribution in DCRM1 (*emb*) mutants. In wild-type untreated larvae, Dorsal is predominantly cytoplasmic, but becomes translocated into the nucleus after infection with bacteria. In contrast, in *emb*^{2} mutants, Dorsal is predominantly nuclear even in untreated animals, and its nuclear accumulation is only marginally increased after bacterial infection (Fig. 2 B). A similar phenotype was also observed for the other *Drosophila* Rel homologue, Dif (unpublished data). The nuclear accumulation of Dorsal and Dif in unchallenged *emb* mutant larvae indicates that Rel proteins are continuously shuttling in and out of the nucleus and that their nuclear export requires DCRM1.

DNup88 anchors DNup214 at the nuclear envelope

How, then, does DNup88 affect the levels of CRM1-mediated nuclear export? Human Nup88 is localized at the cytoplasmic side of the NPC (Cronshaw et al., 2002), where it binds to another nucleoporin, CAN/Nup214 (Fornerod et al., 1997b). The two nucleoporins are in complex with CRM1 because anti-Nup214 antibodies can coprecipitate Nup88 and CRM1 (Fornerod et al., 1997b). First, we tested whether the *Drosophila* Nup214 homologue binds to Nup88 directly by GST pull-down experiments and the yeast two-hybrid system (Belgareh et al., 1998). As expected from previous experiments in other systems, the two proteins were found to bind directly to each other in both assays (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200304046/DC1).

To assess the fate of Nup214 in *mbo* larvae, we generated a polyclonal antiserum against an NH_2-terminal fragment of the protein. On Western blot of Schneider cell extracts, the antiserum recognized a band of ~210 kD, whose intensity became severely reduced after treatment of the cells with double-stranded RNA deriving from the DNup214 cDNA (Fig. 3 C). Staining of wild-type larvae with the anti-DNup214 antiserum and analysis by confocal microscopy showed that DNup214 is localized at the nuclear rim (Fig. 3 A). This localization was disturbed in *mbo* mutants, DNup214 was found predominantly inside the nucleus, and later when the mutants were arrested at a prolonged 3rd instar stage (Uv et al., 2000), the signal became weaker, suggesting that DNup214 became degraded. The mislocalization of DNup214 in *mbo* mutants does not reflect a general breakdown in NPC structure because overexpression of DNup88 in *mbo* mutants restored the localization of DNup214 at the nuclear envelope, and analysis of *mbo* mutants with an antibody recognizing several phenylalanine-glycine repeat–containing nucleoporins (mAb414) or a serum raised against the nuclear basket component Nup153 did not reveal any alterations in the amount or

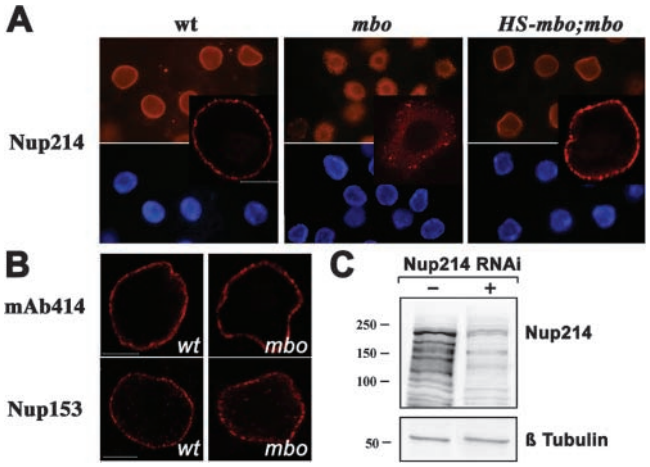

Figure 3. **DNup214 is mislocalized in *mbo* mutants.** (A) DNup214 localization in fat body cells of wild-type, *mbo* mutants, and *mbo* larvae overexpressing DNup88. The top row shows DNup214 staining (red), and the bottom row shows DAPI. Confocal sections are shown in the insets. (B) Confocal sections of fat body cells from wild-type and *mbo* larvae stained with anti-mAb414 and anti-Nup153. Bars (A and B), 2 μm. (C) Western blot of S2 cells before (−) and after (+) Nup214 RNAi probed with the anti-DNup214 antibody. β-Tubulin is a loading control.

distribution of these proteins (Fig. 3 B). These results indicate that DNup88 binds to DNup214 and is selectively required for its localization at the nuclear envelope.

CRM1 is mislocalized in mbo mutants

CRM1-mediated protein export involves binding of the exporter to the cargo in the nucleus in the presence of RanGTP, translocation through the NPC, and cargo release at the cytoplasmic face of the pore through RanGAP-mediated hydrolysis of RanGTP to RanGDP (Floer and Blobel, 1999; Komeili and O'Shea, 2001). In wild-type tissues, high levels of DCRM1 are found along the nuclear rim, whereas lower levels are found in the nucleus (Fig. 4 A). In *mbo* mutants, the DCRM1 staining at the nuclear rim was absent, and the protein appeared exclusively localized in the nucleus (Fig. 4 A). As in wild-type tissues, DCRM1 was excluded from a central area inside the nucleus. Overexpression of DNup88 in *mbo* mutants rescued the mutant phenotypes and restored the localization of DCRM1 on the nuclear rim (Fig. 4 A). To further examine whether the *mbo* phenotype results from mislocalization of CRM1 rather than an alteration in the amount of DCRM1 protein, we analyzed extracts of wild-type and *mbo* mutants by Western blot. The levels of DCRM1 were the same in larvae of the two genotypes (Fig. 4 C), arguing that DNup88 may control NES-mediated nuclear export by sequestering CRM1 at the nuclear pore. The requirement of DNup88 for retention of CRM1 on the nuclear rim may be indirect because DNup214 is also mislocalized in *mbo* mutants. Therefore, we tested whether DNup88 may directly interact with CRM1 by GST pull-down experiments and in the yeast two-hybrid system. In both assays, the NH_2-terminal region of DCRM1 (encompassing aa 1–561) can bind to the COOH-terminal part (aa 504–702) of DNup88 (Fig. 5). Using the same assays, we tested whether the DNup88 fragments could bind to the fly Importin-β homologue, Ketel (Lippai et al., 2000). No interactions were detected between DNup88 and Ketel (five different fragments or the full-length protein), arguing that the DNup88–CRM1 interaction is specific (unpublished data). Thus, DNup88 alone may bind to a fraction of CRM1 and anchor it at the nuclear membrane, or it may act in a complex with Nup214 to facilitate CRM1 retention.

Is the DNup88 requirement selective for CRM1, or does it also bind to other transport factors and influence their activity? Importin-β and several other transport factors have been found enriched along the nuclear envelope (Gorlich et al., 1995, 1997). We analyzed the localization of DImportin-β (Lippai et al., 2000) and DNXF-1, an mRNA exportin (Herold et al., 2000; Wilkie et al., 2001), in *mbo* mutants and their wild-type siblings. The localization of both proteins at the nuclear envelope and in the nucleus was indistinguishable between wild-type and *mbo* larvae, whereas CRM1 was clearly mislocalized in *mbo* mutants reared and stained in parallel (Fig. 4 B). A major fraction of RanGAP,

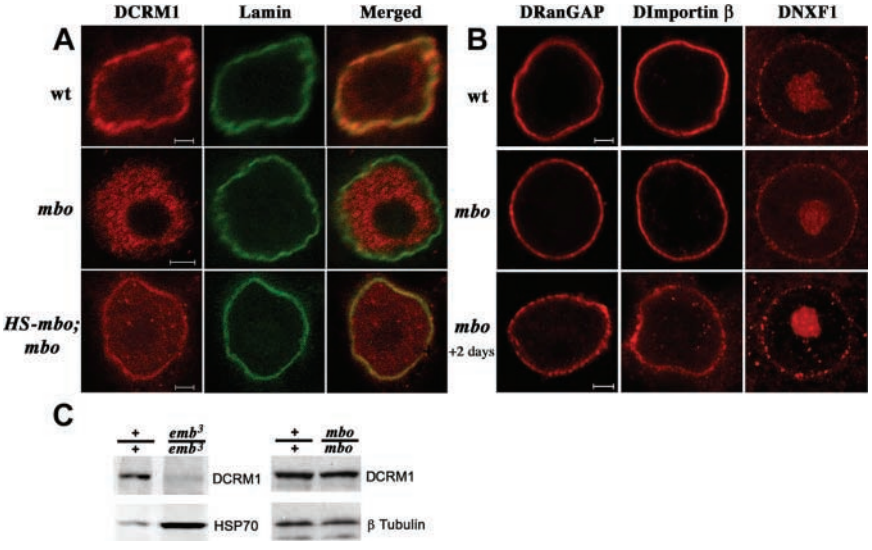

Figure 4. **DCRM1 is mislocalized in *mbo* mutants.** (A) Confocal analysis of DCRM1 localization in fat body cells of wild-type, *mbo* mutants, and *mbo* larvae overexpressing DNup88. In each row, the left panel shows DCRM1 (red) and the middle panel shows lamin (green). To the right is the overlay. (B) Confocal sections of fat body cells from wild-type, early and late *mbo* mutant 3rd instar larvae stained for DRanGAP, DImportin-β, and DNXF-1. Localization and levels of these proteins are not changed in early *mbo* 3rd instar larvae (4 d after egg laying). In 6-d-old mutants, the nuclear rim staining appears reduced and punctuated. Bars (A and B), 2 μm. (C) Western blot of larval extracts from wild-type, emb^3, and *mbo* mutants probed for DCRM1. β-Tubulin and HSP70 are loading controls.

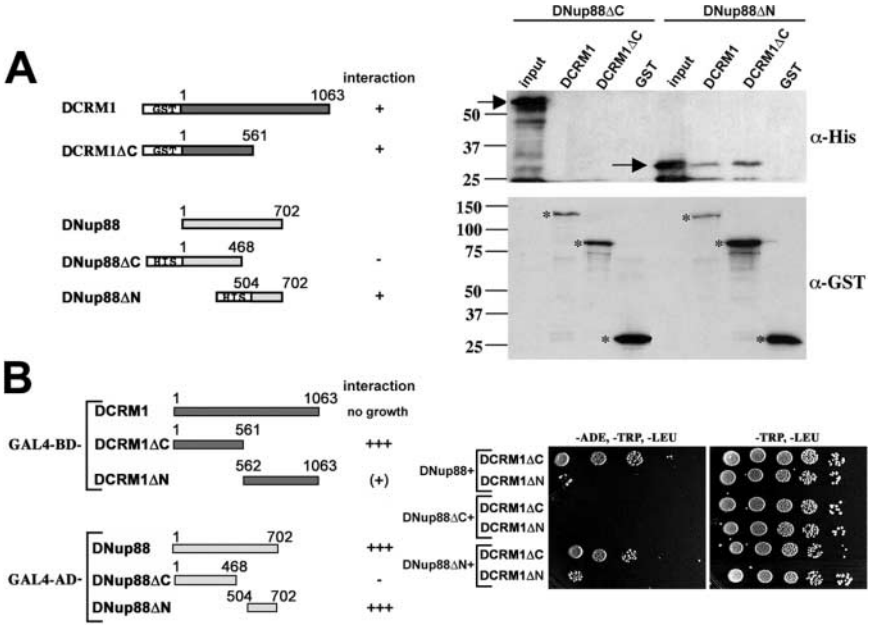

Figure 5. **DNup88 interacts with DCRM1 in vitro.** (A) GST-fusions of DCRM1 and His_6 fusions of DNup88 were used in a GST pull-down assay. The binding is analyzed by Western blot with α-His_6 antibodies (top right). Arrows point to the products of the DNup88 deletions. A quantitation of the same blot with GST antibody (asterisk) is shown at the bottom. (B) DCRM1 interacts with DNup88 in the yeast two-hybrid system. 10-fold serial dilutions of strain PJ69-4A expressing the indicated protein combinations were spotted on synthetic complete medium lacking either adenine, tryptophan, and leucine (−ADE, −TRP, −LEU) or tryptophan and leucine (−TRP, −LEU). Growth in the absence of ADE indicates protein–protein interaction.

the RanGTPase-activating protein responsible for the dissociation of exportins from their cargoes in the cytoplasm, is bound to the cytoplasmic filaments through interactions with RanBP2/Nup358 (Mahajan et al., 1997; Matunis et al., 1998). As for Importin-β and NXF-1, the localization of RanGAP (Merrill et al., 1999) was not changed in *mbo* mutants (Fig. 4 B). We also analyzed *mbo* larvae at a later stage, as the mutants arrest for about 1 wk at the 3rd instar larval stage before they die. In these animals RanGAP, Importin-β, and NXF-1 were still enriched at the nuclear rim, but the staining appeared punctuated and weaker (Fig. 4 B). Because the defect in CRM1 localization appears earlier, and NLS-EGFP import or mRNA export are not affected in these mutants, we conclude that the late changes on RanGAP, Importin-β, and NXF-1 localization may be a secondary effect after the mislocalization of CRM1 and DNup214.

The varying levels of DNup88 in different cell types during *Drosophila* development and *mbo* mutants show several cell type–specific developmental defects and fail to mount an effective immune response (Uv et al., 2000). The nuclear concentration of the EGFP-NES also shows a consistent spatial and temporal variation during larval development. Western blot analysis of the amounts of Nup88 in the gut of 2nd and 3rd instar larvae revealed a correlation between the amount of Nup88 and the effectiveness of EGFP-NES export during development (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200304046/DC1). We propose that DNup88 functions at the NPC to tether CRM1 and to attenuate its recycling back to the nucleus for another round of export. However, overexpression of Nup88 in wild-type larvae did not generate any obvious phenotype, suggesting that its CRM1-binding ability may be modulated by another factor or by posttranslational modification (Uv et al., 2000). To assess the proposed inhibitory role of Nup88 in the export of other endogenous CRM1 cargoes, we performed a genetic interaction experiment using *mbo* and the two *emb* alleles. *emb* larvae die at the 2nd instar stage (~70 h after egg laying). Removal of one copy of the *mbo* gene from *emb* homozygous mutants prolonged the life span of 20% of the individuals to the 3rd instar larval stage (~120 h after egg laying; Table S1, available at http://www.jcb.org/cgi/content/full/jcb.200304046/DC1). This prolonged survival is accompanied by eversion of the anterior spiracles, a morphological characteristic of progression through larval development, arguing that DNup88 is a general NES export attenuator. The surprising function of DNup88 in anchoring CRM1 at the nuclear envelope and down-regulating the levels of NES protein export suggests an additional level of control in the activation and duration of cellular responses to signaling.

Materials and methods

Drosophila strains

HS-EGFP, HS-NLS-EGFP, and HS-EGFP-NES fly strains were generated by P-element–mediated transformation (Spradling, 1986). A description on the cloning schemes for the different constructs is available online at http://www.jcb.org/cgi/content/full/jcb.200304046/DC1.

Cell lines

S2 cells were transfected as described previously (Ausubel et al., 1993). Stable transformant lines were selected for 4–5 wk in supplemented cell medium containing 300 μg/ml hygromycin B (Invitrogen).

LMB treatment

Dorsal-EGFP and EGFP-NES expression was induced by addition of 0.3 mM $CuSO_4$ to the culture medium for 13 h. LMB (Sigma-Aldrich) was used at a final concentration of 10 nM for 2 h. Salivary glands were dissected and incubated at RT in Ringer's solution with or without 10 nM LMB. After 2 h, the glands were fixed and stained.

Immunostaining of larvae and Western blots

Larvae were heat induced for 45 min at 37°C and analyzed after 3 h. Antibody stainings of larval tissues were performed as described previously (Patel, 1994). Dorsal translocation experiments were done as described previously (Uv et al., 2000). Primary antibodies were used at dilutions as follows: anti-DCRM1 (directed against aa 1–306 of DCRM1) 1:1,000; anti-DNup214 (directed against aa 160–620 of DNup214) 1:10,000; anti-NXF1 (Herold et al., 2001) 1:500; anti-Nup153 (Cordes et al., 1993) 1:300; anti-mAb414 (BabCO) 1:5,000; anti-GFP (Molecular Probes, Inc.) 1:1,000; anti-Dorsal (Gillespie and Wasserman, 1994) 1:1,000; anti-Lamin Dm1 (ADL 84; Stuurman et al., 1995) 1:500; anti-Ketel (Lippai et al., 2000)

1:1,000; and anti-DRanGAP (Merrill et al., 1999) 1:1,000. Stainings were viewed using a fluorescent microscope (Carl Zeiss MicroImaging, Inc.). Openlab v3.1.4 software (Improvision) was used for image acquisition. Laser-scanning microscopes (from Leica or Carl Zeiss MicroImaging, Inc.) were used for confocal imaging. Acquired images were processed with LSM510 software (Carl Zeiss MicroImaging, Inc.). For Western blots, antibody dilutions were used as follows: anti-DNup214 1:1,000; anti-β-tubulin (Amersham Biosciences) 1:1,000; and anti-Hsp70 (Sigma-Aldrich) 1:1,000.

Binding assays

All constructs were generated by PCR amplification from cDNA clones. For the pull-down assay, fragments were cloned either in pGEX-5X or in pRSET vectors. Protein expression and binding reactions were performed as described previously (Uv et al., 1994). A protocol is available online at http://www.jcb.org/cgi/content/full/jcb.200304046/DC1.

Online supplemental material

Supplemental information contains a Materials and methods section and corresponding references, figures, and a table. Fig. S1 shows quantitation of the ratio of nuclear/cytoplasmic NLS-EGFP and EGFP-NES in wild-type and *mbo* mutants. Western blot illustrates the amounts of DNup88 in different wild-type larval stages. Fig. S2 shows DNup88–DNup214 binding in an in vitro pull-down assay and the yeast two-hybrid system. Table S1 describes genetic interaction of *emb* and *mbo* mutants. Online supplemental material available at http://www.jcb.org/cgi/content/full/jcb.200304046/DC1.

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