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E3 ubiquitin ligase HECW2 targets PCNA and lamin B1

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ABSTRACT

Lamins constitute the major architectural proteins of the nuclear lamina that help in maintaining nuclear organization. Mutations in lamins are associated with diverse degenerative diseases, collectively termed laminopathies. HECW2, a HECT-type E3 ubiquitin ligase, is transcriptionally upregulated in HeLa cells expressing Emery-Dreifuss muscular dystrophy-causing-lamin A mutants. However, the role of HECW2 upregulation in mediating downstream effects in lamin mutant-expressing cells was previously unexplored. Here, we show that HECW2 interacts with two lamin A-binding proteins, proliferating cell nuclear antigen (PCNA), *via* a canonical PCNA-interacting protein (PIP) motif, and lamin B1. HECW2 mediates their ubiquitination and targets them for proteasomal degradation. Cells expressing lamin A mutants G232E and Q294P, in which HECW2 is upregulated, show increased proteasomal degradation of PCNA and lamin B1 most likely mediated by HECW2. Our findings establish HECW2 as an E3 ubiquitin ligase for PCNA and lamin B1 which regulates their levels in laminopathic cells. We also found that HECW2 interacts with wild-type lamin A and ubiquitinates it and this interaction is reduced in case of lamin mutants G232E and Q294P. Our findings suggest that interplay among HECW2, lamin A, PCNA, and lamin B1 determines their respective homeostatic levels in the cell and dysregulation of these interactions may contribute to the pathogenicity of laminopathies.

1. Introduction

The nuclear lamina is an electron dense proteinaceous meshwork that lies beneath the nucleoplasmic face of the inner nuclear membrane. Lamins are the major architectural components of the lamina in metazoan nucleus and belong to the type V intermediate filament superfamily. Nuclear lamins are divided into two types on the basis of their expression patterns and biochemical characteristics: A-type and B-type lamins. A-type lamins are encoded by a single gene *LMNA* which gives rise to 4 isoforms, namely, lamin A, C, C2, and AΔ10. B-type lamins include lamin B1 and lamins B2 and B3 which are encoded by *LMNB1* and *LMNB2*, respectively. Lamins play a pivotal role in providing structural and mechanical support to the nucleus. They are involved in a multitude of nuclear functions such as chromatin organization, DNA replication and repair, transcription and gene regulation. More than 300 different mutations identified in *LMNA* gene are associated with 17 distinct degenerative diseases, collectively termed laminopathies. These include muscular dystrophies such as Emery-Dreifuss muscular dystrophy (EMD), limb-girdle muscular dystrophy 1B (LGMD1B), dilated cardiomyopathy (DCM), lipodystrophies, peripheral neuropathies, and accelerated aging syndromes such as Hutchinson-Gilford progeria syndrome (HGPS) [1–8].

Many of the laminopathies are characterized by abnormal nuclear morphology with increased nuclear blebbing, defective nucleocytoskeletal coupling and mechanotransduction, loss of peripheral heterochromatin, and defects in DNA replication and repair. Previous reports have revealed that lamin mutations lead to mislocalization of the inner nuclear membrane protein emerin and nesprin-1α and their subsequent degradation by the proteasome [9]. Mouse embryonic fibroblasts (MEFs) lacking lamin A show mislocalization of emerin [10], and increased proteasomal degradation of retinoblastoma protein (pRb) and DNA repair protein p53-binding protein 1 (53BP1) [11,12]. Mislocalization or loss of several other proteins such as transcription factors sterol regulatory element-binding protein 1 (SREBP1), MOK-2 and inhibitor of growth protein 1 (ING1), pRb, nuclear pore complex protein Nup153, ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR) protein kinases has been reported in cells expressing muscular dystrophy- and HGPS-causing lamin mutants [13–17]. Expression of EMD-causing lamin mutants or knockdown of endogenous lamins in HeLa cells induces proteasomal degradation of heterochromatin marker proteins HP1α and β. In these mutant cells, three distinct E3 ubiquitin ligase components, namely, F-box substrate adaptor protein FBXW10, RING finger E3 ligase RNF123 and HECW2, are transcriptionally upregulated [18,19].

HECW2 is a HECT (homologus to E6-AP carboxy terminus)-type E3 ubiquitin ligase belonging to the NEDD4 (neural precursor cell

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expressed developmentally down-regulated protein 4) family. The domain organization of HECW2 shows that it contains a C2 domain at its N-terminus which is known to be involved in phospholipid/Ca^{2+} signaling, two WW domains which are thought to be the substrate-binding domains and a HECT domain at its C-terminus which contains the catalytic cysteine residue required for substrate ubiquitination [20,21]. HECW2 is reported to mediate ubiquitination and subsequent stabilization of tumor suppressor p73 [22], and is known to be regulated proteasomally by the anaphase-promoting complex (APC) [23]. HECW2 is also known to mediate proteasomal degradation of DNA damage checkpoint signaling kinase, ATR, in lamin-misexpressing cells [17]. Mass spectrometry screening for HECW2-interacting proteins identified a number of lamin-associated proteins such as lamin B1, prelamin A, emerin, HP1 isoforms, and proliferating cell nuclear antigen (PCNA) as potential substrates of HECW2 [23].

The homotrimeric sliding clamp, PCNA, is the processivity factor for DNA polymerase δ which is essential for elongation phase of replication [24]. Lamins play an important role in DNA replication as disruption of lamin organization directly inhibits DNA synthesis [25–27]. Lamin A colocalizes with sites of DNA synthesis in early S phase while lamin B1 foci overlap with PCNA foci at DNA synthesis sites in mid to late S phase [28,29]. Introduction of dominant negative lamin A and B mutants causes lamin aggregation and mislocalization of PCNA and the large subunit of replication factor C (RFC) to these aggregates [30–33]. Furthermore, both lamin A/C and lamin B1 directly bind PCNA *via* their immunoglobulin-like (Ig-like) fold at the C-terminus [34].

In the present study, we have investigated the function of E3 ubiquitin ligase HECW2 in regulating PCNA and lamin B1 in cells expressing EMD-causing lamin A mutants. We have analyzed interactions between HECW2 and PCNA and lamin B1, followed by studying the effects of HECW2 overexpression on PCNA-associated functions. We have subsequently examined the effect of HECW2 upregulation on PCNA and lamin B1 in cells expressing the lamin A mutants G232E and Q294P. We demonstrate that EMD-causing lamin A mutants promote proteasomal degradation of PCNA and lamin B1 by upregulating HECW2. Our findings provide important insights into the role of HECW2 in the proteasomal degradation of key nuclear proteins in lamin mutant-expressing cells.

2. Materials and methods

2.1. Plasmids and shRNAs

GFP-tagged wild-type lamin A and mutant lamin A constructs have been described earlier [35,36]. The full-length HECW2 construct was provided by Dr. Akira Nakagawara (Chiba University, Japan). GFP- and FLAG-tagged HECW2 were generated by sub-cloning the full-length coding sequence (CDS) into pEGFP-C1 mammalian expression vector (Clontech) and pFLAG-C2 mammalian expression vector (generated in the lab by replacing the GFP cassette of pEGFP-C1 with 3X FLAG tag), respectively. Point mutations in the PIP box of HECW2 (PIP3A) and in the HECT domain (C1540A) were generated using QuikChange Site-Directed Mutagenesis kit (Stratagene Cloning System) according to the manufacturer's instructions (Supplementary Table 1). Full-length PCNA was cloned from cDNA of HEK293T cells (obtained from HEK293T total RNA by reverse transcription using Superscript II reverse transcriptase kit (Invitrogen) as per the manufacturer's protocol) into pEGFP-C1 and pFLAG-C2 mammalian expression vectors (Supplementary Table 1). HA-tagged ubiquitin construct cloned in pCI vector was obtained from Dr. Takeshi Inoue (University of Tokyo, Japan). The shRNA sequences targeting HECW2 were cloned into pcDNA 6.2-GW/EmGFP-miR vector (Life Technologies). The targeting sequences used were as follows: (shRNA 2) 5′ –CAGGGAAGTTAAAGTTAATTT– 3′, (shRNA 3) 5′ –GGA GGCCTGATCATGTTTATT– 3′ [23], and the scrambled control had 5′ –GAAATGTACTGCGCGTGGAGAC– 3′ sequence. All the constructs were confirmed by sequencing.

2.2. Cell culture and treatments

HEK293T cells and HeLa cells were cultured in DMEM supplemented with 10% FBS and penicillin-streptomycin and ARPE-19 cells were cultured in DMEM/F12 supplemented with 10% FBS and penicillin-streptomycin at 37 °C in humidified conditions with 5% CO_2. Transient transfections were carried out using Lipofectamine 2000 or 3000 (Life Technologies) as per the manufacturer's protocol. The following chemicals were used at the mentioned concentrations: 6 μM MG132 (Calbiochem), 20 ng/ml Leptomycin B (Sigma), 100 μg/ml cycloheximide (Sigma). UV irradiation was carried out after removing the media and subjecting the cells to a controlled dose of UV using a UV cross-linker (Amersham Life Sciences).

2.3. Antibodies

Primary antibodies used in this study were those against actin (ab3280, Abcam), GAPDH (MAB374, Millipore), PCNA (ab29, Abcam), HECW2 (SAB4502511, Sigma and ab92711, Abcam), GFP (ab290, Abcam), FLAG (F3165, Sigma), lamin A/C (sc-6215, Santa Cruz, ab58529, Abcam and ab8984, Abcam), lamin B1 (ab16048, Abcam), HA (11583816001, Roche and sc-805, Santa Cruz), Ubiquitin (sc-8017, Santa Cruz and ab7780, Abcam), γ-H2Ax (sc-8656, Santa Cruz), NF-κβ p65 (sc-109, Santa Cruz), β-catenin (sc-7963, Santa Cruz), BrdU (Na-61, Calbiochem), cleaved caspase-3 (#9661S, Cell Signaling Technology), cyclin B1 (C23420, BD Transduction laboratories), cyclin D1 (Santa Cruz), cyclin A and E (Santa Cruz), caspase-3 (NB100-56708, Novus Biologicals), emerin (ab40725, Abcam), fibrillarin (sc-25397, Santa Cruz), normal rabbit IgG and normal mouse IgG (sc-2025 and sc-2027, Santa Cruz). Secondary antibodies used for western analysis were donkey anti-rabbit, sheep anti-mouse (both from GE Healthcare), donkey-anti goat (Millipore), goat-anti rabbit and goat-anti mouse (Abcam). Secondary antibodies used for immunofluorescence were Cy3-conjugated sheep anti-mouse, Cy3-conjugated donkey anti-rabbit and Cy5-conjugated anti-mouse (Jackson ImmunoResearch).

2.4. Immunoblotting analysis

For immunoblotting experiments, cells were lysed in 3X Laemmli buffer (180 mM Tris (pH 6.8), 6% SDS, 15% glycerol, 7.5% β-mercaptoethanol, and 0.01% bromophenol blue). Images were captured using Vilber-Lourmat Chemicapt. Densitometry analysis for western blotting was performed using ImageJ software (NIH) and images were processed in Adobe Photoshop CS3. The intensity values plotted or mentioned are average values from the number of biological replicates indicated in the legend.

2.5. Immunofluorescence assays

Cells plated on coverslips were washed with PBS followed by fixation with 3.7% formaldehyde at room temperature (RT) for 10 min or in ice-cold methanol at −20 °C for 15 min. Formaldehyde-fixed cells were subjected to a round of permeabilization with 0.5% Triton X-100 in PBS for 6 min. Cells were then washed with PBS followed by blocking with 0.5% gelatin in PBS for 1 h. Cells were then incubated with the primary antibody diluted in PBS for 1 h at RT followed by staining with secondary antibody diluted in PBS for 1 h at RT. Cells were then washed with PBS and the coverslips were mounted in Vectashield mounting medium containing DAPI (Vector Laboratories). For PCNA staining, cells were methanol-fixed and processed as above. Images were acquired on Leica TCS SP8 confocal microscope using Leica Application Suite X (LAS X) software. Images were processed in Adobe Photoshop CS3 and quantified by counting the cells manually. DAPI was used as an internal control for all the analyzed images.