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Research Article

Highly homologous HERC proteins localize to endosomes and exhibit specific interactions with hPLIC and Nm23B

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Abstract. Small HERC proteins are defined by the presence of one RCC1-like domain and a HECT domain. Having evolved out of one common ancestor, the four members of the family exhibit a high degree of homology in genomic organization and amino acid sequence, thus it seems possible that they might accomplish similar functions. Here we show that small HERC proteins interact with each other and localize to the same cellular structures, which we identify as late endosomes and lysosomes. We demonstrate interaction of HERC3 with the ubiquitin-like proteins hPLIC-1 and hPLIC-2 and we establish interaction of HERC5 with the metastasis suppressor Nm23B. While hPLIC proteins are not ubiquitinated by HERC3, HERC5 plays an important role in ubiquitination of Nm23B. In summary, although small HERC proteins are highly homologous showing the same subcellular distribution, they undergo different molecular interactions.

Keywords. Ubiquitin ligase, HERC, homology, endosome, hPLIC, Nm23B.

Introduction

The ubiquitin-proteasome system regulates protein degradation, receptor internalization and trafficking, signal transduction, cell cycle control, apoptosis, and DNA repair [1–5]. Ubiquitin is covalently linked to proteins due to the sequential action of three types of enzymes known as E1, E2, and E3 with the respective ability to activate, conjugate and transfer the ubiquitin moiety to the target protein. E3 ligases are the key components of the ubiquitin system providing specificity by direct interaction with substrates. Two classes of E3 ligases can be differentiated, homologous to E6AP C terminus (HECT) domain proteins and really interesting new gene (RING) E3 s [4]. The human HECT and regulator of chromosome condensation 1 (RCC1)-containing (HERC) proteins represent a subfamily of HECT domain E3 s consisting of two large and four small proteins [6]. Hallmark of HERC proteins is the presence of two domains, the RCC1-like domain (RLD) and the HECT domain. Whereas the small family members HERC3, 4, 5, and 6 contain only one RLD, the larger members HERC1 and 2 consist of several RLDs and other functional domains.

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HERC proteins mainly localize to membranous and vesicular structures, suggesting a role in intracellular trafficking. HERC1 binds to ADP-ribosylation factor 1 (ARF1) and clathrin [7, 8], and HERC3 partly co-localizes with the vesicular marker Rab5 [9]. The mouse *herc2* gene is located in the *jdf2/rjs* chromosomal region involved in genetic disorder resulting in neuromuscular vesicle defects and abnormal acrosome formation in spermatogenesis, which is consistent with a role in secretory trafficking pathways [10, 11]. A very recent paper also links HERC4 to spermiogenesis and male fertility in mice [12]. HERC5 was initially identified as cyclin E-binding protein-1 (Ceb-1) [13] and has later been shown to be up-regulated in inflammation [14]. Although HERC5 localization is similar to other small HERCs, its association with cyclins proposes a role for HERC5 in cell cycle regulation [6, 13].

As RCC1 catalyzes guanine nucleotide exchange on the small GTPase Ran [15], it was suggested that HERC proteins could function as guanine nucleotide exchange factors (GEFs). However, only the RLD1 of HERC1 has guanine nucleotide release activity dependent on the presence of phosphatidylinositol-4,5-bis-phosphate [16]; for the RLD2 of HERC1 and the RLD of HERC3 no such function was detectable at all [8, 9]. Therefore, it remains elusive if RLDs in HERC actually confer GEF activity. Current available data rather suggest a role in basal interactions with other proteins, as shown for HERC1 RLD2 binding to clathrin and Hsp70 [7, 8].

HERC1, HERC3, and HERC5 are able to bind ubiquitin via the HECT domain [9, 14, 17]; however, no target proteins of HERC ubiquitination could be identified so far. In addition to transfer ubiquitin, HERC5 is required for conjugation of the ubiquitin-like protein interferon-stimulated gene 15 (ISG15) [18, 19]. The HERC1 HECT domain binds the glycolytic isoenzyme M2-pyruvate kinase, but the physiological significance of this interaction is unclear [20]. Recently, HERC1 was identified to interact with tuberous sclerosis complex protein TSC2, leading to its destabilization [21]. Although it is speculated that HERC1 could be the responsible E3 ligase for ubiquitination and degradation of TSC2, a final proof of that hypothesis is missing.

Human proteins linking integrin-associated proteins and cytoskeleton (hPLIC) are ubiquitin-like proteins containing an amino-terminal ubiquitin-like (UBL) and a C-terminal ubiquitin-associated (UBA) domain. hPLIC-1 and hPLIC-2 interact via these domains with the 19S cap of proteasomes and polyubiquitinated proteins, as well as E3 ubiquitin ligases E6AP, βTRCP and Nedd4 to influence degradation of various proteasomal targets like p53, IκBα and presenilin [22–24]. A recent publication identifies endocytic proteins Hrs and Eps15 as hPLIC-1 interaction partners [25]. hPLIC-1 also regulates $GABA_A$ receptor stability by counteracting its degradation via the ubiquitin-proteasome system, benefiting receptor recycling back to the plasma membrane [26] and hPLIC-2 has very recently been shown to influence G protein-coupled receptor (GPCR) endocytosis [27].

Non-metastatic cells 23B (Nm23B) belongs to a family of eight highly conserved proteins possessing nucleoside diphosphate kinase (NDPK) activity [28]. Nm23 isoforms are critically involved in cellular proliferation and differentiation, and have also received attention for their potential roles in oncogenesis and tumor metastasis [29, 30]. Nm23B resembles the transcription factor PuF, which regulates the expression of the proto-oncogene *c-myc* and the *platelet-derived growth factor-α* (*pdgf-α*) gene by binding to pyrimidine-rich sequences in respective promoters [31, 32]. Nm23B is also associated with estrogen receptor β, thereby influencing estrogen-induced gene transcription and cell migration [33]. The role of NDPK in GTP synthesis suggests an involvement of Nm23 enzymes in GTP-requiring processes, thus Nm23A plays a role in dynamin-mediated endocytosis [34], whereas Nm23B is involved in integrin rearrangement [35] and thromboxane receptor endocytosis [36].

In our previous work we demonstrated that small HERC proteins evolved by gene duplication out of a single member and exhibit significant similarities in their genomic organization, protein sequence and subcellular protein distribution [6]. In this work, we extended the pattern of similarity between small HERC proteins by showing co-localization and interaction of the members. Also, we provide evidence that they localize to endosomal compartments. Using yeast two-hybrid screening, we identified molecular interaction partners of HERC proteins, showing that despite their identical subcellular distribution, they bind to different proteins. We revealed interaction of HERC3 with hPLIC-1 and hPLIC-2, and of HERC5 with the metastasis suppressor Nm23B. Finally, we have shown that HERC5 is involved in ubiquitination of Nm23B.

Materials and methods

Plasmids. KIAA0032/HERC3 [37] and KIAA1593/HERC4 [38] cDNA were provided by the Kazusa DNA Research Institute. His-HERC3, myc-HERC3, myc-HERC4, myc-HERC6, and flag-HERC5 have been described previously [6]. HERC3 was cloned into pEGFP-C1 (BD Biosciences) and pcDNA3.1-

flag [14] through an *Eco*RI-*Bgl*II polymerase chain reaction (PCR) fragment corresponding to nucleotides (nt) 167–1263 of HERC3 cDNA fused to *Bgl*II-*Stu*I-digested nt 1264–3372. YFP-HERC3 was obtained by exchanging the GFP cassette of GFP-HERC3 to YFP through *Pin*AI-*Ssp*BI restriction. His-HERC4 and flag-HERC4 were generated by amplifying a cDNA fragment using primers with restriction sites *Eco*RI-*Hin*dIII corresponding to nt 249–1367 and fusion to *Hin*dIII-*Eco*RI-digested nt 1368–3490. For flag-HERC6 cloning, two fragments [6] were ligated *Bam*HI-*Not*I into pcDNA3.1-flag. pGBKT7-HERC3-HECT was obtained by cloning a PCR fragment containing nt 2120–3372 via *Eco*RI-*Sma*I restriction into pGBKT7 (BD Biosciences). All hPLIC expression vectors including pCMV4-flag-hPLIC-2, pCMV4-flag-hPLIC-2-UBL, pCMV4-flag-hPLIC-2-ΔUBA, pCS2(+)-flag-hPLIC-1, and pCS2(+)-flag-hPLIC-1-UBL were kindly provided by P. M. Howley [22], pcDNA3-Nm23B-myc was a generous gift of J. L. Parent [36]. HERC3 C1018A and HERC6 C985A mutations were obtained by site-directed mutagenesis (Stratagene) according to manufacturer. All PCRs were performed with High Fidelity DNA Polymerase (Roche) and verification of constructs was carried out by sequencing using ABI Prism Dye-Terminator Kit (Perkin Elmer).

Cell culture and transfection. HEK293 and HeLa cells were maintained in Dulbecco's modified Eagle's medium (HyClone) supplemented with 10% fetal calf serum (HyClone) at 37°C and 5% CO_2. HMEC were cultured in endothelial basal medium (Cambrex) containing 10 ng/ml human recombinant epidermal growth factor (EGF) (Collaborative Biomedical Products), 1 μg/ml hydrocortisone (Sigma), 0.5 mM dibutyryl cyclic adenosine monophosphate (Fluka) and 10% fetal calf serum (HyClone) at 37°C and 5% CO_2. Transfection of cells was achieved using calcium phosphate or Lipofectamine Plus (Invitrogen) when cell density was about 70%.

Immunofluorescence. HMEC or HeLa cells grown on glass-cover slips were transiently co-transfected with indicated plasmids. Cells at 20 h post transfection were fixed in 3% paraformaldehyde for 15 min. After permeabilization in 0.5% Triton X-100 for 10 min, cells were either used for direct immunofluorescence to detect GFP, CFP or YFP tags, or incubated with polyclonal anti-myc (Upstate), monoclonal anti-EEA1 (BD Biosciences), or monoclonal anti-LBPA (kind gift of J. Gruenberg) antibodies followed by Alexa568- or Alexa488-conjugate (Molecular Probes). Samples were analyzed by a Zeiss laser-scanning confocal microscope with LSM 510 software equipped with a 60× objective. pECFP-RhoB was purchased from BD Biosciences and pEGFP-bos-CD63 was provided by J. Gruenberg.

Immunoprecipitation. HEK293 co-transfected with expression plasmids were harvested in lysis buffer containing 0.5% Nonidet P-40, 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM ethylenediamine tetra-acetic acid (EDTA), supplemented with protease inhibitor (Roche). After incubation for 30 min at 4°C, lysates were clarified by centrifugation and supernatants were incubated with anti-hemagglutinin (HA) or anti-flag affinity matrices (Sigma). Of the lysates, 1/20 was kept for expression control. After 2h incubation at 4°C, samples were washed, and immunoprecipitated proteins were eluted by addition of sodium dodecyl sulfate (SDS)-sample buffer. Lysates and precipitated proteins were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. Proteins were detected with anti-flag M2 (Stratagene) and anti-myc 4A6 (Upstate) antibodies, followed by horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Jackson ImmunoResearch) and ECL plus (Amersham Biosciences).

Yeast two-hybrid analysis. AH109 yeast strain was transformed with the bait plasmid pGBKT7-HERC3-HECT according to the lithium yeast transformation protocol [39]. This clone was then transformed with a human HeLa cDNA library (BD Biosciences), and approximately 8.6×10^6 transformants were grown on restrictive media lacking leucine, tryptophan, and adenine. Clones showing positive interaction ($n=796$) were isolated and grown under more stringent conditions in the presence of 35 mM 3-amino-1,2,4-triazole on medium minus leucine, tryptophan, adenine, and histidine. Of these, 52 positive clones were analyzed by PCR and sequencing [40], and cDNA sequences were analyzed by BLAST database alignments at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). pACT plasmids containing library inserts from positive colonies were isolated via phenol/chloroform extraction and transformed into DH5α bacterial strain. Plasmids were extracted from DH5α and re-transformed into yeast with either the bait or pGBKT7 empty vector to confirm the interaction.

***In vivo* ubiquitination assay.** HEK293 were transfected with expression constructs together with pMT107 encoding polyhistidine-tagged ubiquitin (kind gift of D. Bohmann [41]). Where indicated, cells were treated for 5 h with 50 μM MG132 (Calbiochem). Cells were harvested in phosphate-buffered saline, pelleted, resuspended in buffer A (6 M guani-

dine-HCl, 0.1 M Na_2HPO_4/NaH_2PO_4, 10 mM imidazole, pH 8.0), sonicated for 15 s at 25 % amplitude, and cleared by centrifugation for 10 min at room temperature. Of these lysates 1/20 was kept for expression control. Ubiquitinated proteins included in lysates were affinity-purified with Ni-NTA beads (Qiagen) for 3 h at room temperature, washed twice in buffer A and A/TI, once in buffer TI (25 mM Tris/HCl, 20 mM imidazole, pH 6.8), resolved by SDS-PAGE, and analyzed by Western blotting with anti-flag M2 or anti-myc 4A6 antibodies.

Protein stability assay. HEK293 cells were transfected with Nm23B-myc and either pcDNA3.1 (mock) or flag-HERC5. At 24 h after transfection, 100 µg/ml cycloheximide (CHX) was added and cells were harvested at each indicated time point. Total cell lysates were analyzed by Western Blotting with flag- or myc-specific antibodies. As control for equal loading of protein lysates, Western blots were stained with β-actin AC-15 antibody (Sigma-Aldrich).

Results

HERC family members co-localize and interact. We have recently shown that small HERC proteins display a similar cytosolic distribution [6] and therefore we contemplated that they might co-localize. To assess this possibility we transfected HeLa cells with green fluorescent protein (GFP) tagged-HERC3 together with myc-HERC4, flag-HERC5, or myc-HERC6. After staining with myc- or flag-specific antibodies, localization of proteins was monitored by confocal microscopy. As demonstrated in Figure 1A, GFP-HERC3 extensively co-localized with myc-HERC4 and 6 as well as flag-HERC5. The high extent of co-localization let us speculate that HERC proteins might interact with each other. To confirm this hypothesis, co-immunoprecipitation was carried out in human embryonic kidney 293 (HEK293) cells transfected with either flag-HERC6 or HERC6 mutant C985A (flag-HERC6CA) where the cysteine residue responsible for binding of ubiquitin was replaced by an alanine, and myc-HERC3 or 4. Both HERC6 and HERC6CA bound HERC3 and 4 (Fig. 1B), indicating interaction between proteins of the small HERC family independent of their ubiquitin ligase activity. In summary we show that small HERC proteins co-localize and interact with each other.

Small HERC proteins exhibit endosomal localization. Previous studies on HERC3 revealing cytosolic distribution partly co-localizing with the vesicle-associated proteins ARF and Rab5 [9] suggest that the

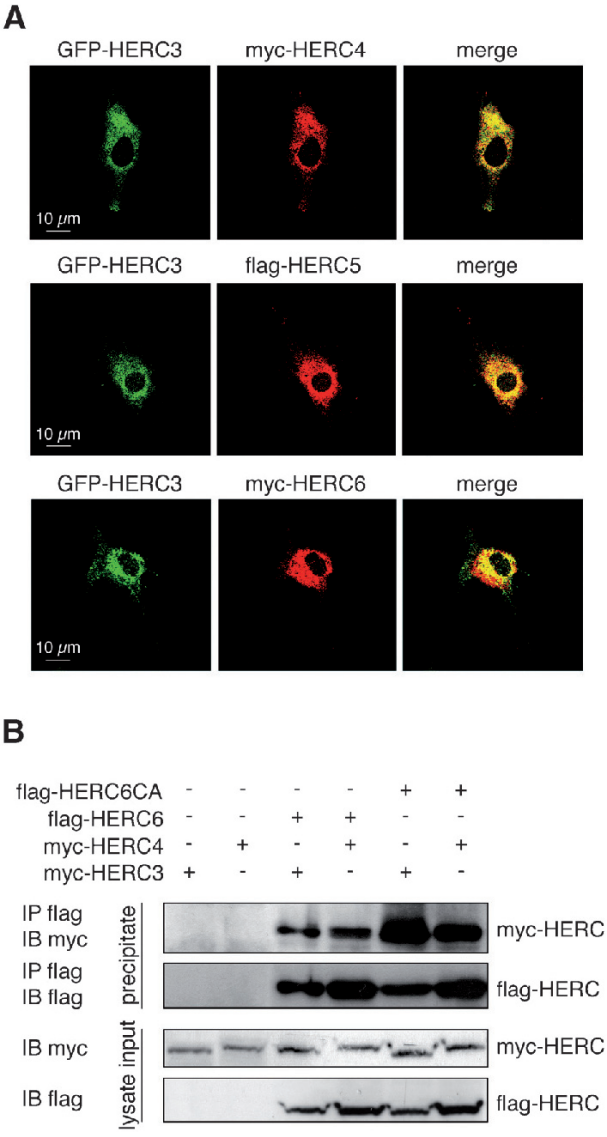

Figure 1. HERC proteins co-localize and interact. (*A*) HeLa were transiently transfected with GFP-HERC3 (green) and myc-HERC4, flag-HERC5 or myc-HERC6 (all red). Myc- and flag-tagged proteins were detected with anti-myc and anti-flag antibodies followed by Alexa568-labelled anti-mouse immunoglobulin secondary antibody, resulting in a double labeling of transfected cells. Localization of proteins was monitored by confocal microscopy. (*B*) Myc-HERC3 and HERC4 together with flag-HERC6 and HERC6 C985A (flag-HERC6CA) were transfected into HEK293. HERC6 was precipitated with flag-affinity matrix and binding of myc-tagged proteins was examined with myc-specific antibody. Co-precipitates were analyzed by Western blotting with anti-myc antibody. cl, cell lysate; IB, immunoblotting; IP, immunoprecipitation.

observed punctate localization pattern of small HERC proteins represents endosomal structures. Therefore, we co-localized yellow fluorescent protein (YFP)-tagged HERC3 with the endosomal marker protein RhoB carrying a cyan fluorescent protein (CFP) tag in human microvascular endothelial cells (HMEC) (Fig. 2A). We chose this cell type on the

basis of previous data obtained in our laboratory indicating that all small HERC proteins are expressed at a decent level in microvascular endothelial cells [6, 14]. Also the larger size of endothelial cells compared to HeLa cells allows better evaluation of vesicular structures. Shown representative for all small HERC proteins, HERC3 largely co-localized with RhoB pointing at HERCs to be endosome-related proteins. Co-localization was also performed for other cellular compartments such as the Golgi apparatus, the endoplasmatic reticulum, mitochondria, and peroxisomes, but none of these substantially co-localized with small HERC proteins (data not shown).

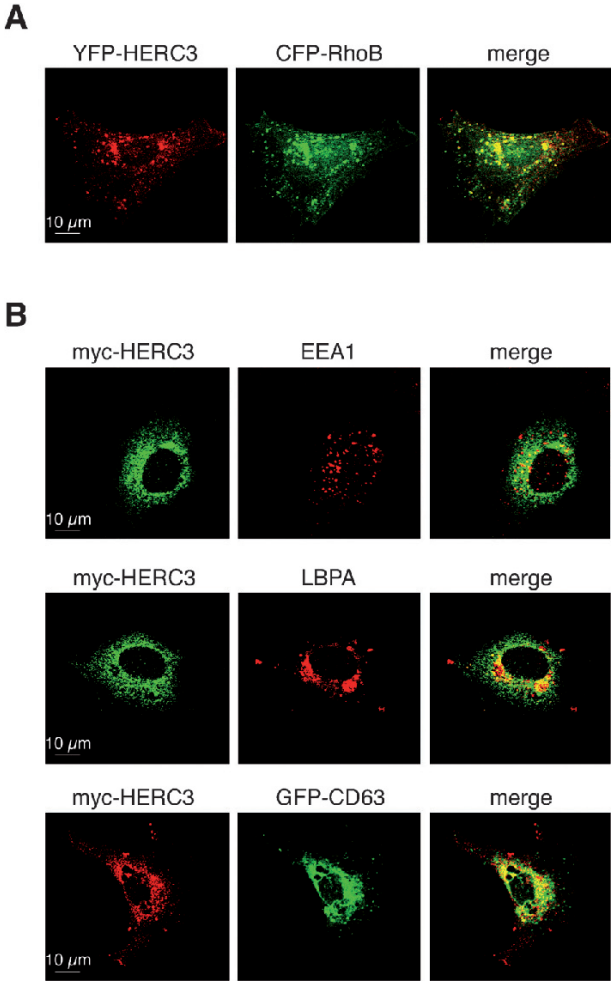

Figure 2. HERC proteins localize to endosomal compartments. (*A*) HMEC were transiently transfected with YFP-HERC3 (red) and the endosomal marker CFP-RhoB (green) and analyzed by direct immunofluorescence via confocal microscopy. (*B*) Co-localization of transiently transfected myc-HERC3 (green and red) with endogenous early endosomal marker EEA1 (red) and late endosomal marker LBPA (red), as well as co-transfected late endosomal and lysosomal marker GFP-CD63 (green) was carried out in HMEC. Cells were fixed, permeabilized, stained with antibodies against EEA1, LBPA, and myc, and analyzed via confocal microscopy.

To further characterize the location of HERC proteins in the endosomal pathway, myc-tagged HERC3 was co-localized in HMEC with early endosomal antigen 1 (EEA1), lysobisphosphatidic acid (LBPA), a lipid component of multivesicular bodies, and GFP-tagged CD63, a component of late endosomal and lysosomal membranes. EEA1 and LBPA were stained with specific monoclonal antibodies, and myc-HERC3 was labeled with rabbit polyclonal myc-antibody. EEA1 showed a punctate staining pattern consistent with the cytoplasmic distribution of early endosomes, while LBPA and GFP-CD63 were located mostly in perinuclear regions. Again representative for all small HERCs, HERC3 mainly resided around the nucleus, showing most co-localization with GFP-CD63 (Fig. 2B). Our data demonstrate that HERC proteins localize to endosomal compartments, especially to late endosomes and lysosomes.

hPLIC-1 and 2 are novel interaction partners of HERC3. To identify interaction partners of HERC proteins we performed yeast two-hybrid screening using the yeast strain AH109 transformed with pGBKT7-HERC3-HECT and a human HeLa cDNA library. Independent clones ($n = 8.6 \times 10^6$) were screened, resulting in 52 positives growing on selective yeast medium. Clones were isolated and sequenced. BLAST searches in cDNA databases revealed that five inserts overlapped with the C terminus of the ubiquitin-like protein hPLIC-2 including the putative collagen-like domain (CLD) and the UBA-domain (Fig. 3A). Co-transformation of hPLIC-2 prey vector with empty bait vector excluded unspecific interaction with the GAL4-binding domain (Fig. 3A). RLD only- and HECT only-containing constructs of all small HERC proteins were co-transformed with hPLIC-2 revealing an interaction with HECT domains of HERC3, HERC4, and HERC6 in yeast (data not shown).

To investigate interaction of hPLIC-2 with HERC proteins in human cells, we performed co-immunoprecipitation experiments. HEK293 were transfected with myc-tagged HERC3, 4, 6 and flag-hPLIC-2 full-length or flag-hPLIC-2 lacking the UBA domain (Δ). To assess if HERC ubiquitin ligase activity is important for interaction, we also co-expressed a HERC3 active cysteine1018-to-alanine mutant (HERC3CA). HERC proteins were pulled down with myc-specific antibody and the presence of hPLIC-2 was detected with flag-antibody. As a result, both full-length hPLIC-2 and hPLIC-2ΔUBA, were co-precipitated with HERC3, indicating that the HERC binding site in hPLIC-2 is not solely composed of the UBA domain (Fig. 3B). HERC3CA was readily precipitated by

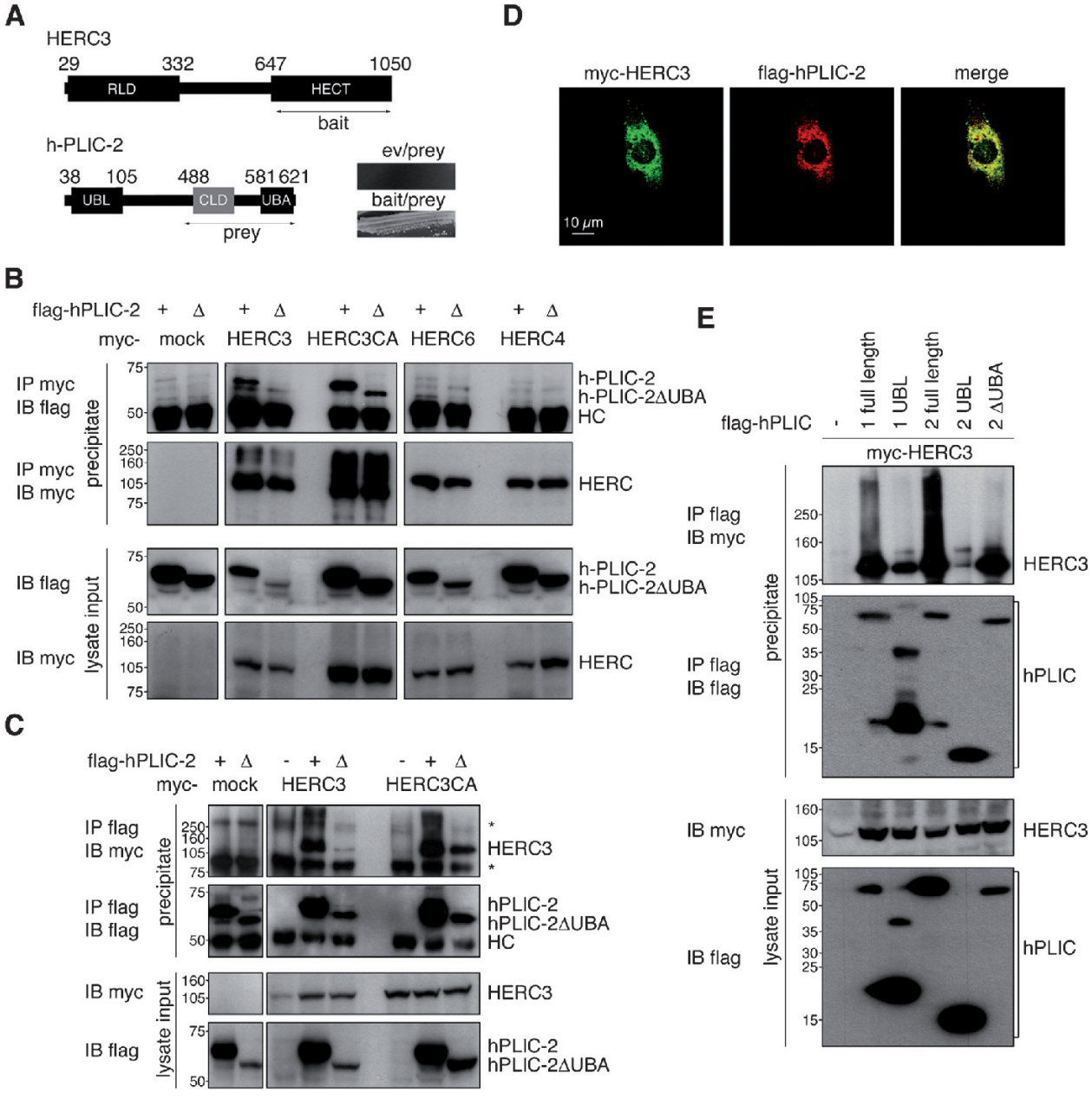

Figure 3. HERC3 interacts with the ubiquitin-like proteins hPLIC-1 and hPLIC-2. (*A*) Yeast two-hybrid screening was performed with the HECT domain of HERC3 (bait) to screen a HeLa cDNA library, thereby identifying the C-terminal UBA domain of hPLIC-2 (prey). The interaction was confirmed using selective yeast media. (*B*) Co-immunoprecipitation was carried out in HEK293 transiently expressing flag-hPLIC-2 or hPLIC-2ΔUBA together with myc-HERC3, HERC3 C1018A (HERC3CA), HERC4 or HERC6. HERC proteins were pulled down with myc antibody and the presence of hPLIC-2 was detected by Western blotting with flag-specific antibody. (*C*) Co-immunoprecipitation as in (*B*), but with precipitating hPLIC-2 and detecting HERC3 in Western blotting. (*D*) For co-localization, myc-HERC3 and flag-hPLIC-2 were transiently expressed in HeLa, followed by antibody staining, and confocal microscopy. (E) HEK293 transfected with myc-HERC3 and flag-hPLIC-1, hPLIC-1 UBL, hPLIC-2, hPLIC-2 UBL, or hPLIC-2ΔUBA were lysed, hPLIC proteins were precipitated with flag-agarose and HERC3 was detected in Western blotting with myc-specific antibody. ev, empty vector; RLD, RCC1-like repeats; HECT, homologous to E6AP C terminus; UBL, ubiquitin-like domain; CLD, collagen-like domain; UBA, ubiquitin-associated domain; HC, heavy chain; *, unspecific band.

hPLIC-2, suggesting that binding of HERC3 to hPLIC-2 is independent of its ubiquitin ligase activity. Interestingly, in contrast to the yeast experiment, no binding of hPLIC-2 to HERC4 or HERC6 was monitored, showing that the interaction between HERC3 and hPLIC-2 is specific (Fig. 3B). Endorsing these results, hPLIC-2 as well as hPLIC-2ΔUBA co-precipitated HERC3 and HERC3CA via pull down with flag-agarose (Fig. 3C). Substantial co-localization of hPLIC-2 and HERC3 in HeLa cells further

supported interaction between the two proteins (Fig. 3D).

Comparison of identified yeast clones suggested the C-terminal 220 amino acids of hPLIC-2 including the UBA domain and the CLD as putative E3 binding site. As HERC3 interacted with hPLIC-2ΔUBA in human cells (Fig. 3B, C), we determined possible binding to the CLD. We tested HERC3 interaction with hPLIC-1, which is 80% identical to hPLIC-2, but does not harbor the CLD [22]. As shown in Figure 3E, HERC3 bound with the same affinity to hPLIC-1 as to hPLIC-2, ruling out the involvement of CLD in the interaction. Since the N-terminal UBL domain of hPLIC-1 and hPLIC-2 interacts with the HECT ubiquitin ligase E6AP [22], we next examined HERC3 binding to this domain. HERC3 was pulled down by both UBL constructs, although with markedly lower affinity than with full-length or ΔUBA plasmids (Fig. 3E). To summarize, we show that among small HERC proteins HERC3 specifically binds to at least two distinct sites in hPLIC-1 and hPLIC-2.

hPLIC-2 UBA domain binds to poly-ubiquitinated HERC3. When co-precipitating HERC3 with full-length hPLIC-1 or hPLIC-2, but not with hPLIC-2ΔUBA or the UBL constructs, a high molecular weight smear was detected as seen commonly with ubiquitinated proteins (Fig. 3C, E). The UBA domain was shown earlier to bind to polyubiquitin chains [42], hence it seemed reasonable that the hPLIC-2 UBA domain could recruit ubiquitinated HERC3. To address this question, we performed an experiment with histidine-tagged ubiquitin to pull down ubiquitinated proteins under denaturating conditions with nickel-nitrilotriacetic acid (Ni-NTA) beads. To evaluate the implication of the proteasome, we added the proteasome inhibitor MG132. Ubiquitinated forms of HERC3 detected with myc-specific antibody were slightly enriched by addition of MG132; however, when hPLIC-2 was co-expressed, polyubiquitinated HERC3 substantially increased even in the absence of MG132 (Fig. 4). In contrast, hPLIC-2ΔUBA had less effect on recruitment of ubiquitinated HERC3. As HERC3 was shown to be an active E3 ligase [9], it seemed possible that hPLIC-2 could be ubiquitinated by HERC3. However, no ubiquitinated hPLIC-2 was detected with flag-specific antibody (Fig. 4), signifying that hPLIC-2 is not a substrate of HERC3. In conclusion, expression of hPLIC-2 leads to an UBA domain-dependent accumulation of ubiquitinated HERC3.

HERC5 interacts with the metastasis suppressor Nm23B. In addition to hPLIC-2, yeast two-hybrid screening with pGBKT7-HERC3-HECT led to isolation of two clones that we identified as tumor metastasis suppressor Nm23B. To determine the specificity of the interaction in yeast, we re-transformed the isolated Nm23B plasmid with either empty vector (ev) or different HERC constructs (Fig. 5A). As indicated by growth on selective yeast media, Nm23B bound specifically to the HECT domain of HERC proteins. Thereby interaction with HERC3, HERC5, and HERC6 was more pronounced than with HERC4.

To further investigate binding of HERC and Nm23B, HEK293 cells were transfected with expression vectors containing flag-HERC or Nm23B-myc. Lysates of transfected cells were used for co-immunoprecipitation with anti-flag antibody, followed by immunoblotting with an anti-myc antibody. The blots showed that Nm23B interacted specifically with HERC5, whereas binding was not detected with HERC3, HERC4, or HERC6 (Fig. 5B). Interaction between HERC5 and Nm23B was further supported by considerable co-localization in HeLa cells (Fig. 5C). To summarize, Nm23B was found in yeast to interact with all small HERC proteins. However, in human cells interaction was specific for HERC5.

HERC5 is required for ubiquitination of Nm23B. Having established an interaction between HERC5

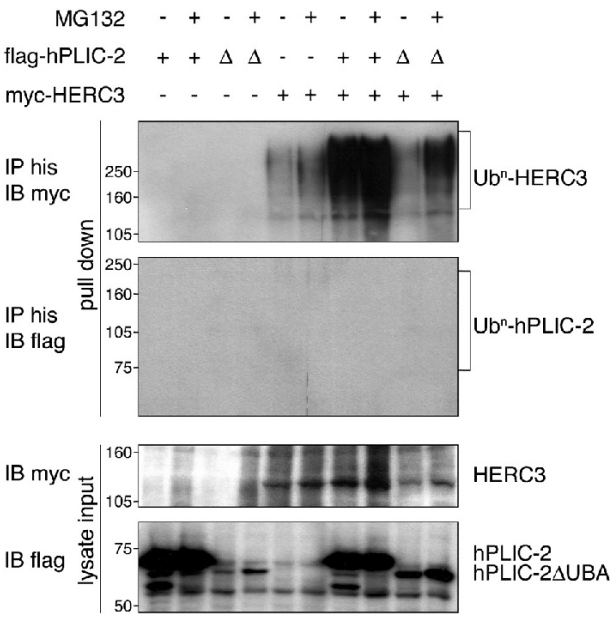

Figure 4. hPLIC-2 binds poly-ubiquitinated HERC3 via the UBA domain. HEK293 were transfected with myc-HERC3, flag-hPLIC-2, flag-hPLIC-2ΔUBA together with his-ubiquitin and where indicated cells were treated with MG132. Cells were lysed in buffer containing guanidine hydrochloride, his-ubiquitin-coupled proteins were pulled down with Ni-NTA-conjugated beads and precipitates were probed with myc- and flag-specific antibodies for the presence of HERC3 and hPLIC-2, respectively.

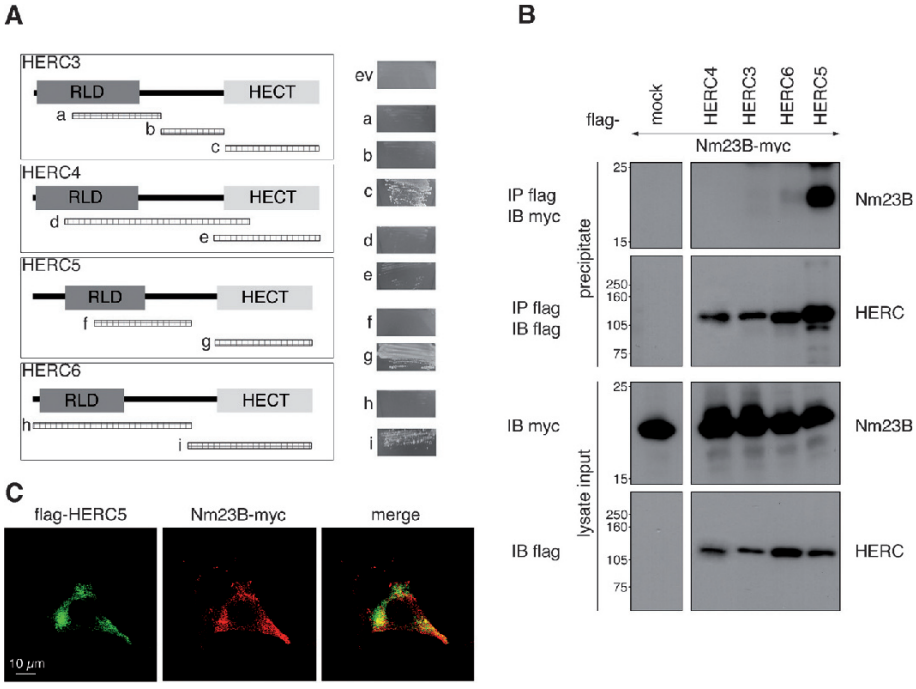

Figure 5. HERC5 specifically binds the metastasis suppressor Nm23B. (*A*) Interaction of different HERC baits (a–i) with Nm23B in yeast was determined by growth on selective media. As a control, Nm23B was co-expressed with pGBKT7 empty vector (ev). (*B*) Co-immunoprecipitation of HERC proteins with Nm23B was performed in HEK293 transfected with plasmids for flag-HERC and Nm23B-myc. HERC were precipitated with flag-agarose and the presence of Nm23B was tested with myc antibody. (*C*) HeLa were transfected with indicated expression vectors and immunostained for HERC5 and Nm23B with myc and flag antibodies.

and Nm23B we wanted to know, if HERC5 is capable of directing Nm23B ubiquitination. We transfected HEK293 cells with flag-HERC5, Nm23B-myc and histidine-tagged ubiquitin constructs and purified whole-cell extracts over Ni-NTA beads. Precipitates were analyzed for the presence of ubiquitinated Nm23B by immunoblotting with anti-myc antibody. In absence of HERC5 no ubiquitinated Nm23B was detected, but co-expression of HERC5 efficiently stimulated Nm23B ubiquitination (Fig. 6A). To investigate whether Nm23B is targeted by HERC5 for proteasomal degradation, we treated cells with MG132. Addition of MG132 did not lead to a further increase of ubiquitinated Nm23B (Fig. 6A). Also ubiquitinated Nm23B did not appear as a characteristic high-molecular weight smear normally seen for proteins targeted for degradation by the proteasome, suggesting that ubiquitination of Nm23B by HERC5 serves a function other than marking for destruction. To endorse this theory we examined protein stability of Nm23B in absence and presence of HERC5. Cells were transfected either with Nm23B-myc alone or with Nm23B-myc together with flag-HERC5 and treated with cycloheximide (CHX) for 0, 4, 8, 12, and 16 h. Independent of HERC5 co-expression, Nm23B protein levels remained unchanged even after 16 h CHX treatment (Fig. 6B). In summary, Nm23B ubiquitination is mediated by HERC5. Stable Nm23B protein in presence of HERC5 as well as proteasome-independent ubiquitination suggest that ubiquitination of Nm23B serves a different purpose than marking it for degradation.

Discussion

In an earlier study we have shown that small HERC proteins have evolved from a common ancestral gene and are therefore closely related [6]. In the present study we show co-localization of all small HERC proteins with late endosomes and lysosomes. Further, we demonstrate interaction between family members; however, by identifying specific interactions of HERC3 with hPLIC-2 and HERC5 with Nm23B, we provide evidence that despite their similarities, small HERC proteins may perform different functions. Finally, we show that HERC5 is necessary for ubiquitination of Nm23B.

Localization of small HERC proteins to vesicular structures is in line with earlier findings. Whereas HERC1 was shown to localize to the Golgi region, HERC3 was mostly co-localized with the early endosomal marker Rab5 [8, 9]. In agreement with these data we did not find any co-localization of small HERC proteins with Golgi membranes [9]; however, while we generally confirm localization to the endocytic pathway, we observe greater co-localization with late endosomes and lysosomes than with early endosomes. One possible reason for this discrepancy might be that Cruz et al. [9] used specific antibodies to stain endogenous HERC3, whereas we performed immunofluorescence with over-expression of HERC proteins. But considering that both endogenous and exogenous HERC3 show the same localization pattern [6], it is unlikely that this accounts for the difference in HERC co-local-

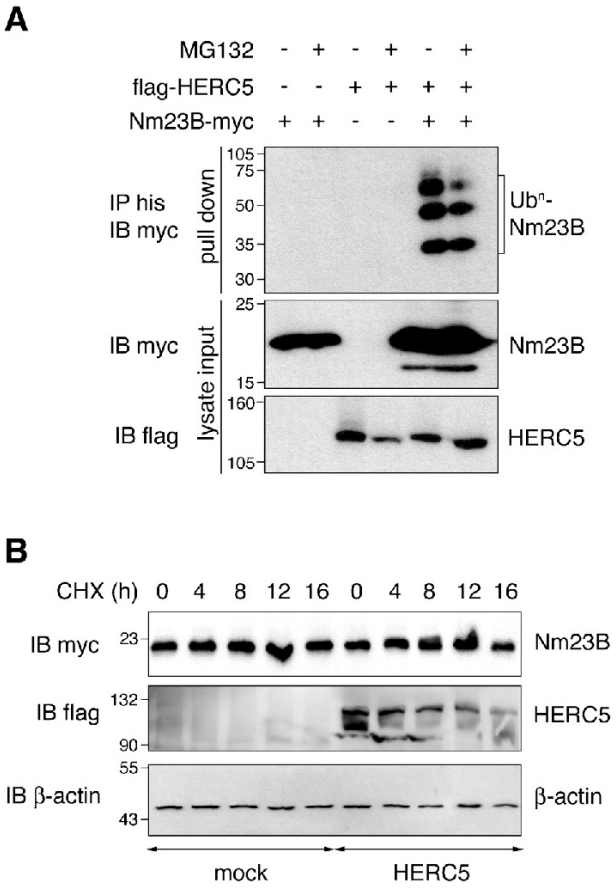

Figure 6. Expression of HERC5 results in ubiquitination of Nm23B without influencing its protein stability. (*A*) Flag-HERC5, Nm23-myc and his-ubiquitin were transiently expressed in HEK293. Prior to lysis under denaturing conditions, cells were treated with MG132. Ubiquitinated proteins were captured with Ni-NTA beads and Nm23B was detected in Western blotting with myc antibody. (*B*) HEK293 were transfected with Nm23B-myc and flag-HERC5. At 24 h after transfection, cells were treated with CHX for the indicated times. Lysates were subject to SDS-PAGE and expression of Nm23B and HERC5 was monitored by immunoblotting with myc and flag antibodies, respectively. As loading control, expression of β-actin was monitored.

ization. Another possibility might be that different cell lines and compartmental markers were used in the studies. However, as HERC proteins are mainly located around the nucleus, localization to late endosomal and lysosomal compartments seems reasonable.

HERC proteins are not the first HECT E3 ligases localizing to the endocytic pathway, as a considerable number especially of the WW-containing Nedd4 subfamily play important roles in receptor endocytosis. Yeast Rsp5p and its mammalian homolog Nedd4 were both shown to associate with endosomal compartments and regulate stability, *e.g.*, of the insulin-like growth factor I receptor, the vascular endothelial growth factor receptor-2 and epithelial sodium channels [43–46]. ITCH mediates the ubiquitination and subsequent endocytosis of the CXC chemokine receptor 4 and transient receptor potential channels [47, 48]. As HERC E3 ligases appear to be located at late endosomes and lysosomes, a role in sorting of membrane proteins into the lumen of multivesicular bodies rather than in internalization is expected. HERC3 was shown earlier to be cytosolic and only present in small amounts in membrane fractions [9]. As neither a transmembrane region nor a signal sequence enabling HERC proteins to integrate into or enter vesicular membranes can be found by bioinformatical analyses, an association at the cytoplasmic site of vesicles seems plausible.

Interaction between all small HERC proteins might indicate assembly to dimers or multimers, although this would be rather unexpected as HECT E3 ligases are mainly known to act as monomers. In contrast to RING E3 ligases, which are well known to multimerize, only few HECT E3 ligases function as dimers. Interestingly, most of them have assigned functions in receptor endocytosis. Association of ITCH with the RING E3 ligase Cbl contributes negatively to epidermal growth factor receptor down-regulation by impairing its ubiquitination [49], and recruitment of the RING E3 ligase RNF11 by Smurf2 and ITCH promotes AMSH degradation [50]. Currently there is no example of an interaction between two members of the HECT E3 ligase family. To rule out the possibility that over-expression leads to agglomeration of HERC proteins, co-precipitation of endogenous proteins will be necessary. However, we have not yet succeeded in generating antibodies against HERC proteins functioning in Western blotting. Neither immunization of rabbits with purified truncated HERC-constructs nor with synthetic peptides resulted in HERC-specific immunogenic antisera. Also commercially available antibodies failed to detect HERC proteins in Western blotting. As soon as appropriate antibodies are available it will be possible to investigate the potential physiological interaction of the family members with each other in more detail.

We identified hPLIC-2 and Nm23B as interaction partners for HERC proteins via yeast two-hybrid screening. In human cells, interactions were specific for hPLIC-2 and HERC3, and Nm23B and HERC5; however, in yeast all small HERC proteins were capable of binding to each partner. The variance between the two systems might be explained by the use of different HERC constructs. While co-immunoprecipitation in HEK293 was carried out with full-length proteins, yeast two-hybrid interaction was observed with truncated HECT domain proteins. Since small HERC proteins possess most homology in the HECT domain [6], use of HECT only constructs might result in insufficient specificity.

As demonstrated for E6AP [22], we show interaction of HERC3 with the UBL domain of hPLIC; however, our studies in yeast and also in human cells suggest binding of HERC3, at least in part, also to the UBA domain. Presence of the UBA domain increased the amount of bound HERC3 to hPLIC and co-precipitating HERC3 with full-length hPLIC, in contrast to hPLICΔUBA, resulted in the detection of high molecular weight HERC3 bands. As the UBA domain of hPLIC binds to poly-ubiquitinated proteins [51] and it was shown earlier that HERC3 is ubiquitinated [9], we speculated that the UBA domain of hPLIC binds to ubiquitinated HERC3. Indeed, like inhibition of the proteasome, co-expression of hPLIC-2 led to accumulation of ubiquitinated HERC3, which was not achieved by a variant of hPLIC-2 without the UBA domain. Therefore, it is possible that hPLIC UBA inhibits degradation of ubiquitinated HERC3 either by generally blocking the proteasome or by specifically capturing HERC3, thereby leading to its accumulation. Interference of hPLIC with proteasomal degradation of ubiquitinated proteins was shown earlier, but since the ubiquitin-independent proteasomal target ornithine decarboxylase is normally degraded in presence of hPLIC, a total block of the proteasome by hPLIC UBA can be excluded [22, 23]. It can rather be assumed that degradation of proteasomal substrates like HERC3 is negatively influenced by a specific interaction of the UBA domain with polyubiquitin chains linked to target proteins, by a yet unknown recognition mechanism.

Association between hPLIC and non-ubiquitinated HERC3 was detected using hPLIC proteins without UBA domains. Such proteins are artificial, but it seems possible that a change of conformation of hPLIC under certain conditions allows for binding of poly-ubiquitin to the UBA domain in one case and binding of proteins to the UBL domain in another. A possible mechanism for this was provided by studies on HHR23A, which like hPLIC contains one UBL domain, but two UBA domains [52, 53]. By itself, HHR23A adopts a closed conformation defined by the interaction of the UBL with the two UBA domains. Binding of the proteasomal subunit S5a disrupts the HHR23A inter-domain interactions and thereby causes it to adopt an open conformation [52]. *Vice versa*, binding of polyubiquitin to the UBA domains promotes the association of the UBL domain with ligands such as proteasome subunits [53].

The biological functions of small HERC proteins are poorly understood. Since both interaction partners, Nm23B and hPLIC, are partly implicated in endocytosis and vesicular trafficking and HERC proteins are located at endocytic structures, it seems reasonable to speculate that HERC proteins might also play their part in regulating vesicular transport. Interestingly, both Nm23B and hPLIC associate with GPCRs. While hPLIC proteins regulate endocytosis of V2 vasopressin receptor, beta-2 adrenergic receptor and CXC chemokine receptor 4 [27, 54], Nm23B supports internalization of the thromboxane A2 receptor [36]. It has been extensively shown that ubiquitination critically influences endocytosis, trafficking and lysosomal degradation of receptor tyrosine kinases [2], but for GPCRs no such strong connection could be established so far. In contrast to receptor tyrosine kinases, it has been suggested that the ubiquitin-proteasome system plays a crucial role in GPCR degradation [55]. hPLIC-2 was shown to bind to E3 ligases βTRCP and E6AP as well as proteasomes, suggesting that proteasomes, E3 ligases and hPLIC proteins exist together in a complex [22]. According to that model we were able to show co-immunoprecipitation of HERC3 with hPLIC-2 and the proteasomal subunits Rpn10 and HC-2 (data not shown). Involvement of hPLIC in proteasomal degradation [56, 57] as well as in receptor trafficking points at a possible role as shuttling factors linking proteasomes to the endocytic pathway. As HERC3 was found in a complex with hPLIC-2 and proteasomal subunits and was shown to localize to endosomal compartments, it might also participate in linking these two cellular functions.

In addition to its implication in receptor endocytosis, Nm23B regulates transcription of the c-myc gene and therefore proliferation of cells [32]. Recently, Nm23B was shown to be up-regulated by amplification of N-myc in neuroblastoma, thereby influencing Cdc42 function, a crucial regulator of cell differentiation [58]. As HERC5 binds to cyclins and was shown to have high mRNA expression levels in proliferating cells and tissues [6, 13], it seems possible that it has like Nm23B a role in proliferation and cell cycle regulation. In contrast to all other HERC family members, HERC5 contains an extremely positively charged N terminus harboring a nuclear localization signal. We found that, although full-length HERC5 protein is mainly located in the cytoplasm, a N-terminally truncated protein was solely located in the nucleus (data not shown). Therefore, nuclear translocation of HERC5 seems plausible, although the stimulus remains elusive. Like HERC5, the majority of Nm23B appears to be cytosolic; however, dependent on the cell-cycle phase, it can also be found in the nucleus [59, 60].

We have shown that expression of HERC5 leads to ubiquitination of Nm23B, but that Nm23B protein level is not influenced by co-expression of HERC5. Therefore, we think that ubiquitination of Nm23B directed by HERC5 influences its function rather than

marking it for destruction. This is also supported by the fact that the pattern of ubiquitinated Nm23B resembles three sharp bands and not the characteristic poly-ubiquitin smear observed for proteins targeted for degradation. As conjugation of tetra-ubiquitin represents the minimum signal for efficient proteasomal targeting of substrates [61], it is unlikely that ubiquitinated Nm23B is recognized by the proteasome. Accordingly, inhibition of the proteasome did not lead to an increase in Nm23B ubiquitination. Importantly, many components of the endocytic machinery as well as transcription factors are ubiquitinated without being degraded [62–64].

To finally prove that HERC5 acts as E3 ligase for ubiquitination of Nm23B, it would be necessary to establish an *in vitro* system with purified recombinant proteins. However, as also observed by others [18, 19], the generation of recombinant functionally active full-length HERC proteins is problematic and we have not been successful with that approach. Nevertheless, the ability of HERC5 to interact with both ubiquitin [14] and Nm23B is consistent with HERC5 being the E3 ligase directing Nm23B ubiquitination.

In summary, we find that members of the small HERC family co-localize and interact with each other, but otherwise undergo different molecular interactions. Whereas hPLIC proteins seem to influence HERC3 ubiquitination, HERC5 is implicated in ubiquitination of Nm23B. Identification of hPLIC and Nm23B as HERC-binding proteins constitutes an important step forward in determining function of HERC proteins; however, further work will be necessary to unravel the molecular consequences of the shown interactions.

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