Nedd4 Family-interacting Protein 1 (Ndfip1) Is Required for the Exosomal Secretion of Nedd4 Family Proteins*

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The ability to remove unwanted proteins is an important cellular feature. Classically, this involves the enzymatic addition of ubiquitin moieties followed by degradation in the proteasome. Nedd4 proteins are ubiquitin ligases important not only for protein degradation, but also for protein trafficking. Nedd4 proteins can bind to target proteins either by themselves or through adaptor protein Ndfip1 (Nedd4 family-interacting protein 1). An alternative mechanism for protein removal and trafficking is provided by exosomes, which are small vesicles (50–90-nm diameter) originating from late endosomes and multivesicular bodies (MVBs). Exosomes provide a rapid means of shedding obsolete proteins and also for cell to cell communication. In the present work, we show that Ndfip1 is detectable in exosomes secreted from transfected cells and also from primary neurons. Compared with control, Ndfip1 increases exosome secretion from transfected cells. Furthermore, while Nedd4, Nedd4-2, and Itch are normally absent from exosomes, expression of Ndfip1 results in recruitment of all three Nedd4 proteins into exosomes. Together, these results suggest that Ndfip1 is important for protein trafficking via exosomes, and provides a mechanism for cargoing passenger proteins such as Nedd4 family proteins. Given the positive roles of Ndfip1/Nedd4 in improving neuronal survival during brain injury, it is possible that exosome secretion provides a novel route for rapid sequestration and removal of proteins during stress.

The ability to dispose of unwanted proteins is an important function during cellular homeostasis in health or disease. The best studied pathway for disposing of unwanted proteins involves the addition of ubiquitin chains to target proteins followed by degradation of the complex in the proteasome. A major class of enzymes involved in target recognition in this pathway is the E3 ligases with HECT (homology to the E6-associated protein C terminus domain) domains (1). Nedd4 and Nedd4-2 are archetypal members of this family, with ability to bind and ubiquitinate proteins containing PPXY motifs (2, 3). In the nervous system, Nedd4-mediated ubiquitination is required for down-regulating voltage-gated K+ and Na+ channels (4, 5), axon-guidance proteins (6), and TrkA neurotrophin receptor (7). In addition, Nedd4 family proteins (e.g. Nedd4, Nedd4-2, and Itch) can also ubiquitinate target proteins that are bound to Nedd4 adaptors. One such adaptor is Ndfip1 (Nedd4 family-interacting protein 1) originally identified in a screen for Nedd4-binding partners (8, 9). Ndfip1 contains three transmembrane domains and is localized in the Golgi and post-Golgi vesicles. Through their transmembrane domains, Ndfip1 can bind membrane proteins and in doing so, either recruit or inhibit the interaction of membrane proteins with cytosolic Nedd4. In the brain, both Ndfip1 and Nedd4 are co-expressed in the same neuron, and their interaction has been shown to be crucial for increasing the survival of cortical neurons during injury (10).

Besides proteasomal degradation of targeted proteins, Nedd4 proteins also participate in a number of cellular trafficking activities including viral budding, protein sorting, and cell signaling (for reviews, see Refs. 11, 12). The execution of these functions requires the participation of multivesicular bodies (MVBs) and their intraluminal vesicles (ILVs). These organelles control the sorting of ubiquitinated proteins for recycling to the plasma membrane, or alternatively, for destruction in the lysosome (13). The ILVs are formed by inward budding and scission of vesicles from the limiting membrane of MVBs into their lumen. Interestingly, MVBs can also fuse with the plasma membrane, leading to the release of their ILVs (50–90-nm diameter) into the extracellular space. The released vesicles are known as exosomes (see reviews in Refs. 14, 15) and are recoverable by centrifugation at 100,000 × g. It is now clear that exosomal release provides a cellular means of shedding unwanted proteins as in maturing reticulocytes (16), but accumulating evidence also indicates that released exosomes can also mediate cell-to-cell communication, antigen presentation, and oncogenic protein propagation (17, 18).

Although hundreds of proteins are carried within exosomes and their composition is allied to the cell of origin (19), there is little information on the mechanisms leading to exosomal transport of the hundreds of proteins. Recent evidence point to the involvement of ESCRT (endosomal sorting complex required for transport) proteins in sorting ubiquitinated proteins to specific endosome compartments prior to exosome

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§The abbreviations used are: MVB, multivesicular bodies; HA, hemagglutinin; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; ER, endoplasmic reticulum; TEM, transmission electron microscopy.
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budding and scission (20). Nedd4-mediated ubiquitination appears to be a necessary step for recruitment of viral proteins (Gag, LMP2A) to the late endosome before ESCRT-mediated vesicular budding within MVBs (21–23). So while Nedd4 can regulate exosome biogenesis via ubiquitination of proteins in the early steps of the pathway, there is no evidence that Nedd4 proteins are themselves recruited into exosomes. In the present study, we provide evidence to show that Nedd4 family proteins and their adaptor protein Ndfip1 are released in exosomes. While Ndfip1 is constitutively secreted in exosomes, its presence in exosomes is necessary for the exosomal secretion of Nedd4 family proteins.

EXPERIMENTAL PROCEDURES

Antibodies—Antibodies used were rabbit polyclonal anti-Ndfip1, mouse anti-FLAG M2 (Sigma), mouse anti-GM130, and anti-flotillin (BD Transduction Laboratories, San Jose, CA), mouse anti-KDEL (Stressgen, Ann Arbor, MI), mouse anti-Alix (Cell Signaling, Danvers, MA), mouse anti-β actin (Sigma), mouse anti-HA (Roche, Basel, Switzerland), goat anti-mouse-HRP, and goat anti-rabbit-HRP (Millipore, Billerica, MA).

Cell Culture—Human embryonic kidney cells (HEK293T cells) were grown to 90% confluence in 10-cm culture dishes or 6-well plates with 15 ml or 2 ml of medium (10% fetal calf serum, 50 units of penicillin, 50 μg streptomycin, 4 mM L-glutamate in Dulbecco’s modified Eagle’s medium, Invitrogen, Carlsbad, CA), respectively, and transfected with indicated plasmids using Lipofectamine 2000 according to the instructions of the manufacturer (Invitrogen). Construction of FLAG-tagged pcDNA3-based constructs harboring Ndfip1, Nedd4, Nedd4-2, and Itch have been previously described (9, 24). pFLAG-CMV2-Itch was provided by Dr. A. Angers (University of Montréal), pMT123-ubiquitin-HA was provided by Dr. D. Bohmann (University of Rochester). In addition, the following construct was also used: pEF-N-FLAG-CrmA (cytokine response modifier A). BFA (10 μg/ml) and Exo1 (100 μM) (Sigma) were added as indicated 24 h after transfection for another 24 h.

Immunoblotting—HEK293T cells were harvested at indicated time points, washed with ice-cold PBS and lysed in 1 ml of RIPA100 buffer (50 mM Tris, pH 8, 100 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.2% SDS, Protease Inhibitor mixture (Roche) for 30 min on ice. Cell debris was removed by centrifugation (3000 rpm, 15 min, 4 °C). 10 μl of the cell lysates were subject to SDS-PAGE. Supernatants were harvested at indicated time points, and debris was removed by centrifugation (3000 rpm, 15 min, 4 °C). Equal amounts of supernatant volumes (20 μl) from equal amounts of parent cells (2 × 10⁶) were subject to SDS-PAGE. Western blots were incubated with monoclonal anti-FLAG, anti-GM130, anti-actin, anti-flotillin, polyclonal anti-Ndfip1 antiserum, and anti-HA antibody. Primary antibodies were detected by a goat anti-mouse-HRP or a goat anti-rabbit-HRP secondary. The silver staining procedure was performed according to the manufacturer’s instructions (SilverSnap Kit, Pierce).

Immunoprecipitation—One 10-cm plate with 2 × 10⁶ HEK293T cells was plated for each immunoprecipitation. FLAG-tagged proteins were immunoprecipitated from cell lysates and supernatants using anti-FLAG M2 Affinity Gel (Sigma). The recovered proteins were washed three times with PBS plus Protein Inhibitor Mixture (Roche), resuspended in Laemmli buffer and used for SDS-PAGE.

Primary Cortical Cultures—Primary cortical cultures were performed as previously described (25). After 7 days in culture, BFA (10 μg/ml) and cycloheximide (10 μg/ml, Sigma) were added for 6 h. Neurons were fixed with 4% paraformaldehyde and antibody staining was performed as previously described (25).

Isolation of Exosomes—Exosomes were isolated from the supernatant of primary cortical neurons and HEK293T cells after 7 and 2 days in culture, respectively. Supernatants were cleared of debris by centrifugation for 10 min at 200 × g and again for 20 min at 20,000 × g. Exosomes were recovered from the cleared supernatant by centrifugation for 1 h at 100,000 × g. The exosome pellet was washed with ice-cold PBS plus Protein Inhibitor Mixture (PIC, Roche) and centrifuged again for 1 h at 100,000 × g. The pellet was resuspended in PBS plus PIC. The amount of protein in the exosome preparation was determined by BCA assay (Pierce). To generate green fluorescent labeled exosomes, HEK 293T cells were labeled with PKH67 (Sigma), plated and exosomes were harvested 48 h later. Fluorescence of labeled exosomes was measured with a Victor3 plate reader (PerkinElmer Life Sciences, Waltham, MA) at 485/535-nm wavelength.

Electron Microscopy—Transmission electron microscopy was performed using standard protocols. In brief, exosomes were fixed in 100 μl of 2.5% (w/v) glutaraldehyde (Sigma) in PBS. 5 μl of exosome preparation was applied to a 200-mesh copper grid supported with formvar/carbon (ProSciTech, Kirwan, Australia), and the grid was air-dried. Grids were washed, negatively stained with 3% saturated aqueous uranyl acetate, and viewed with a transmission electron microscope (Siemens Elmiskop 102, Siemens, Munich, Germany).

RESULTS

Ndfip1 Is Secreted into the Supernatant of Cultured Cells—To determine if Ndfip1 is released from cultured cells, we transiently overexpressed FLAG-tagged Ndfip1 in HEK293T cells using Lipofectamine 2000 followed by collection of culture media (12, 24, and 48 h after transfection) for Western blotting against the FLAG tag. Secreted Ndfip1-Flag was detected in the culture medium by Western blotting 24 h after transfection, with the greatest signal intensity detected after 48 h (Fig. 1A). The predicted molecular weight of Ndfip1-FLAG is 27 kDa, and a similar band of equivalent molecular mass was seen in cell lysates harvested at 12, 24, and 48 h (Fig. 1A) and the supernatant suggesting that Ndfip1-FLAG is released into the media as full-length protein. To demonstrate that the release of Ndfip1 was not an artifact of cell transfection and protein overexpression in HEK293T cells, a variety of control proteins were transfected and examined for their secretion into culture supernatant. Control Nedd4 family proteins were selected for their known capacity to bind Ndfip1 (e.g. Nedd 4, Nedd 4-2, and Itch) (Fig. 1B) (9). We also chose proteins whose molecular masses are similar to Ndfip1 (e.g. CrmA and Bcl-2) (Fig. 1C). In these experiments, Ndfip1 is clearly detected in Western blots of the
Ndfip1 secretion is mediated by the classical ER-Golgi Pathway—To determine whether Ndfip1 secretion is mediated by the classical ER-Golgi pathway, secretion of Ndfip1 by cultured cells was monitored in the presence or absence of Brefeldin A (BFA) or Exo1. Brefeldin A and Exo1 are inhibitors of protein transport from the ER to the Golgi, by virtue of their ability to induce the rapid release of ADP-riboseylation factor (ARF) 1 from the Golgi membranes (26). Treatment of transfected HEK293T cells with either BFA or Exo1 drastically reduced Ndfip1 secretion into the supernatant (Fig. 2, A). These results indicate that Ndfip1 secretion into the culture supernatant is an intrinsic characteristic of Ndfip1.

Ndfip1 Secretion Is Mediated by the Classical ER-Golgi Pathway—To determine whether Ndfip1 secretion is mediated by the classical ER-Golgi pathway, secretion of Ndfip1 by cultured cells was monitored in the presence or absence of Brefeldin A (BFA) or Exo1. Brefeldin A and Exo1 are inhibitors of protein transport from the ER to the Golgi, by virtue of their ability to induce the rapid release of ARF 1 from the Golgi membranes (26). Treatment of transfected HEK293T cells with either BFA or Exo1 drastically reduced Ndfip1 secretion into the supernatant (Fig. 2, A), suggesting that Ndfip1 secretion involves the classical ER-Golgi pathway. Support for this is seen in the increased intensity of Ndfip1 staining in the cell following treatment with BFA for 6 h (Fig. 2, B and C). After BFA treatment, Ndfip1 shows co-localization with KDEL, a marker for the ER (27), suggesting that Ndfip1 is not transported from the ER to the Golgi and is accumulated in the ER (Fig. 2C). Ndfip1 is known to be up-regulated after stress induced by starvation and trauma (10). Therefore, increased Ndfip1 staining may be due to increased protein synthesis, rather than BFA blockade of Ndfip1 transport. To test this, new protein synthesis of Ndfip1 was prevented by cycloheximide during treatment with BFA (Fig. 2D). Immunostaining of these cells showed persistent Ndfip1 increase, suggesting that the increase is due to Ndfip1 accumulation from BFA treatment, rather than from increased protein synthesis.

Ndfip1 Is Localized in Exosomes—Ndfip1 has three transmembrane domains and previous results indicate that it localizes to the Golgi apparatus (9, 10). However, these experiments also report Ndfip1 immunostaining outside the Golgi membranes, suggesting transient associations with other cellular organelles (10). To test the possibility that Ndfip1 is transported to the extracellular milieu as membrane-bound protein within exosomes, a number of experiments were carried out using HEK293T cells transfected with Ndfip1, and primary untransfected cortical neurons expressing endogenous Ndfip1. Using standard protocols to isolate exosomes from cells and neurons (28, 29), supernatant was harvested from HEK293T cells 48 h after transfection or from primary cortical neurons.
Ndip1 and Nedd4 in Exosomes

To confirm that Ndip1 is present in exosomes and not in general membrane debris, Western blotting was also carried out using antibodies to GM130, a known Golgi membrane protein. The negative results for GM130 suggest that the exosomal preparations are not contaminated with membrane debris. Further evidence that the loaded samples contain only exosomes was directly provided by transmission electron microscopy (TEM) following uranyl acetate staining of exosome preparation from primary neurons. The TEM analysis indicates the sample consists of microvesicles with shapes and diameters (50–100 nm) consistent with exosomes (Fig. 3, C1–C4). The localization of exosomes in primary cortical neurons was examined using antibodies to Alix, a component of the ESCRT complex in MVBs and a marker for ILVs and exosomes (31). These experiments showed that immunostaining for Alix was co-extensive with Ndip1 in primary cortical neurons (Fig. 3, D1–D3), further suggesting that Ndip1 in these neurons localizes to ILVs.

Ndip1-FLAG but not in the control cells (from three independent experiments). The exosome marker flotillin confirms the presence of exosomal proteins in both Ndip1 and control lanes (Fig. 3A). Differential centrifugation of samples from untransfected primary cortical neurons demonstrates that endogenous Ndip1 is present in the exosomal fraction, confirmed by the flotillin marker. Lack of staining with the Golgi marker GM130 suggests that the exosomes are not contaminated by other cellular organelles.

Overexpression of Ndip1 Increases Exosomal Protein Levels—Our results suggest that increasing Ndip1 levels may provide a novel mechanism for up-regulating certain binding proteins such as Nedd4, Nedd4-2, and Itch in exosomes. However, it is unclear whether or not Ndip1 can up-regulate the overall level of exosomal proteins. To approach this question in a semi-quantitative fashion, we examined protein levels in exosomes following Ndip1 transfection, compared with control transfection with CrmA. Following transfection in HEK293T cells, cell lysates and exosomes were prepared and equal volumes loaded onto SDS-PAGE gels followed by silver staining. The results indicate higher staining intensities for exosomal proteins in the Ndip1 lanes (Fig. 4B, lanes 3 and 4), compared with the CrmA lanes (Fig. 4B, lanes 1 and 2). As a control, the respective cell lysate lanes indicate comparable similar intensities between all four lanes, highlighting that the exosomal results are semiquantitative. This was further confirmed by comparing protein levels (by BCA assay) present in exosomes harvested from Ndip1-expressing cells versus controls (Bcl-2 expressing cells) (Fig. 4C). Thus, increasing Ndip1 expression in HEK293T cells results in higher protein levels in exosome preparations. Alternatively, these results may also suggest higher production of

(From embryonic day 15 mouse embryos) 7 days after plating. Following clearing of cell debris by centrifugation (20,000 × g for 20 min), exosomes were pelleted (100,000 × g for 1 h) and analyzed by Western blotting for Ndip1-FLAG in transfected cells. The results clearly demonstrate the presence of Ndip1 in these exosome preparations, while the vector-alone samples did not contain Ndip1 (Fig. 3A). Exosome content in both vector-alone and transfected preparations was confirmed by the presence of flotillin, a lipid raft protein known to be localized in exosomes (30). A similar result was also obtained from Western blots of exosomes prepared from the supernatant of untransfected primary neurons (Fig. 3B), suggesting that Ndip1 presence in exosomes is not the result of Ndip1 overexpression or a consequence of transfection.

FIGURE 3. Ndip1 is present in exosomes of transfected HEK293T cells and untransfected primary cortical neurons. A, in transfected HEK293T cells, exosomes isolated by differential centrifugation exhibit the presence of Ndip1-FLAG but not in the control cells (from three independent experiments). The exosome marker flotillin confirms the presence of exosomal proteins in both Ndip1 and control lanes. B, differential centrifugation of samples from untransfected primary cortical neurons demonstrates that endogenous Ndip1 is present in the exosomal fraction, confirmed by the flotillin marker. Lack of staining with the Golgi marker GM130 suggests that the exosomes are not contaminated by other cellular organelles. C, transmission electron microscopy of exosomal preparations confirms their purity, as demonstrated by microvesicles 50–100-nm diameter. Higher power views are provided in C2–C4. D, cellular localization of Ndip1 (red) is co-extensive with the exosome marker Alix (green, arrow). Scale bars, 100 nm (C1–4); 5 μm (D3).
Overexpression of Ndfip1 causes recruitment of Nedd4, Nedd4-2, and Itch into exosomes.

A, immunoprecipitation using anti-FLAG antibodies with Ndfip1 or control Bcl-2 expressing cells transfected with either Nedd4, Nedd4-2 or Itch. Cell lysate blots confirm efficient transfection of all plasmids (cell lysate lanes 1–6). In exosomal preparations, Nedd4, Nedd4-2, or Itch is only present when co-transfected with Ndfip1 (exosome lanes 2, 4, and 6) but not with Bcl-2 (exosome lanes 1, 3, and 5) (from three independent experiments). B, duplicates from one of three independent experiments showing that cell lysates have equivalent levels of proteins. In contrast, exosomes from Ndfip1-transfected cells show elevated protein levels (exosome lanes 3 and 4), compared with CrmA control-transfected cells (exosome lanes 1 and 2). Equal volumes of exosomal preparations harvested from the same amount of parent cells were loaded. C, results of four experiments comparing protein levels in exosomes of control versus Ndfip1-transfected cells (*, p < 0.05). D, dye-labeled exosomes from Ndfip1-transfected cells show greater fluorescent intensities compared with exosomes from Bcl-2-transfected cells (*, p < 0.05). C and D depict the average of four independent experiments, error bars are S.E.; a.u., arbitrary units.

Overexpression of Ndfip1 Increases the Level of Ubiquitinated Proteins in Exosomes—One of the proposed functions for Ndfip1 is ubiquitination of substrate proteins in association with Nedd4 ligases (12). This notion is strongly supported by parallel studies conducted in yeast where Bsd2p (Ndfip1 homologue) is known to recruit Rsp5p (Nedd4 homologue) for ubiquitination of transmembrane proteins (33). Given that Ndfip1 in the current study has been shown to be present in exosomes, and that Ndfip1 is capable of increasing protein levels in exosomes, it is of interest to examine whether or not Ndfip1 up-regulation in exosomes is associated with increased protein ubiquitination. To address this question, HEK293T cells were transfected with Ubiquitin-FLAG alone or together with Ndfip1 to study levels of ubiquitination. Western blotting was performed with an anti-HA antibody to visualize protein ubiquitination. The results demonstrate that Ndfip1 overexpression slightly increases protein ubiquitination in cell lysates (Fig. 5A, compare lanes 1 and 2). However, while control cells (without Ndfip1) have only low levels of ubiquitinated proteins in exosomes (Fig. 5A, lane 3), Ndfip1 drastically increases the level of ubiquitinated proteins in exosomes (Fig. 5A, compare lanes 3 and 4).

One possible interpretation for the above results is due to increased Ndfip1 ubiquitination in exosomes. To examine this question, the ubiquitination of Ndfip1 in cell lysate was compared with that in exosomes. Following co-transfection of Ubiquitin-FLAG with either Ndfip1-FLAG or CrmA-FLAG, a control protein, immunoprecipitation with FLAG beads was carried out followed by blotting with anti-HA antibodies (for ubiquitination) or with anti-FLAG antibodies (to visualize CrmA-FLAG or Ndfip1-FLAG). In cell lysates, Ndfip1-FLAG was clearly ubiquitinated (Fig. 5B, lane 2), while CrmA-FLAG was ubiquitinated to a lesser extent (Fig. 5B, lane 1). In exosomes, there is clearly no ubiquitination of Ndfip1-FLAG (Fig. 5B, lane 4), despite evidence that Ndfip1 has been successfully immunoprecipitated (Fig. 5B, lane 4). Together, these results raise the conclusion that increased expression of Ndfip1 leads to the accumulation of ubiquitinated proteins in exosomes.

**DISCUSSION**

Degradation of proteins that are no longer required is part and parcel of protein cycling in cellular metabolism. A number...
of protein degradation pathways have been identified, including lysosomal and non-lysosomal pathways. Traditionally, the latter requires target protein ubiquitination and subsequent degradation in the 26S proteasome. Major players in this process are Nedd4 proteins, E3 ligases important for identifying and ubiquitinating specific target substrates in health and in disease (4, 24). Through its adaptor Ndfip1, the range of potential targets for Nedd4 binding and ubiquitination is dramatically expanded to embrace proteins lacking the recognition PPXY motifs (9). Proof of principle studies using yeast homologues suggest that Ndfip1 and Nedd4 are key players for removing damaged and misfolded proteins during cellular stress (33). Indeed, we recently demonstrated that Ndfip1 and Nedd4 proteins are up-regulated in stressed neurons during brain injury, suggesting that in higher organisms, a parallel mechanism is operative for removing toxic proteins in times of stress (10).

Recently, an additional cellular mechanism for removing unwanted proteins by exosomal transport of proteins to the extracellular environment has been identified (15). In reticulocytes, exosomal release provides a mechanism for getting rid of obsolete cell surface membranes during red blood cell maturation (16). With this in mind, we set out to explore cellular trafficking of Ndfip1, and whether or not it might be involved in the exosomal transport of proteins, suggesting a novel mechanism for Ndfip1-mediated transport of unwanted proteins. Our results suggest this to be the case, and confirm that that Ndfip1 is secreted into the supernatant of cells overexpressing this protein. In addition, Ndfip1 is secreted from cultured primary cortical neurons, suggesting that Ndfip1 secretion is not an artifact of protein overexpression, but may be germane to cells that normally express this protein. Our experiments also indicate that the secretion of Ndfip1 utilizes the classical ER to Golgi pathway.

What might be the possible incentives for a Golgi-embedded protein to be transported to MVBs and ultimately secreted in exosomes? The concept of a Golgi protein being secreted in exosomes is nothing new (19), however in the current study, its effects on exosomal content generally and specifically is somewhat surprising. In general terms, overexpression of Ndfip1 was found to increase exosome release from cells, contributing to more protein secretion, compared with the control. In addition, the general level of protein ubiquitination in exosomes was also increased. Together, these two attributes would suggest that Ndfip1 may play a major role in mediating exosomal transfer of other ubiquitinated proteins. The presence of ubiquitinated proteins in MVBs and exosomes has previously been reported (19, 34), but its physiological significance remains unclear. Possible explanations include the requirement for proteins to be polyubiquitinated prior to exosomal sorting in MVBs or alternatively, ubiquitination is necessary for directing exosomal cargo to lysosomes for degradation. A third explanation is that ubiquitination of cargo proteins is an important step for exosomal release by intraluminal budding. Support for this argument is provided by the demonstration that the Gag polyprotein of HTLV-1 virus can utilize Nedd4 for their ubiquitination prior to their binding to Tsg101, and consequently leading to their assembly and budding in the MVBs (21).

**FIGURE 5.** Overexpression of Ndfip1 in HEK293T cells is associated with elevated levels of protein ubiquitination in exosomes. **A**, transfection of Ubiquitin-HA alone results in low level protein ubiquitination in exosomes (lane 3). Co-transfection of Ubiquitin-HA with Ndfip1-FLAG causes increased signal intensity, suggesting increased ubiquitination in exosomes (lane 4) as a consequence. Loading control for actin indicates similar sample loads for exosomes and lysates. The presence of Ndfip1 in the transfected samples is confirmed with anti-FLAG antibodies. **B**, ubiquitination of either Ndfip1 or control CrmA is only seen in cell lysates (lanes 1 and 2). Ndfip1-FLAG in exosomes is not ubiquitinated (lane 4) although Ndfip1 is clearly present. * indicates a nonspecific band is present in all lanes. The results represent three independent experiments.
It remains unclear whether or not Nedd4 family proteins are involved in ubiquitinating exosomal proteins but their presence in exosomes (as a consequence of Ndfip1) suggests guilt by association. If that is the case, then increased ubiquitination in exosomes is mechanistically dependent on the presence of Ndfip1. Together, they would participate in regulating protein trafficking. On the other hand, it is possible that ubiquitination of proteins by Nedd4 family proteins occurs in MVBs (not exosomes), followed by protein sorting to lysosomes or exosomes. We have previously shown that Nedd4 protein is localized to MVBs (35). In this light, the current data would suggest that termination or reversal of Nedd4-mediated ubiquitination in MVBs may require Nedd4 to be removed into exosomes, and this is regulated by Ndfip1. During neuronal injury, a large number of potentially harmful substances accumulate inside and outside neurons with the capacity to induce cell death (36). These include metal cations and excitotoxins that may trigger ionic imbalances and metabolic overload.

We have previously observed that increasing neuronal concentrations of Ndfip1 is associated with cell survival (10), and that this process may involve Ndfip1/Nedd4-mediated ubiquitination and removal of proteins that facilitate entry of metal cations and neurotransmitters, including metal transporters and glutamate receptors. Ion transporters and membrane channels are abundant in exosomes (19), and glutamate receptors have been identified in exosomes from primary neurons (28). In this context, it is reasonable to hypothesize that exosomal transport of Ndfip1 and its associated cargo proteins would provide an additional and rapid route for removal of unwanted proteins to enhance neuronal survival.

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REFERENCES

1. Huibregtse, J. M., Scheffner, M., Beaudenon, S., and Howley, P. M. (1995) Proc. Natl. Acad. Sci. U. S. A. **92**, 2563–2567
2. Staub, O., Dho, S., Henry, P., Correa, J., Ishikawa, T., McGlade, J., and Rotin, D. (1996) *EMBO J.* **15**, 2371–2380
3. Pirozzi, G., McConnell, S. J., Uveges, A. J., Carter, J. M., Sparks, A. B., Kay, B. K., and Fowlkes, D. M. (1997) *J. Biol. Chem.* **272**, 14611–14616
4. Fotia, A. B., Ekberg, J., Adams, D. J., Cook, D. I., Poronnik, P., and Kumar, S. (2004) *J. Biol. Chem.* **279**, 28930–28935
5. Ekberg, J., Schuetz, F., Boase, N. A., Conroy, S. J., Manning, J., Kumar, S., Poronnik, P., and Adams, D. J. (2007) *J. Biol. Chem.* **282**, 12135–12142
6. Myat, A., Henry, P., McCabe, V., Flintoft, L., Rotin, D., and Tear, G. (2002) *Neuron* **35**, 447–459
7. Arevalo, J. C., Waite, J., Rajagopal, R., Beyna, M., Chen, Z. Y., Lee, F. S., and Chao, M. V. (2006) *Neuron* **50**, 549–559
8. Jolliffe, C. N., Harvey, K. F., Haines, B. P., Parasivam, G., and Kumar, S. (2000) *Biochem. J.* **351**, 557–565
9. Harvey, K. F., Shearwin-Whyatt, L. M., Fotia, A., Parton, R. G., and Kumar, S. (2002) *J. Biol. Chem.* **277**, 9307–9317
10. Sang, Q., Kim, M. H., Kumar, S., Bye, N., Morganti-Kossman, M. C., Gunnersen, J., Fuller, S., Howitt, J., Hyde, L., Beisbarth, T., Scott, H. S., Silke, J., and Tan, S. S. (2006) *J. Neurosci.* **26**, 7234–7244
11. Ingham, R. J., Gish, G., and Pawson, T. (2004) *Oncogene* **23**, 1972–1984
12. Shearwin-Whyatt, L., Dalton, H. E., Foot, N., and Kumar, S. (2006) *Bioessays* **28**, 617–628
13. Gruenberg, J., and Stemmark, H. (2004) *Nat. Rev. Mol. Cell. Biol.* **5**, 317–323
14. Denzer, K., Kleijmeer, M. J., Heijnen, H. F., Stoorvogel, W., and Geuze, H. J. (2000) *J. Cell Biol.* **113**, 3365–3374
15. van Niel, G., Porto-Carreiro, I., Simeos, S., and Raposo, G. (2006) *J. Biol. Chem.* **140**, 13–21
16. Johnstone, R. M., Adam, M., Hammond, J. R., Orr, L., and Turbide, C. (1987) *J. Biol. Chem.* **262**, 9412–9420
17. Johnstone, R. M. (2006) *Blood Cells Mol. Dis.* **36**, 315–321
18. Al-Nedawi, K., Meehan, B., Micallef, J., Lhotak, V., May, L., Guha, A., and Rak, I. (2008) *Nat. Cell Biol.* **10**, 619–624
19. Pitsikas, T., Shen, R. F., and Knepper, M. A. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101**, 13368–13373
20. Urbe, S., Sachse, M., Row, P. E., Preisinger, C., Barr, F. A., Strous, G., Klumperman, J., and Clague, M. J. (2003) *J. Cell Biol.* **161**, 4169–4179
21. Blot, V., Perugi, F., Gay, B., Prevost, M. C., Briant, L., Tangy, F., Abriel, H., Staub, O., Dokhelar, M. C., and Pique, C. (2004) *J. Cell Biol.* **177**, 2357–2367
22. Segura-Morales, C., Pescia, C., Chatellard-Causse, C., Sadoul, R., Bertrand, E., and Basuyu, E. (2005) *J. Biol. Chem.* **280**, 27004–27012
23. Ikeda, M., and Longnecker, R. (2007) *Virology* **360**, 461–468
24. Fotia, A. B., Dinudom, A., Shearwin, K. E., Koch, J. P., Korbmacher, C., Cook, D. I., and Kumar, S. (2003) *Faesel. J.* **17**, 70–72
25. Putz, U., Harwoll, C., and Nedivi, E. (2005) *Nat. Neurosci.* **8**, 322–331
26. Feng, Y., Yu, S., Lasell, T. K., Jadhav, A. P., Macia, E., Chardin, P., Melancon, P., Roth, M., Mitchison, T., and Kirchhausen, T. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100**, 6449–6474
27. Vaux, D., Tooze, J., and Fuller, S. (1990) *Nature* **345**, 495–502
28. Faure, J., Lachenal, G., Court, M., Hirlinger, J., Chatellard-Causse, C., Blot, B., Grange, J., Schoehn, G., Goldberg, Y., Boyer, V., Kirchhoff, F., Raposo, G., Garin, J., and Sadoul, R. (2006) *Mol. Cell. Neurosci.* **31**, 642–648
29. Trajkovic, K., Hsu, C., Chiantia, S., Rajendran, L., Wenzel, D., Wieland, F., Schwille, P., Brugger, B., and Simons, M. (2008) *Science* **319**, 1244–1247
30. de Gassart, A., Geminard, C., Felee, B., Raposo, G., and Vidal, M. (2003) *Blood* **102**, 4336–4344
31. de Gassart, A., Geminard, C., Hoekstra, D., and Vidal, M. (2004) *Traffic* **5**, 896–903
32. Morelli, A. E., Larregina, A. T., Shufesky, W. J., Sullivan, M. L., Stolz, D. B., Papworth, G. D., Zahorchak, A. F., Logar, A. J., Wang, Z., Watkins, S. C., Falco, L. D., Jr., and Thomson, A. W. (2004) *Blood* **104**, 3257–3266
33. Hettema, E. H., Valdez-Taubas, J., and Pelham, H. R. (2004) *EMBO J.* **23**, 1279–1288
34. Buschow, S. I., Lieheber, J. M., Wubbolts, R., and Stoorvogel, W. (2005) *Blood Cells Mol. Dis.* **35**, 398–403
35. Shearwin-Whyatt, L. M., Brown, D. L., Wylie, F. G., Stow, J. L., and Kumar, S. (2004) *J. Cell Sci.* **117**, 3679–3689
36. Vink, R., Nimmo, A. J., and Cernak, I. (2001) *Clin. Exp. Pharmacol. Physiol.* **28**, 919–921