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Oxidative Stress Inhibits Nuclear Protein Export by Multiple Mechanisms That Target FG Nucleoporins and Crm1

Noah Crampton,* Mohamed Kodiha,* Sanhita Shrivastava, Rehan Umar, and Ursula Stochaj

Department of Physiology, McGill University, Montreal, QC, Canada H3G 1Y6

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Nuclear transport of macromolecules is regulated by the physiological state of the cell and thus sensitive to stress. To define the molecular mechanisms that control nuclear export upon stress, cells were exposed to nonlethal concentrations of the oxidant diethyl maleate (DEM). These stress conditions inhibited chromosome region maintenance-1 (Crm1)-dependent nuclear export and increased the association between Crm1 and Ran. In addition, we identified several repeat-containing nucleoporins implicated in nuclear export as targets of oxidative stress. As such, DEM treatment reduced Nup358 levels at the nuclear envelope and redistributed Nup98. Furthermore, oxidative stress led to an increase in the apparent molecular masses of Nup98, Nup214, and Nup62. Incubation with phosphatase or *β*-*N*-acetyl-hexosaminidase showed that oxidative stress caused the phosphorylation of Nup98, Nup62, and Nup214 as well as *O*-linked *N*-acetylglucosamine modification of Nup62 and Nup214. These oxidant-induced changes in nucleoporin modification correlated first with the increased binding of Nup62 to the exporter Crm1 and second with the reduced interaction of Nup62 with other FxFG-containing nucleoporins. Together, oxidative stress up-regulated the binding of Crm1 to Ran and affected multiple repeat-containing nucleoporins by changing their localization, phosphorylation, *O*-glycosylation, or interaction with other transport components. We propose that the combination of these events contributes to the stress-dependent regulation of Crm1-mediated protein export.

INTRODUCTION

Oxidants play a key role in cell physiology under normal and disease conditions. For example, oxidative stress generated by a shift in the cellular redox potential is a critical factor in diabetes, many neurodegenerative disorders, and ischemia of the heart and brain (Dröge, 2002; Filippo *et al*., 2006; Valko *et al*., 2007; Doyle *et al*., 2008; Forbes *et al*., 2008). At the cellular level, an excess of oxidants will trigger a response that may affect intracellular trafficking of macromolecules, including protein transport in and out of the nucleus. Severe oxidative stress can lead to apoptosis, whereas cells survive milder forms of oxidant exposure (Kodiha *et al*., 2004; 2008a). Such a milder form of oxidative stress can be induced with diethyl maleate (DEM), a compound that depletes glutathione. DEM inhibits classical nuclear import in several model organisms (Stochaj *et al*., 2000; Kodiha *et al*., 2008a). In HeLa cells, DEM alters the intracellular distribution of importin-*α* and CAS, but has little effect on the Ran GTPase concentration gradient across the nuclear envelope (Kodiha *et al*., 2008a). It is not known whether DEM-induced stress affects nuclear protein export, and potential oxidant-sensitive targets of the export apparatus remain to be identified.

Several pathways participate in the export of proteins from the nucleus to the cytoplasm; common to all of these pathways is the interaction between nucleoporins and soluble transport factors (Madrid and Weis, 2005; Terry *et al*., 2007). One of the best-characterized routes of nuclear export is mediated by the importin-*β* family member chromosome region maintenance-1 (Crm1), a carrier that recognizes nuclear export signals (NES) that are rich in hydrophobic residues (Hutten and Kehlenbach, 2007). Crm1-dependent protein export is initiated by the generation of a trimeric export complex in the nucleus that contains Crm1, RanGTP, and the NES-containing cargo. After the translocation across the nuclear pore complex (NPC), nuclear export is terminated by the disassembly of export complexes on the cytoplasmic side of the NPC, a process that requires GTP hydrolysis on Ran.

In addition to soluble components, Crm1-mediated export relies on several nucleoporins (Nups) and the nucleoporin-associated RanGAP1 (Madrid and Weis, 2005; Hutten and Kehlenbach, 2007; Terry *et al*., 2007). One-third of the ~30 nucleoporins contain repeats; they are particularly important for transport as the repeats provide docking sites for carriers during their movement across the NPC. Among these nucleoporins, Nup62, Nup214, Nup358, and Nup153 with FxFG repeats and Nup98 with GLFG repeats have well-established roles in nuclear trafficking. At the NPC, specific nucleoporins associate to generate functional mod-

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* These authors contributed equally to this work.

Address correspondence to: Ursula Stochaj (ursula.stochaj@mcgill.ca).

Abbreviations used: BSA, bovine serum albumin; Crm1, chromosome region maintenance-1; DAPI, 4′6-diamidino-2-phenylindole; DSP, dithiobis(succinimidylpropionate); NE, nuclear envelope; NES, nuclear export signal; NPC, nuclear pore complex.

ules (Schwartz, 2005). However, the localization within the NPC is not necessarily static, and nucleoporins that are mobile or contain flexible domains are essential for nuclear transport (Griffis *et al*., 2002; reviewed in Tran and Wente, 2006). As such, interactions between flexible nucleoporin domains or segments containing FxFG repeats are believed to support the translocation of cargo across the nuclear pore (Paulillo *et al*., 2005; Stochaj *et al*., 2006). Besides functioning at the NPC, several nucleoporins can also be detected in other cellular compartments, where they may have additional roles (Griffis *et al*., 2002; Joseph *et al*., 2004, 2008; Andres-Hernando *et al*., 2008). Interestingly, a recent publication demonstrated aging-dependent changes in the composition of NPCs; this was linked to an increased diffusion channel of the nuclear pore and possibly oxidative damage of nucleoporins (D'Angelo *et al*., 2009). The impact of oxidative stress on active nuclear transport, however, was not analyzed in these studies.

Nucleoporins that participate in Crm1-mediated nuclear export are located in different modules of the NPC. Nup62 is present at the nuclear and cytoplasmic sides of the central channel, but Nup214 and Nup88 are at the base of cytoplasmic NPC filaments, where they provide a platform for the disassembly of export complexes (Fornerod *et al*., 1997; Hutten and Kehlenbach, 2006; reviewed in Hutten and Kehlenbach, 2007). Nup358, at the tip of cytoplasmic filaments, may have multiple functions in Crm1-dependent export. Thus, Nup358 serves as a binding partner for sumoylated RanGAP1, which stimulates the Ran GTPase activity and thereby facilitates the dissociation of Crm1 export complexes. Nup358 was also proposed to support Nup214 and Nup88 in generating a stage for the dissociation of export complexes (Bernad *et al*., 2004). Other studies, however, suggest that Nup358 is not critical for the export of Crm1-containing complexes in vitro (Hutten *et al*., 2008). Regardless of the possible role of Nup358 in the disassembly of export complexes, it is still feasible that Nup358 enhances nuclear export by promoting the efficient recycling of cargo-free Crm1 (Hutten and Kehlenbach, 2007). The role of Nup62 and Nup214 in Crm1-dependent export has been substantiated by several in vitro studies, which demonstrated the binding of Crm1 to Nup62 and Nup214; these interactions require RanGTP (Kehlenbach *et al*., 1999; Lindsay *et al*., 2001; Hutten and Kehlenbach, 2006). At present, it is not clear whether in growing cells any of these processes are regulated by stress.

Nup98, the only nucleoporin with GLFG repeats in mammalian cells, plays an important role in nuclear export of macromolecules. Nup98 participates in Crm1-dependent export of the human immunodeficiency virus protein Rev and of multiple types of RNA (Powers *et al*., 1997; Zolothukin and Felber, 1999; Bachi *et al*., 2000; Griffis *et al*., 2002). Nup98 is mobile and can be detected in both the cytoplasm and the nucleus; within the nucleus Nup98 associates with unique compartments, called GLFG bodies (Griffis *et al*., 2003). It has not been analyzed whether Nup98 is sensitive to oxidant exposure.

In this contribution, we determined in growing cells the effects of oxidant exposure on nuclear protein export. Crm1 and nucleoporins Nup358, Nup214, Nup62, and Nup98 were identified as targets of oxidative stress. We demonstrate that stress altered the binding of Crm1 to several nucleoporins and Ran, as well as nucleoporin phosphorylation and *N*-acetylglucosamine (GlcNAc) modification. Moreover, the interactions of Crm1 with nucleoporins and among nucleoporins changed in response to oxidant exposure. These stress-dependent changes correlated with the inhibition of protein export from the nucleus.

MATERIALS AND METHODS

Growth of HeLa Cells and Stress Exposure

HeLa cells were grown, transiently transfected, and treated with 2 mM DEM for 4 h as described previously (Kodiha *et al*., 2008a). A plasmid encoding green fluorescent protein (GFP)-tagged Nup98 was kindly provided by Dr. M. Powers (Griffis *et al*., 2002).

Indirect Immunofluorescence

Immunofluorescent staining followed published procedures (Kodiha *et al*., 2008a,b,c, 2009). Antibodies against RanGAP1 were a generous gift of F. Melchior (ZMBH, Heidelberg, Germany), and antibodies against Crm1 kindly provided by G. Grosveld (St. Jude Children's Research Hospital, Memphis, TN) were used for indirect immunofluorescence. Nup214 antibodies (A01) were from Santa Cruz Biotechnology (Santa Cruz, CA), Abnova (Taipei, Taiwan), or Bethyl Laboratories (Montgomery, TX); all other antibodies were purchased from Santa Cruz Biotechnology. Procedures for immunofluorescent staining and digitonin treatment of fixed cells to visualize Crm1 on the cytoplasmic side of the nuclear envelope have been published previously (Kodiha *et al*., 2008b). The detection of Nup98 was described in detail (options 2 and 4 in Kodiha *et al*., 2009). Images were acquired for sections <0.7 μm with an LSM510 confocal microscope (Carl Zeiss International, Toronto, ON, Canada) and a 63× objective (numerical aperture 1.4). For each condition, fluorescence signals were measured for at least 50 cells (Kodiha *et al*., 2008c). Nuclear and cytoplasmic or nuclear envelope fluorescence was quantified with the MetaXpress multiwavelength cell scoring or multiwavelength translocation module (Kodiha *et al*., 2008c).

Western Blotting

Procedures for Western blotting and quantification of enhanced chemiluminescence (ECL) signals were described recently (Quan *et al*., 2006). For antigens that migrated close to the heavy or light chains of immunoglobulin G (IgG) TrueBlot horseradish peroxidase-conjugated secondary antibodies (eBioscience, San Diego, CA) were used as recommended by the manufacturer.

Phosphatase Treatment and Removal of O-GlcNAc Moieties

Control or DEM-treated HeLa cells were boiled in 40 mM Tris-HCl, pH 8.0, 1% SDS, 50 mM dithiothreitol, 1 mM NaN_3, 2.5 mM NaF, and a cocktail of protease inhibitors (aprotinin, pepstatin, and leupeptin, each at 1 μg/ml) for 10 min. DNA was sheared by vortexing with glass beads during the incubation period, and insoluble material was removed by centrifugation (5 min at 13,000 rpm; microfuge). Supernatants were diluted 1:5 in water and precipitated with 10% trichloroacetic acid for 20 min on ice. Sediments obtained after 1-min centrifugation (11,000 rpm) were resuspended in 100 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, and 0.025% Triton X-100 and incubated with 200 U/ml calf intestinal phosphatase (CIP; New England Biolabs, Ipswich, MA) for 1 h at 37°C. Controls were incubated without CIP. Treatment with β-*N*-acetyl-hexosaminidase (here referred to as hexosaminidase) was carried out essentially as published previously (Zachara, 2008). In brief, samples were boiled 5 min in 1% SDS, vortexed with glass beads, and centrifuged 5 min at 13,000 rpm (microfuge). Supernatants were diluted 1:2 into 100 mM sodium citrate, pH 4.5, containing 8% Triton X-100 and twofold concentrated protease inhibitors (Roche Diagnostics, Laval, QC, Canada). Samples were incubated with or without 100 U/ml hexosaminidase for 20 h at 37°C and then analyzed by Western blotting.

Protein Cross-Linking and Immunoprecipitation

Control and DEM-treated HeLa cells were washed with prewarmed phosphate-buffered saline (PBS) and incubated with 200 μM of the cleavable cross-linker dithiobis[succinimidylpropionate] (DSP; Pierce Chemical, Rockford, IL) for 15 min at 37°C. Samples were washed in PBS and stored at −70°C until use. All subsequent incubations were at 4°C. Proteins were solubilized in PBS/10 mM Tris-HCl, pH 7.4, containing 0.1% SDS, 1% NP-40, 1 mM NaN_3, 2.5 mM NaF, 20 mM β-glycerophosphate, and protease inhibitors (Roche Diagnostics) and vortexed with glass beads. Supernatants (5 min at 13,000 rpm; microfuge) were preadsorbed for 30 min with protein G-Sepharose (Santa Cruz Biotechnology) and cleared by centrifugation. Supernatants were incubated for 1 h with antibodies, then protein G-Sepharose was added, and samples were incubated overnight. Resins were washed three times with PBS/1 mM NaN_3, bound material was released by incubation with twofold concentrated gel sample buffer for 15 min at 95°C and analyzed by Western blotting. Upon this treatment, Nup214 migrated at a molecular mass of ~150 kDa. This was observed with three different antibodies against Nup214. Note

that for all of the coimmunoprecipitations analyzed, the same samples were probed with both antibodies that recognize the interacting components.

Immunoprecipitation under Denaturing Conditions

Denatured proteins were immunoprecipitated with antibodies specific for Nup62 or Nup214 (catalog nos. sc-1915 and sc-26055, Santa Cruz Biotechnology; catalog no. 1-97, Abnova), Ran (catalog no. sc-1156, Santa Cruz Biotechnology), and mab414 (BAbCO, Richmond, CA) essentially as described previously (Kodiha *et al*., 2004).

Exit of Crm1 from the Nucleus

Nuclear exit of endogenous Crm1 was measured for control and DEM-treated cells as described previously (Kodiha *et al*., 2008b). In brief, after a 4-h incubation with ethanol or DEM, cells were digitonin-permeabilized and incubated with transport buffer, an energy-regenerating system, 1 mM guanosine triphosphate, and 50 μg/ml RanQ69L for the times indicated. Cells were fixed and Crm1 was detected by indirect immunofluorescence as described above. Pixel intensities for Crm1 located at the nuclear envelope or in the nuclear interior were measured following published procedures (Kodiha *et al*., 2008b).

Statistics

Results are shown as means ± SD. Student's *t* test (two-tailed) for unpaired samples was used to identify significant differences, which are indicated in the figures with *$p < 0.05$ or **$p < 0.01$. For multiple comparisons with one-way analysis of variance (ANOVA), *$p < 0.05$ specifies significant differences.

RESULTS

Oxidative Stress Inhibits Crm1-dependent Nuclear Export

To measure the effect of DEM treatment on the intracellular localization of Crm1-cargo, conditions were chosen for which most of the cells remained viable (Kodiha *et al*., 2008a). We generated monomeric citrine (mCit)-NES as a fluorescent reporter; this protein contains mCit fused to the leucine-rich NES of protein kinase A inhibitor. Unlike most endogenous cargoes, mCit-NES has the advantage of not containing nuclear or cytoplasmic retention signals or other regulatory sequence elements that control its localization. With a molecular mass of ~28 kDa, mCit-NES can diffuse across NPCs and is therefore a valuable tool to examine the activity of the nuclear protein export apparatus. In transiently transfected HeLa cells with active nuclear export, mCit-NES was predominantly cytoplasmic (Figure 1A). However, upon incubation with DEM, mCit-NES redistributed within the cell, and levels of the reporter protein increased significantly in the nucleus. Previous studies showed that under the conditions used here, nuclear envelopes remain intact (Sánchez *et al*., 2005; Kodiha *et al*., 2008a). Thus, DEM treatment inhibited mCit-NES nuclear export, rather than damaging the barrier function of the nuclear membranes. In control experiments, the reporter protein mCit distributed throughout the nucleus and cytoplasm under all conditions, demonstrating that changes in mCit-NES localization were not mediated by the fluorescent tag (data not shown).

To obtain quantitative information about the stress-induced changes in nuclear export, fluorescence signals in nuclear and cytoplasmic compartments were measured. The nuclear/cytoplasmic ratios of fluorescence intensities were calculated (see *Materials and Methods* for details), and the ratio obtained for control cells was defined as 1. Compared with controls, stressed samples showed a significant increase in the nuclear/cytoplasmic fluorescence of mCit-NES, which is due to nuclear export inhibition (Figure 1A).

In a parallel set of experiments, we measured the effect of leptomycin B (LMB) on the distribution of mCit-NES (Figure 1B). Like oxidant treatment, LMB efficiently redistributed mCit-NES throughout the cell; quantification of the redistribution revealed that LMB had a more pronounced effect than DEM (Figure 1B). This was to be expected, because

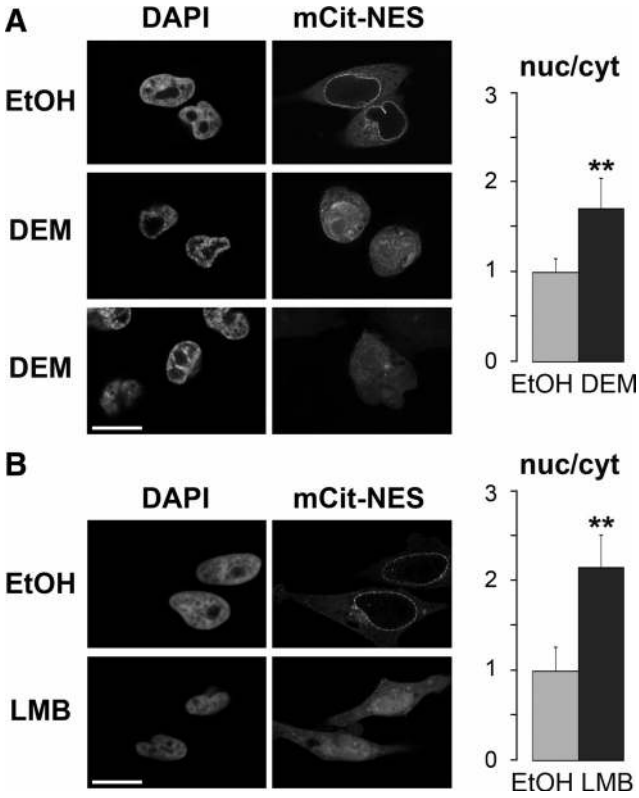

Figure 1. Oxidative stress inhibits Crm1-mediated nuclear export. (A) HeLa cells transiently synthesizing the reporter protein mCit-NES were incubated in the solvent ethanol or 2 mM DEM for 4 h at 37°C as indicated. Green fluorescence of the reporter protein and 4′,6-diamidino-2-phenylindole (DAPI) staining of the DNA are shown for control and DEM-treated samples. Two different samples are shown for stressed cells. Images were acquired by confocal microscopy, *intranuclear* and cytoplasmic fluorescence intensities were quantified for at least 50 cells for each condition (see *Materials and Methods* for details). The ratio obtained for nuclear/cytoplasmic fluorescence (nuc/cyt) in control cells was defined as 1. The increase in nuclear/cytoplasmic fluorescence in stressed cells indicates the inhibition of nuclear export. (B) LMB prevents nuclear export of mCit-NES. Transiently transfected cells were incubated with ethanol or 100 nM LMB dissolved in ethanol for 4 h at 37°C. Cells were fixed, stained with DAPI, and nuc/cyt ratios were quantified for the mCit-NES distribution as described for part A. Student's *t* test, **$p < 0.01$. Bar, 20 μm.

under the conditions used in our studies LMB will prevent binding of cargo to the carrier Crm1 (Bernard *et al*., 2004).

Oxidative Stress Reduces Significantly Nup358 Levels at the Nuclear Envelope but Increases the Amounts of Nup214, Nup62, and Crm1

To begin to define the stress-induced changes at the nuclear envelope that may contribute to nuclear export inhibition, we monitored whether DEM altered the association of nucleoporins or Crm1 with the nuclear envelope (NE). To achieve this, we recently developed procedures for quantitative indirect immunofluorescence and the measurement of fluorescence at the NE (Kodiha *et al*., 2008c). Staining with antibodies against Nup358 revealed a drastic reduction of Nup358 at the NE of stressed cells (Figure 2A). By contrast, signals at the NE were increased for Crm1, Nup214, and Nup62, and there was no significant change for RanGAP1. To our knowledge, this is the first quantitative image-based

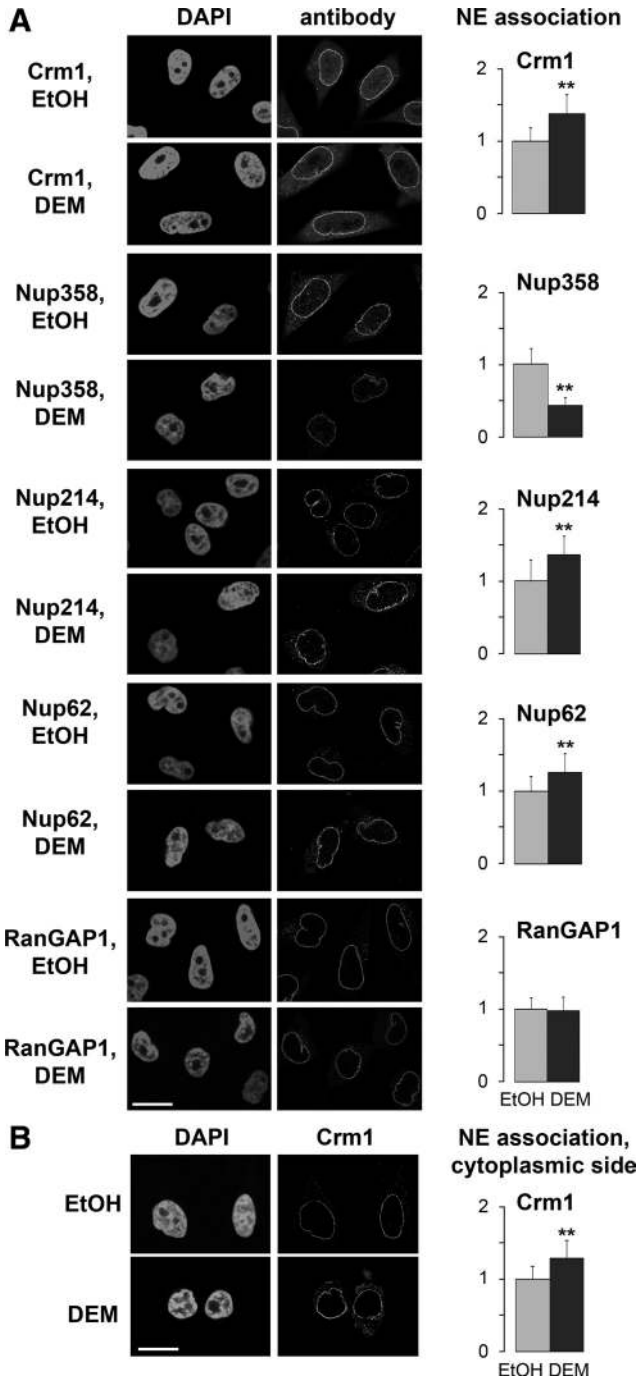

Figure 2. Oxidative stress changes the levels of Crm1 and multiple nucleoporins at the nuclear envelope. (A) The association of Crm1, Nup358, Nup214, Nup62, and RanGAP1 with nuclear envelopes was quantified under control and stress conditions by using the multiwavelength translocation module. A drastic reduction in nuclear envelope fluorescence is observed for Nup358. (B) Stress-induced changes in Crm1 associated with the cytoplasmic side of the NE. Control and DEM-treated cells were fixed and semipermeabilized with digitonin (Kodiha *et al.*, 2008b) to detect Crm1 at the cytoplasmic face of the NE. Bar, 20 μm.

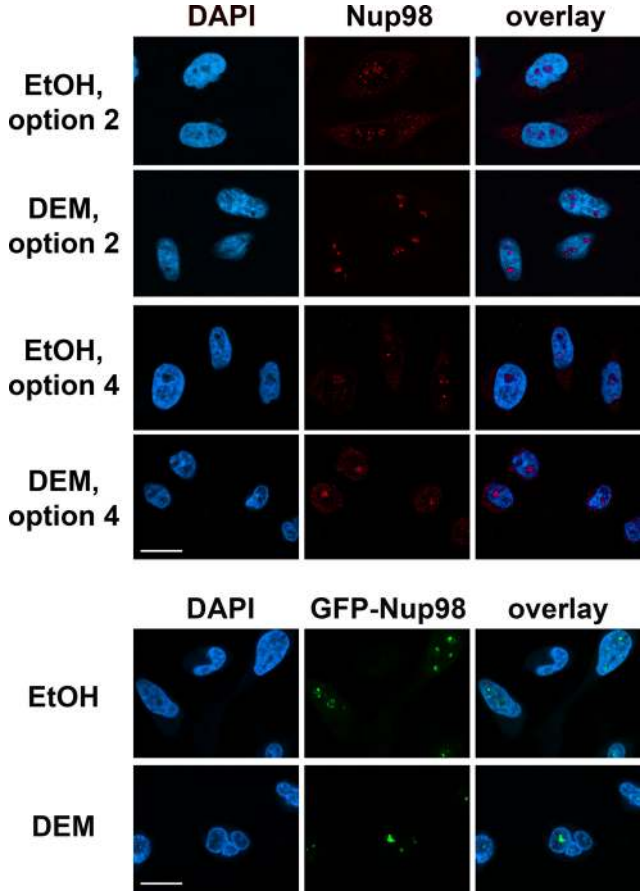

Figure 3. DEM treatment induces the redistribution of Nup98. Control and stressed cells were monitored for the distribution of Nup98 with two different staining methods (option 2 and 4; Kodiha *et al.*, 2009). Both procedures show the relocation of Nup98 in response to oxidative stress. Note that a larger portion of Nup98 associates with nucleoli after DEM treatment. Similar results were obtained for transiently transfected HeLa cells synthesizing GFP-Nup98. Bar, 20 μm.

analysis that documents changes in nucleoporin levels at the NE.

Crm1 interacts with NPCs on the nuclear as well as cytoplasmic side of the NE, where the disassembly of protein export complexes takes place. We therefore analyzed the effect of DEM on Crm1 levels at the cytoplasmic side of the NE (see *Materials and Methods* for details). Crm1 binding to the cytoplasmic face of the nuclear membrane was elevated by stress, similar to what was observed for overall NE binding of the carrier (Figure 2B).

Treatment with Oxidant Triggers the Relocation of Nup98

Nup98 is present in multiple cellular compartments, and several protocols have been developed to detect Nup98 in different locations (Kodiha *et al.*, 2009). In Figure 3, we applied two different staining protocols (option 2 and 4 from Kodiha *et al.*, 2009) to determine the distribution of Nup98. Both methods demonstrated that after DEM incubation, Nup98 redistributed within the cell. In response to stress, the most prominent changes were observed within or adjacent to nucleoli. Dot-like immunostaining in the nucleus and nucleolus was specific to Nup98, as it was diminished by silencing with Nup98 small interfering RNA (Supplemental Figure 1). Similar to endogenous Nup98, a GFP-Nup98 reporter protein that has been generated by others to characterize Nup98 (Grifis *et al.*, 2002), altered its localization in response to oxidative stress (Fig-

ure 3). Collectively, our analyses demonstrate that oxidative stress modulates the distribution of several repeat-containing nucleoporins.

Effect of Oxidative Stress on the Levels of Nuclear Transport Factors

We have chosen mild oxidative stress for the experiments described here, and the majority of cells remained viable under these conditions (Kodiha *et al*., 2008a). Nevertheless, since many nucleoporins are prone to degradation when cells are exposed to environmental changes (Patre *et al*., 2006), we wanted to compare the levels of transport factors in control and DEM-treated cells. To this end, crude extracts were analyzed by Western blotting with antibodies against Crm1, nucleoporins, or RanGAP1. ECL signals were quantified using actin as reference, and signals obtained under control conditions were defined as 1 (Supplemental Figure 2). We further examined how the de novo protein synthesis contributed to protein levels under control and stress conditions by comparing cells that were incubated in the absence or presence of cycloheximide (Supplemental Figure 2). In response to oxidative stress and without cycloheximide, none of the transport factors displayed significant changes in concentration. Cycloheximide treatment revealed that de novo protein synthesis was important to replenish the pools of Crm1, Nup358, and Nup98 in stressed cells. Together, these experiments show that there was no direct correlation between the cellular levels of transport factors and their association with the NE.

Nuclear Envelope Association of Cargo-Free Crm1 in Control and Stressed Cells

We further tested whether DEM incubation altered the interaction of cargo-free Crm1 with the NE. To this end, cells were treated with LMB under conditions established previously to study the empty carrier (Bernad *et al*., 2004). It should be noted that for the experiments conducted with LMB a higher concentration of ethanol was present in all samples, as ethanol served as a solvent for LMB. The conditions are therefore not directly comparable with data in Figure 2.

Figure 4 shows that LMB reduced significantly Crm1 binding to the NE, both under control and stress conditions. Oxidative stress increased the amount of Crm1 at the NE, and the association of cargo-free Crm1 with nuclear membranes was somewhat elevated by DEM (Figure 4A).

We next determined whether the conditions used for LMB experiments affected the cellular levels of Crm1. Indeed, quantitative Western blotting revealed that the amounts of Crm1 were reduced when DEM or LMB were present (Figure 4B). To compensate for these changes in carrier concentration, we calculated the *relative* NE association of Crm1; i.e., values obtained for Crm1 binding to the NE were corrected for changes in protein concentration. Data for the *relative* NE association of Crm1 suggest that stress increased binding to the NE both for Crm1 with or without cargo (Figure 4A).

Nup62, Nup214, and Nup98 Are Posttranslationally Modified When Cells Are Exposed to Oxidative Stress

Western blots depicted in Supplemental Figure 2 indicated that DEM treatment decreased the electrophoretic mobilities of Nup62, Nup214, and Nup98. This shift could be caused by oxidant-induced posttranslational modifications of the nucleoporins, such as phosphorylation or *O*-glycosylation. We addressed this question by incubating crude extracts from control and stressed cells with CIP. Phosphatase treatment somewhat reduced the DEM-dependent mobility shift of Nup62 and Nup214. However, the stress-induced shift was not completely reversed (Figure 5A), indicating that other modifications contributed to the change in Nup62 and Nup214 mobility. In contrast to Nup62 and Nup214, phosphatase treatment of Nup98 reversed the stress-induced increase in electrophoretic mobility. Incubation with phosphatase increased the electrophoretic mobility of Nup98 from unstressed samples, suggesting that the nucleoporin is phosphorylated even under normal growth conditions. After phosphatase treatment, Nup98 from both control and stressed cells migrated to a similar position during SDS-polyacrylamide gel electrophoresis, supporting the interpretation that Nup98 phosphorylation plays a major role in the mobility shift caused by DEM.

Nup62 and Nup214 are modified by *O*-linked GlcNAc sugar moieties; for many proteins, this modification is upregulated under a variety of stress conditions (Zachara *et al*., 2004). Two different types of experiments were carried out to monitor potential changes in *O*-GlcNAc modification. First, samples were incubated with β-*N*-acetyl-hexosaminidase (for simplicity referred to as hexosaminidase), an enzyme that removes terminal *O*-GlcNAc moieties, followed by Western blotting with nucleoporin-specific antibodies (Zachara, 2008). Incubation with hexosaminidase increased the electrophoretic mobility of Nup62 for both control and stress samples; this treatment also increased the electrophoretic mobility for Nup214 from stressed cells (Figure 5B).

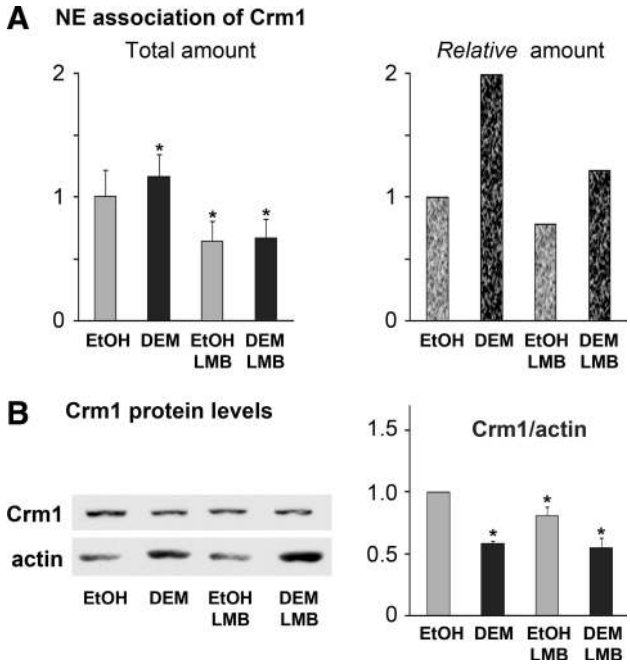

Figure 4. Oxidative stress alters the interaction of cargo-free Crm1 with the NE. The effect of stress on the nuclear envelope association of Crm1 was determined in the absence and presence of LMB. (A) The total amount of Crm1 at the NE was measured as described for Figure 2. The calculation of *relative* amounts of Crm1 at the NE are based on the carrier concentrations under different conditions as determined by Western blotting. (B) Aliquots of crude extracts with comparable amounts of Crm1 were analyzed by Western blotting, and changes in Crm1 protein levels were determined relative to actin. The ratio of Crm1/actin for unstressed control samples was defined as 1. Results are shown for four independent experiments. Statistically significant differences were identified by one-way ANOVA (*$p < 0.05$).

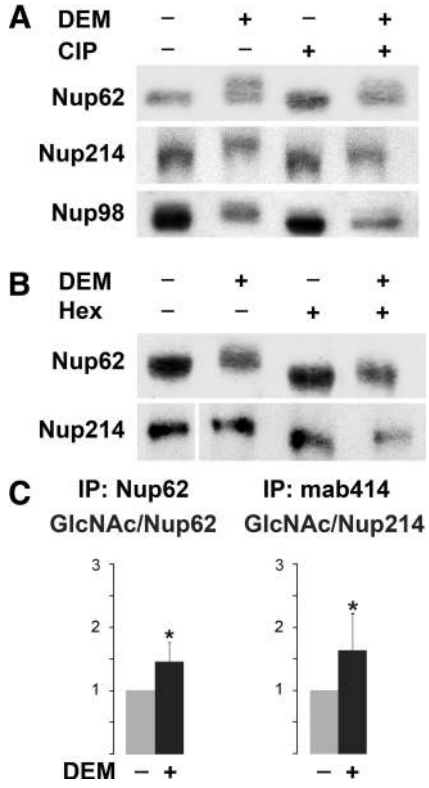

Figure 5. Analysis of stress-induced shifts in Nup214 and Nup62 electrophoretic mobility. (A) Nup62, Nup214, and Nup98 are phosphorylated under normal and stress conditions. Crude extracts prepared from control or DEM-treated cells were incubated with CIP and examined by Western blotting. (B) Oxidative stress increases the *O*-GlcNAc modification of Nup62 and Nup214. Crude extracts from untreated or stressed cells were digested with hexosaminidase (Hex) and analyzed by Western blotting. (C) Stress-induced changes in *O*-GlcNAc modification were quantified for Nup62 and Nup214. Nucleoporins were immunoprecipitated under denaturing conditions, followed by Western blotting with antibodies against *O*-GlcNAc moieties. Filters were stripped and reprobed to measure the amount of nucleoporin. ECL signals were determined by densitometry following standard procedures. For control samples, the ratio GlcNAc/nucleoporin was defined as 1. Note that the *O*-glycosylation of both Nup62 and Nup214 increased in response to DEM treatment. Student's *t* test, $^*p < 0.05$.

In a second set of experiments, we quantified the changes in *O*-GlcNAc modification that were induced by stress. To this end, Nup62 and Nup214 were immunoprecipitated from crude extracts under denaturing conditions, and immunoprecipitates were probed by Western blotting with antibodies against *O*-GlcNAc moieties. Filters were then stripped and reprobed with antibodies that recognize Nup62 or Nup214, respectively. Quantification of ECL signals (Figure 5C) revealed that DEM treatment caused an increase in *O*-GlcNAc modification that was 1.44-fold for Nup62 and 1.62-fold for Nup214. In summary, these results revealed that oxidative stress amplified the posttranslational modification of Nup98, Nup62, and Nup214.

The Interactions Nup62/Crm1, Nup153/Crm1, Nup88/Crm1, Nup88/Nup214, and Ran/Crm1 Are Sensitive to Oxidative Stress

During the translocation of Crm1 through NPCs, the carrier binds to different nucleoporins, and in pull-down experiments, performed in the presence of RanGTP, Crm1 interacts with Nup62 and Nup214 in vitro (Kehlenbach *et al*., 1999; Lindsay *et al*., 2001). Since Crm1/nucleoporin binding is dynamic in living cells, we stabilized these interactions with the cleavable membrane-permeable cross-linker DSP. (It should be noted that reversible cross-linking affected the electrophoretic mobility of several transport factors which gave more diffuse bands upon Western blotting.)

After immunoprecipitation with Crm1-specific antibodies and cleavage of the cross-linker, immunopurified samples were probed for the presence of different binding partners as described in *Materials and Methods* (Figure 6). Nup62 coimmunoprecipitated with Crm1 under these conditions, and the amount of Nup62 that copurified with Crm1 increased modestly in stressed cells. The same result was obtained in reciprocal experiments, when coimmunoprecipitations were carried out with antibodies against Nup62 (Figure 6A). Similarly, we detected an increase in the association of Crm1 with Nup153 (Figure 6B), whereas no drastic changes occurred for the interaction between Crm1 and Nup214 (Figure 6C).

Nup214 and Nup88 are both components of the cytoplasmic NPC filaments, where they contribute to the disassembly of nuclear export complexes. Nup88 binds tightly to Nup214 and together these nucleoporins are at the basis of cytoplasmic NPC filaments (reviewed in Hutten and Kehlenbach, 2007). Furthermore, our previous results showed that a portion of Nup88 relocates to the nucleus in response to oxidative stress, where it associates with high molecular mass complexes (Kodiha *et al*., 2008a). Thus, we tested whether stress also altered the interactions between Nup88, Crm1, and Nup214. Indeed, coimmunoprecipitation experiments revealed that DEM treatment reduced the association between Nup88 and Crm1 as well as Nup88 and Nup214 (Figure 6D).

Crm1-mediated export relies on Ran, and the formation of nuclear export complexes requires RanGTP. Incubation with DEM leaves the nucleocytoplasmic Ran concentration gradient intact (Kodiha *et al*., 2008a); however, it is not known whether Ran interactions are modified upon oxidant exposure. To address this point, Crm1-containing complexes were immunopurified as described above and tested for the presence of Ran (Figure 6E). These experiments revealed that DEM treatment increased significantly the amount of Ran that associated with Crm1.

Data shown in Supplemental Figure 3 further support the results described above. The same Crm1 immunoprecipitation was used to monitor the copurification of different binding partners, and control experiments were carried out side by side with unrelated IgG. These studies demonstrated that under the conditions used for immunopurification, nonspecific precipitation of Crm1, nucleoporins, or Ran was either not detected or minimal (Supplemental Figure 3).

Oxidative Stress Alters the Interactions of Nup62 with Other FxFG Repeat-containing Nucleoporins

We have previously shown that during interphase, members of the FxFG family of nucleoporins interact; in particular, Nup62 associated with Nup153 and Nup214 in growing cells (Stochaj *et al*., 2006). These data together with results from others (Paulillo *et al*., 2005) suggested that associations between FxFG repeat nucleoporins provide a platform to move cargo across the NPC. We now tested whether oxidant treatment altered the interactions between these repeat-containing nucleoporins. As expected, in unstressed cells Nup62 coimmunoprecipitated with Nup214 and Nup153 (Figure 7). However, these interactions were sensitive to oxidative

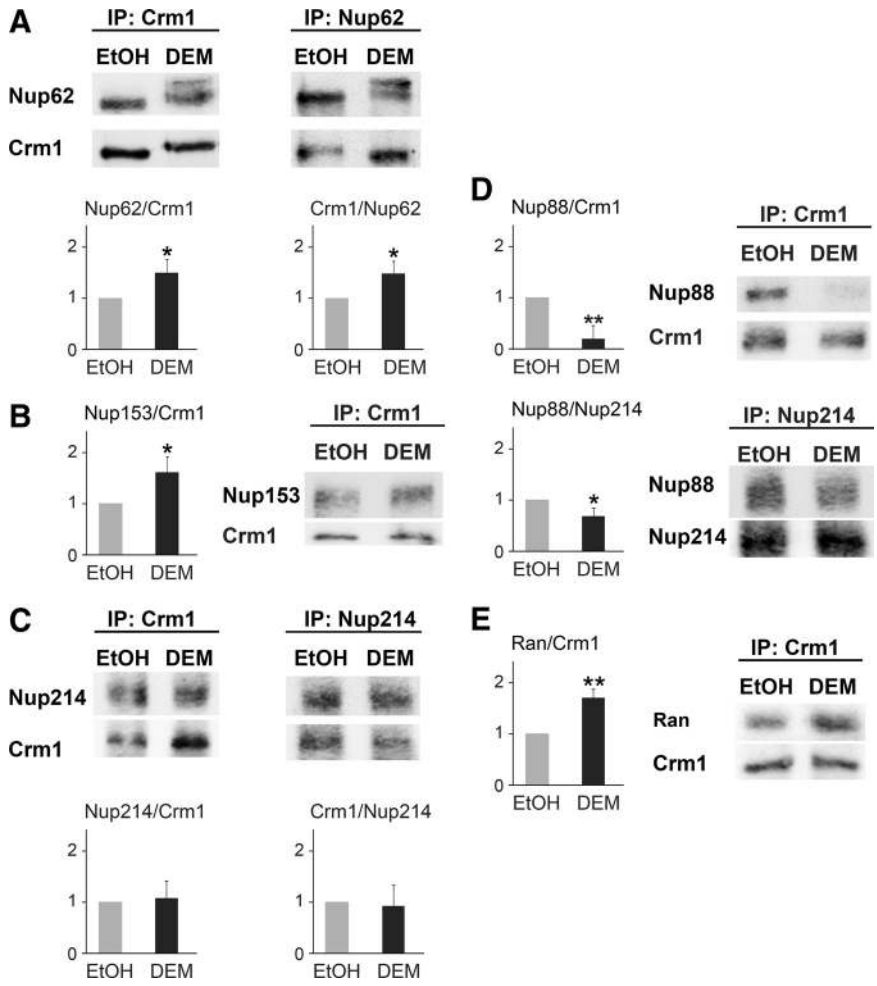

Figure 6. Oxidative stress increases the association between Crm1 and Nup62, Nup153 or Ran, but reduces Nup88/Crm1 and Nup88/Nup214 complexes. Proteins in control (EtOH) and stressed cells (DEM) were covalently cross-linked with DSP. Crude extracts were prepared for immunoprecipitation (IP) with antibodies against the proteins indicated, and immunopurified proteins were analyzed by Western blotting as described in *Materials and Methods*. (A) ECL signals were quantified to determine the ratio Nup62/Crm1 or Crm1/Nup62 for immunoprecipitations with Crm1, and the ratio Crm1/Nup62 for immunoprecipitation with Nup62 specific antibodies. Results are shown for three independent experiments, the ratio of Nup62/Crm1 or Crm1/Nup62 was defined as 1 for control samples. (B and C) Coimmunoprecipitations with antibodies against Crm1, Nup153, or Nup214 were carried out and analyzed as described in A. (D) Immunocomplexes were isolated with antibodies against Crm1 or Nup214 and probed for the presence of Nup88. (E) The interaction between Crm1 and Ran was tested under normal and stress conditions. Significant differences in binding under normal and stress conditions were identified with Student's *t* test, $^{*}p < 0.05$ and $^{**}p < 0.01$. Note that the associations Nup62/Crm1, Nup153/Crm1, and Ran/Crm1 increased in DEM-treated cells, whereas a reduction was observed for Nup88/Crm1 and Nup88/Nup214.

stress, and the interactions among Nup62, Nup214, and Nup153 were reduced significantly in DEM-treated cells.

Oxidative Stress Interferes with the Exit of Crm1 from the Nucleus

To gain insight into the possible impact of DEM treatment on the movement of Crm1 across the nuclear envelope, we measured the exit of the carrier from nuclei. Control and stressed cells were semipermeabilized and incubated in the presence of energy and RanQ69L, a mutant form of Ran that mimics RanGTP. These conditions promote export of importin-β–like carriers from the nucleus (Kodiha *et al*., 2008b, and references therein), and Crm1 exit was monitored by quantification of fluorescence intensities at the NE and in the nuclear interior (Figure 8). Consistent with data shown in Figure 2, Crm1 levels at the NE were higher for stressed cells at 0 min. During the subsequent incubation, additional differences in Crm1 distribution were observed. In unstressed cells, Crm1 levels both at the NE and in the nucleoplasm changed significantly. As such, the association with the NE increased, whereas Crm1 levels in the nuclear interior were diminished. This scenario is consistent with the idea that Crm1 moved from the nucleoplasm to the NE for subsequent release at the cytoplasmic side of the NPC.

By contrast, a different picture emerged for DEM-treated cells (Figure 8). After oxidative stress, Crm1 binding to the NE remained similar for all the time points analyzed. Furthermore, exit of Crm1 from the nucleoplasm was less efficient compared with control cells. (Note that the time course for the loss of Crm1 from the nuclear interior was less steep compared with unstressed cells.) Together, these results suggest that the exit of Crm1 from the nucleus is impaired by oxidative stress.

DISCUSSION

Oxidant Exposure Inhibits Crm1-mediated Nuclear Export and Alters the Binding of Crm1 to the NPC and Ran

In this contribution, we show that oxidative stress inhibits Crm1-dependent nuclear protein export, and we identified several essential components of the export apparatus as targets of oxidative stress.

Crm1 is a key component of nuclear trafficking and the most important carrier for nuclear protein export. Under normal growth conditions, a portion of the carrier is located at the NE, in the nucleus and in the cytoplasm. Our results now demonstrate that Crm1 is sensitive to oxidative stress, which enhanced its association with nuclear membranes. This stress-induced increase of NE binding was also detected for the pool of Crm1 that was present on the cytoplasmic face of the NE, where the disassembly of nuclear export complexes takes place and the recycling of the empty carrier is initiated. Likewise, the stress-dependent up-regulation in NE binding was observed for cargo-free carrier, albeit to a lesser extent. Together, our data support the idea

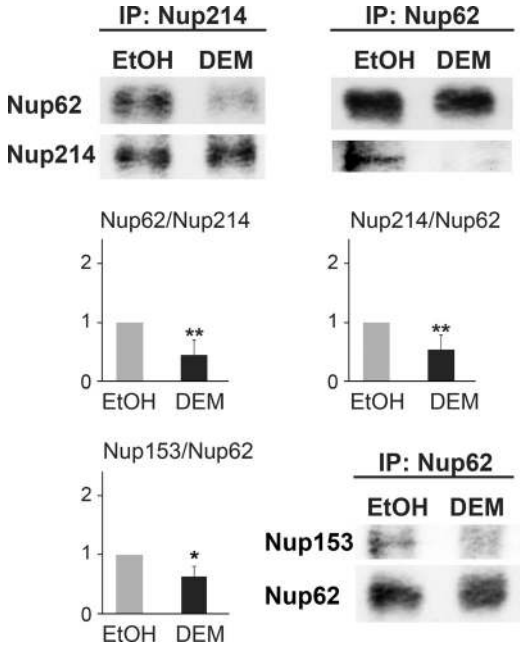

Figure 7. Oxidant treatment alters the interactions of Nup62 with Nup214 and Nup153. DSP–cross-linked proteins from control and DEM-treated samples were immunoprecipitated with antibodies against different nucleoporins as shown in the figure. ECL signals of Western blots were evaluated as described for Figure 6. In these experiments, interactions between Nup62 and Nup214 or Nup153 were reduced by stress.

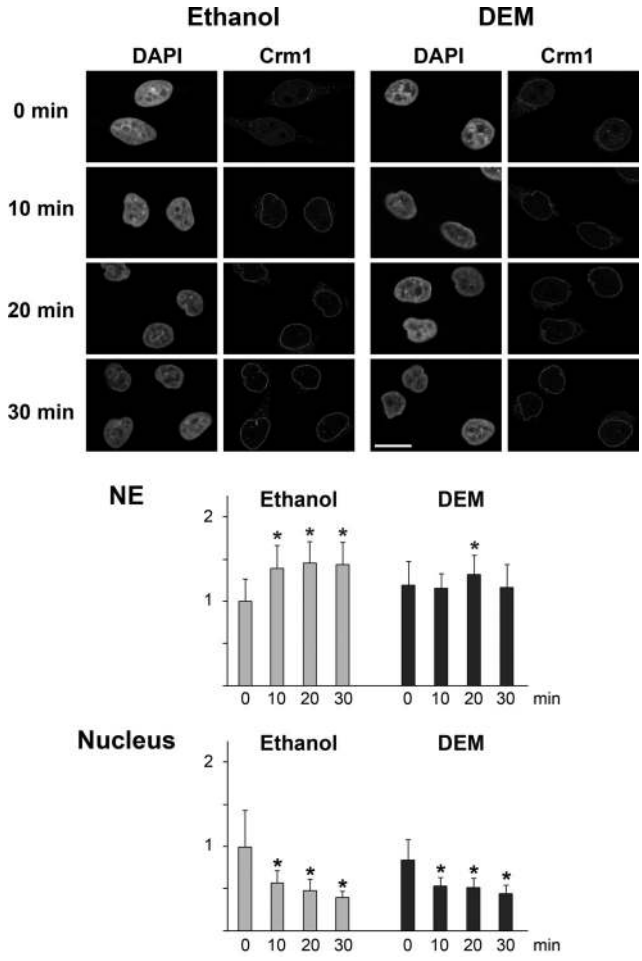

Figure 8. Oxidative stress reduces the exit of Crm1 from the nucleus. HeLa cells incubated with ethanol or DEM were extracted with digitonin and incubated under conditions that facilitate the exit of nuclear carriers from the nucleus (Kodiha *et al.*, 2008b). At the times indicated, samples were fixed and processed for the immunolocalization of Crm1. Crm1 levels at the NE or in the nucleus were measured as pixel intensities/area. Results obtained for ethanol-treated cells at 0 min were defined as 1. Significant changes within each of the four data sets were identified by one-way ANOVA (*$p < 0.05$); 0 min served as the reference. Bar, 20 μm.

that oxidative stress alters the association of Crm1 with the NE, both during nuclear export and the recycling of empty carrier. Ultimately, this change in NE binding may interfere with several steps that are important for nuclear transport, such as the termination of the export reaction and the initiation of new export cycles.

During its passage through the NPC, Crm1 binds to multiple nucleoporins, including Nup62 and Nup214. We demonstrate now that Crm1 interactions with Nup62 increased significantly in stressed cells, whereas no drastic changes were observed in its binding to Nup214. Since Crm1 levels at the NPC are elevated in response to stress, it is conceivable that this is, at least in part, due to the enhanced association between the carrier and Nup62 as well as Nup153.

Unlike the enhanced binding to Nup62 and Nup153, protein complexes containing Crm1/Nup88 or Nup88/Nup214 were significantly reduced in response to oxidant exposure. These diminished interactions are consistent with our previous observation that a portion of Nup88 relocates to the nucleus of stressed cells (Kodiha *et al.*, 2008a), where it may no longer be available as a binding partner for Crm1 or Nup214. The stress-induced changes in associations among Crm1, Nup88, and Nup214 may affect the structure and function of the disassembly platform for Crm1-mediated export, thereby interfering with the unloading of cargo at the cytoplasmic side of the NPC.

Importantly, the oxidant-dependent changes of Crm1 interactions were not limited to its associations with nucleoporins, as DEM treatment enhanced the binding between Crm1 and Ran. This stress-induced increase in Crm1/Ran association may suggest changes in the formation and/or dissociation of Crm1 transport complexes, which may contribute to the inhibition of export. A recent study showed that cysteine residues in Ran undergo oxidation to sulfenic acid in response to oxidative stress induced by hydrogen peroxide (Leonard *et al.*, 2009). Future experiments will have to test whether this Ran modification alters the binding to Crm1 or other nuclear carriers.

Oxidative Stress Targets Several Repeat-containing Nucleoporins

Oxidant treatment affected Nup358, Nup214, and Nup62, all members of the FxFG family of nucleoporins, as well as the GLFG nucleoporin Nup98 (summarized in Table 1). As such, DEM exposure drastically diminished Nup358 levels at the NE; at the same time, Western blot analyses demonstrated that the cellular concentration of Nup358 was not reduced significantly, supporting the idea that oxidative stress caused the release of Nup358 from the NPC.

Previous studies suggested that Nup358 supports Crm1-dependent nuclear export; first as a platform for export complex disassembly, second as a binding site for cargo-free Crm1 during carrier recycling to the nucleus (Bernad *et al.*,

Table 1. Oxidative stress affects multiple components of the nuclear export apparatus

Factor	Changes in response to oxidative stress
Crm1	NE association increased NE association of empty carrier increased Interaction with Nup62 increased Interaction with Nup153 increased Interaction with Ran increased Interaction with Nup88 reduced Exit from the nucleus reduced
Nup358	Levels at the NE drastically reduced
Nup214	NE association increased *O*-GlcNAc modification increased Interaction with Nup62 reduced Interaction with Nup88 reduced
Nup62	NE association increased Phosphorylation and *O*-GlcNAc modification increased Interaction with Crm1 increased Association with Nup214 reduced Association with Nup153 reduced
Nup98	Redistribution in the nucleus Phosphorylation increased

Oxidative stress affects multiple components of the nuclear export apparatus. Results obtained for Crm1 and repeat-containing nucleoporins involved in nuclear export are summarized.

2004). Other data indicate that Nup358 has only a minor role in terminating Crm1-dependent export (Hutten *et al*., 2008). Independent of the contribution of Nup358 to the disassembly of Crm1 export complexes, our results show clearly that Nup358 is a target for oxidative stress. It is feasible that the drastic reduction of Nup358 at the NE of stressed cells altered the recycling of Crm1 and possibly other cellular functions that depend on nuclear Nup358.

Surprisingly, the NE association of RanGAP1, which in its sumoylated form binds to Nup358, was not significantly diminished by stress. One possible explanation could be that under normal growth conditions not all of the RanGAP1 binding sites provided by Nup358 are occupied. If this is the case, a reduction of Nup358 may not necessarily cause an equivalent decrease of RanGAP1 at the NE.

The oxidant-induced changes observed by us were not restricted to Crm1 and FxFG nucleoporins but affected other transport components as well. This included the nucleoporin Nup98, which relocated in part to the nucleoli of stressed cells. Together, these results suggest that multiple components of the transport apparatus, which are involved in different aspects of nuclear transport, are sensitive to stress-induced changes in cell physiology.

Despite the prominent effect on the intracellular distribution of transport factors and nucleoporins in particular, the impact of oxidant exposure was not limited to their localization. In support of this idea, we show here that the posttranslational modifications of Nup214, Nup62, and Nup98 were up-regulated by stress. In particular, GlcNAc modification of Nup214 and Nup62 increased significantly in oxidant-treated cells, and DEM triggered the phosphorylation of Nup98. Moreover, the simultaneous treatment of Nup62 with phosphatase and hexosaminidase suggested that the phosphorylation of Nup62 also increased upon stress (data not shown).

Interestingly, the up-regulation of nucleoporin modification correlated not only with the enhanced binding between Crm1 and Nup62 but also with the reduced interactions among FxFG-repeat nucleoporins. It is conceivable that these stress-induced changes contribute to the inhibition of nuclear export by affecting multiple steps of the transport process. For example, if posttranslational modifications of Nup62 control its binding to Crm1, the stress-induced increase of modifications may stabilize this association. In this scenario, a stabilized Crm1/Nup62 or Crm1/Nup153 interaction may impede the movement of export complexes to the cytoplasm. At the same time, the recycling of empty Crm1 to the nucleus could be slowed down if Crm1/Nup62 and Crm1/Nup153 binding is more stable. Either of these changes can be expected to interfere with Crm1-dependent trafficking.

Although Crm1/nucleoporin binding is essential to nuclear export, we showed that other aspects of nuclear trafficking are sensitive to stress as well. For example, the movement of carrier/cargo complexes and empty carriers across the NPC relies on repeat-containing nucleoporins. Some of these nucleoporins are entirely mobile or have mobile domains (Paulillo *et al*., 2005; Tran and Wente, 2006). In particular, interactions between Nup153, the mobile portion of Nup214, or the Nup62 repeat domain are likely to contribute to the movement of macromolecules across the nuclear pore (Paulillo *et al*., 2005; Stochaj *et al*., 2006). We now demonstrate that the associations among these crucial nucleoporins are regulated by stress.

Collectively, the redistribution and modification of FxFG nucleoporins, Nup98, and Nup88 may change the interac-

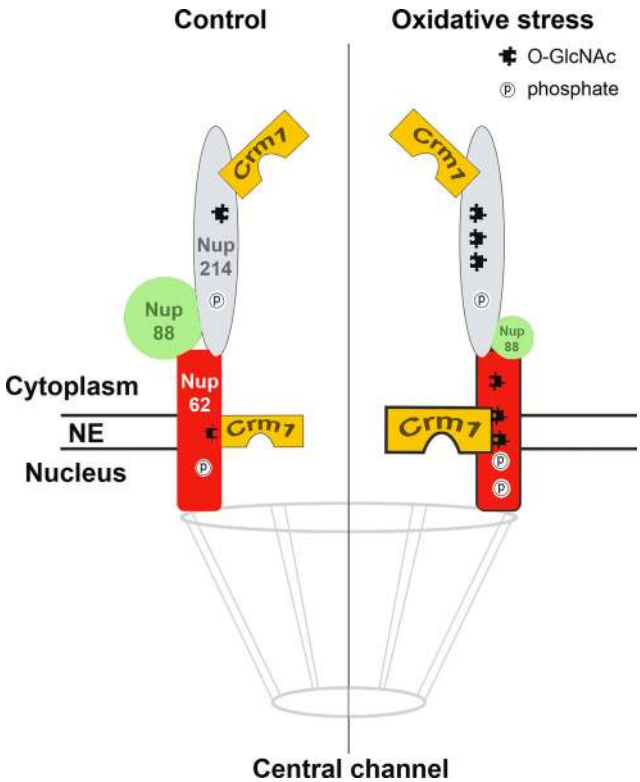

Figure 9. Simplified model for oxidant-induced changes at the NPC. Oxidative stress affects Crm1 (brown), Nup62 (red), and Nup214 (gray) at the NE. Oxidant exposure enhances the interaction between Crm1 and Nup62, while the posttranslational modifications of Nup62 and Nup214 increase. See *Discussion* for details.

tions among nucleoporins. As a consequence, this could alter the proper organization of NPC modules that provide platforms for the translocation or disassembly of transport complexes. One may speculate that such changes will ultimately affect the movement of nuclear carriers across the nuclear envelope. In support of this hypothesis, we demonstrate that Crm1 exit from the nucleus to the cytoplasm is indeed reduced by oxidative stress. Together with the increased association between Crm1 and Ran, this may culminate in the diminished export of cargos to the cytoplasm.

CONCLUSIONS

Results described in the present contribution support the idea that several components of the Crm1 export pathway respond to changes in cell physiology, as exemplified by oxidative stress. Figure 9 depicts a simplified model of the NPC and some of the key interactions analyzed by us. In this model, the stress-induced increase in binding between Crm1 and Nup62 may elevate the steady-state concentration of Crm1 at the NPC. This scenario applies to both Crm1 with and without cargo. In addition, interactions between multiple repeat-containing nucleoporins are significantly reduced in response to oxidant exposure. The observed changes in interactions correlated with the up-regulation of phosphorylation and *O*-glycosylation of nucleoporins. We speculate that one consequence of these changes at the NPC will be a reduction in Crm1-dependent protein export, which will ultimately affect the large number of cargos that are transported by Crm1.

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REFERENCES

Andres-Hernando, A., Lanaspa, M. A., Rivard, C. J., and Berl, T. (2008). Nucleoporin 88 (Nup88) is regulated by hypertonic stress in kidney cells to retain the transcription factor tonicity enhancer-binding protein (TonEBP) in the nucleus. J. Biol. Chem. *283*, 25082–25090.

Bachi, A., *et al*. (2000). The C-terminal domain of TAP interacts with the nuclear pore complex and promotes export of specific CTE-bearing RNA substrates. RNA *6*, 136–158.

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.

D'Angelo, M. A., Raices, M., Panowski, S. H., and Hetzer, M. W. (2009). Age-dependent deterioration of nuclear pore complexes causes a loss of nuclear integrity in postmitotic cells. Cell *136*, 284–295.

Doyle, K. P., Simon, R. P., and Stenzel-Poore, M. P. (2008). Mechanisms of ischemic brain damage. Neuropharmacology *55*, 310–318.

Dröge, W. (2002). Free radicals in physiological control of cell function. Physiol. Rev. *82*, 47–95.

Ebina, H., Aoki, J., Hatta, S., Yoshida, T., and Koyanagi, Y. (2004). Role of Nup98 in nuclear entry of human immunodeficiency virus type 1 cDNA. Microb. Infect. *6*, 715–724.

Filippo, C. D., Cuzzocrea, S., Rossi, F., Marfella, R., and D'Amico, M. (2006). Oxidative stress as the leading cause of acute myocardial infarction in diabetics. Cardiovasc. Drug Rev. *24*, 77–87.

Forbes, J. M., Coughlan, M. T., and Cooper, M. E. (2008). Oxidative stress as a major culprit in kidney disease in diabetes. Diabetes *57*, 1446–1454.

Fornerod, M., van Deursen, J., van Baal, S., Reynolds, A., Davis, D., Murti, K. G., Fransen, J., and Grosveld, G. (1997). 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.

Griffis, E. R., Altan, N., Lippincott-Schwartz, J., and Powers, M. A. (2002). Nup98 is a mobile nucleoporin with transcription dependent dynamics. Mol. Biol. Cell *13*, 1282–1297.

Griffis, E. R., Xu, S., and Powers, M. A. (2003). Nup98 localizes to both nuclear and cytoplasmic sides of the nuclear pore and binds to two distinct nucleoporin subcomplexes. Mol. Biol. Cell *14*, 600–610.

Hutten, S., and Kehlenbach, R. H. (2006). Nup214 is required for CRM1-dependent nuclear protein export in vivo. Mol. Cell. Biol. *26*, 6772–6785.

Hutten, S., and Kehlenbach, R. H. (2007). CRM1-mediated nuclear export: to the pore and beyond. Trends Cell Biol. *17*, 193–201.

Hutten, S., Flotho, A., Melchior, F., and Kehlenbach, R. H. (2008). The Nup358-RanGAP complex is required for efficient importin alpha/beta-dependent nuclear import. Mol. Biol. Cell *19*, 2300–2310.

Joseph, J., Liu, S., Jablonski, S. A., Yen, T. J., and Dasso, M. (2004). The RanGAP1-RanBP2 complex is essential for microtubule-kinetochore interactions in vivo. Curr. Biol. *14*, 611–617.

Joseph, J., and Dasso, M. (2008). The nucleoporin Nup358 associates with and regulates interphase microtubules. FEBS Lett. *582*, 190–196.

Kehlenbach, R. H., Dickmanns, Kehlenbach, A., A., Guan, T., and Gerace, L. (1999). A role for RanBP1 in the release of Crm1 from the nuclear pore complex in a terminal step of nuclear export. J. Cell Biol. *145*, 645–657.

Kodiha, M., Bański, P., Ho-Wo-Cheong, D., and Stochaj, U. (2008b). Dissection of the molecular mechanisms that control the nuclear accumulation of transport factors importin-α and CAS in stressed cells. Cell. Mol. Life Sci. *65*, 1756–1767.

Kodiha, M., Brown, C. M., and Stochaj, U. (2008c). Analysis of signaling events by combining high-throughput screening technology with computer-based image analysis. Sci. Signal. *1*, 12

Kodiha, M., Chu, A., Matusiewicz, N., and Stochaj, U. (2004). Multiple mechanisms promote the inhibition of classical nuclear import upon exposure to severe oxidative stress. Cell Death Differ. *11*, 862–874.

Kodiha, M., Tran, D., Qian, C., Morogan, A., Presley, J. F., Brown, C. M., and Stochaj, U. (2008a). Oxidative stress mislocalizes and retains transport factor importin-α and nucleoporins Nup153 and Nup88 in nuclei where they generate high molecular mass complexes. Biochim. Biophys. Acta *1783*, 405–418.

Kodiha, M., Umar, R., and Stochaj, U. (2009). Optimized immunofluorescence staining protocols to detect the nucleoporin Nup98 in different subcellular compartments. Nat. Netw. Protoc. doi: 10.1038/nprot.2009.16.

Leonard, S. E., Reddie, K. G., and Carroll, K. S. (2009) Mining the thiol proteome for sulfenic acid modifications reveals new targets for oxidation in cells. ACS Chem. Biol. *4*, 783–799.

Lindsay, M. E., Holaska, J. M., Welch, K., Paschal, B. M., and Macara, I. G. (2001). Ran-binding protein 3 is a cofactor for Crm1-mediated nuclear protein export. J. Cell Biol. *153*, 1391–1402.

Madrid, A. S., and Weis, K. (2005). Nuclear transport is becoming crystal clear. Chromosoma *115*, 98–109.

Patre, M., Tabbert, A., Hermann, D., Walczak, H., Rackwitz, H., Cordes, V. C., and Ferrando-May, E. (2006). Caspases target only two architectural components within the core structure of the nuclear pore complex. J. Biol. Chem. *281*, 1296–1304.

Paulillo, E. M., Phillips, E. M., Köser, J., Sauder, U., Ullman, K. S., Powers, M., and Fahrenkrog, B. J. (2005). Nucleoporin domain topology is linked to the transport status of the nuclear pore complex. J. Mol. Biol. *351*, 784–798.

Powers, M. A., Forbes, D. J., Dahlberg, J. E., and Lund, E. (1997). The vertebrate GLFG nucleoporin, Nup98, is an essential component of multiple RNA export pathways. J. Cell Biol. *136*, 241–250.

Quan, X., Tsoulos, P., Kuritzky, A., Zhang, R., and Stochaj, U. (2006). The carrier Msn5p/Kap142p promotes nuclear export of the hsp70 Ssa4p and relocates in response to stress. Mol. Microbiol. *62*, 592–609.

Sánchez, L., Kodiha, M., and Stochaj, U. (2005). Monitoring the disruption of nuclear envelopes in interphase cells with GFP-β-galactosidase. J. Biomol. Tech. *16*, 235–238.

Schwartz, T. U. (2005). Modularity within the architecture of the nuclear pore complex. Curr. Opin. Struct. Biol. *15*, 221–226.

Stochaj, U., Bański, P., Kodiha, M., and Matusiewicz, N. (2006). The N-terminal domain of the mammalian nucleoporin p62 interacts with other nucleoporins of the FXFG family during interphase. Exp. Cell Res. *312*, 2490–2499.

Stochaj, Rassadi, U., R., and Chiu, J. (2000). Stress-mediated inhibition of classical nuclear import pathway and nuclear accumulation of the small GTPase Gsp1p. FASEB J. *14*, 2130–2132.

Tran, E. J., and Wente, S. R. (2006). Dynamic nuclear pore complexes, life on the edge. Cell *125*, 1041–1053.

Terry, L. J., Shows, E. B., and Wente, S. R. (2007). Crossing the nuclear envelope, hierarchical regulation of nucleocytoplasmic transport. Science *318*, 1412–1416.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T., Mazur, M., and Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. Int. J. Biochem. Cell Biol. *39*, 44–84.

Zachara, N. E. (2008). Detection and analysis of (O-linked β-N-acetylglucosamine)-modified proteins. In: The Nucleus, Volume 2, Chromatin, Transcription, Envelope, Proteins, Dynamics, and Imaging, ed. R. Hancock, Totowa, NJ: Humana Press, 227–254.

Zachara, N. E., O'Donnell, N., Cheung, W. D., Mercer, J. J., Marth, J. D., and Hart, G. W. (2004). Dynamic O-GlcNAc modification of nucleocytoplasmic proteins in response to stress. A survival response of mammalian cells. J. Biol. Chem. *279*, 30133–30142.

Zolothukin, A. S., and Felber, B. K. (1999). Nucleoporin Nup98 and Nup214 participate in nuclear export of human immunodeficiency virus type 1 Rev. J. Virol. *73*, 120–127.