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 points 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.
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 ml 1-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-ribosylation 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. 2A), 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 supernatant (Fig. 1A) and can also be visualized following immunoprecipitation with anti-FLAG antibodies (Fig. 1B). Despite almost similar transfection efficiencies (indicated by equivalent protein levels in cell lysate), none of the control proteins were secreted into the supernatant (Fig. 1, B and C). These results indicate that Ndfip1 secretion into the culture supernatant is an intrinsic characteristic of Ndfip1.

**FIGURE 1. Ndfip1 is secreted into supernatant by transfected HEK293T cells.** A, Western blotting shows that Ndfip1 is present in cell lysates and supernatant of transfected HEK293T cells. While Ndfip1 may be seen at all time points in cell lysates, its appearance in supernatant of transfected cells occurs in a time-dependent fashion. B, immunoprecipitation using anti-FLAG antibodies reveals Ndfip1-FLAG in supernatant of transfected cells. The asterisk refers to nonspecific bands present in both lysate and supernatant samples. In contrast, transfection of Ndfip1-binding proteins Nedd4-FLAG, Nedd4-2-FLAG, Itch-FLAG exhibit only transfected proteins in the cell lysate, but not in the supernatant, indicating that these proteins are not secreted. C, immunoprecipitation of non Ndfip1-binding proteins CrmA-FLAG and Bcl-2-FLAG are also not present in the supernatant of transfected HEK293T cells. The results are representative for three independent experiments.

**FIGURE 2. Ndfip1 secretion in exosomes is blocked by Brefeldin A and Exo1.** A, transfected HEK293T cells do not secrete Ndfip1 into the supernatant following treatment with either BFA or Exo1. Ndfip1 was immunoprecipitated from cell lysate and supernatant using anti-FLAG antibodies followed by SDS-PAGE and Western blotting (from three independent experiments). B, in primary cortical neurons, endogenous Ndfip1 (red) is expressed at low levels in a punctate pattern and shows no co-localization with KDEL, an ER marker. C, following treatment of neurons with BFA for 6 h, there is increased staining for Ndfip1 in the cytoplasm around the nucleus. D, addition of cycloheximide to BFA treatment also causes Ndfip1 accumulation. Scale bar: 10 μm (B).
Ndfip1 and Nedd4 in Exosomes

To confirm that Ndfip1 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 Ndfip1 in primary cortical neurons (Fig. 3, D1–D3), further suggesting that Ndfip1 in these neurons localizes to ILVs.

Nedd4, Nedd4-2, and Itch Recruited into Exosomes by Ndfip1 Expression—Ndfip1 release into the supernatant in exosomes is likely to be physiological as it occurs in cultured primary cortical neurons. We therefore asked whether any of the known Ndfip1-binding proteins are co-released with Ndfip1. Using HEK293T cells, we overexpressed either Ndfip1 or control protein Bcl-2 together with certain proteins (Nedd4, Nedd4-2, Itch) that have been shown to bind to Ndfip1 (9). The control transfections with Bcl-2 showed that neither Nedd4, Nedd4-2, nor Itch are present in exosomes (Fig. 4A, exosome lanes 1, 3, and 5) despite their presence in cell lysates (Fig. 4A, cell lysate lanes 1, 3, and 5). In contrast, parallel transfections of Ndfip1 with either Nedd4, Nedd4-2, or Itch showed the presence of Nedd4, or Nedd4-2 or Itch in both lysates (Fig. 4A, cell lysate lanes 2, 4, and 6) and also in exosomes (Fig. 4A, exosome lanes 2, 4, and 6). Together, these experiments indicate that Ndfip1 is sufficient for the exosomal secretion of Nedd4, Nedd4-2, and Itch.

Overexpression of Ndfip1 Increases Exosomal Protein Levels—Our results suggest that increasing Ndfip1 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 Ndfip1 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 Ndfip1 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 Ndfip1 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 comparatively similar intensities between all four lanes, highlighting that the exosomal results are semi-quantitative. This was further confirmed by comparing protein levels (by BCA assay) present in exosomes harvested from Ndfip1-expressing cells versus controls (Bcl-2 expressing cells) (Fig. 4C). Thus, increasing Ndfip1 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 x g for 20 min), exosomes were pelleted (100,000 x g for 1 h) and analyzed by Western blotting for Ndfip1-FLAG in transfected cells. The results clearly demonstrate the presence of Ndfip1 in these exosome preparations, while the vector-alone samples did not contain Ndfip1 (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 Ndfip1 presence in exosomes is not the result of Ndfip1 overexpression or a consequence of transfection.
exosomes in response to Ndfip1. To distinguish between these possibilities, we used a green fluorescent dye (PKH67) that has previously been utilized to label exosomes (32). Following labeling, equivalent exosomal fractions from HEK293T cells were measured for fluorescence intensity using a Fluoro-spectrometer, comparing exosomes from cells expressing either Ndfip1 or Bcl-2 control. The results showed that exosomes from Ndfip1-expressing cells show twice as much fluorescence as the control cells (*, p < 0.05). 4D) can be interpreted that Ndfip1 promotes the secretion of more exosomes rather than more protein carried in the same quantity of exosomes.

**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-HA 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 increased 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-HA 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 PPXYY 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 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).
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 excitotoxic neurotransmitters capable of triggering 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 ubiquitinations of proteins by Nedd4 family proteins occurs in MVBs (not exosomes), followed by protein sorting to lysosomes or exosomes. 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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