A non-proteolytic role for ubiquitin in deadenylation of MHC-I mRNA by the RNA-binding E3-ligase MEX-3C

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The regulation of protein and mRNA turnover is essential for many cellular processes. We recently showed that ubiquitin—traditionally linked to protein degradation—directly regulates the degradation of mRNAs through the action of a newly identified family of RNA-binding E3 ubiquitin ligases. How ubiquitin regulates mRNA decay remains unclear. Here, we identify a new role for ubiquitin in regulating deadenylation, the initial and often rate-limiting step in mRNA degradation. MEX-3C, a canonical member of this family of RNA-binding ubiquitin ligases, associates with the cytoplasmic deadenylation complexes and ubiquitinates CNOT7(Caf1), the main catalytic subunit of the CCR4-NOT deadenylation machinery. We establish a new role for ubiquitin in regulating MHC-I mRNA deadenylation as ubiquitination of CNOT7 by MEX-3C regulates its deadenylation activity and is required for MHC-I mRNA degradation. Since neither proteasome nor lysosome inhibitors rescued MEX-3C-mediated MHC-I mRNA degradation, our findings suggest a new non-proteolytic function for ubiquitin in the regulation of mRNA decay.
Messenger RNA (mRNA) turnover plays a critical role in the regulation of the majority of cellular processes. Up to 50% of the changes seen in gene expression are estimated to occur at the level of mRNA stability\(^1,2\), although how mammalian mRNA decay is regulated remains poorly understood. Although ubiquitin is traditionally associated with protein degradation, we recently identified a role for ubiquitin in the degradation of mRNA\(^3\). Of the more than 600 E3 ubiquitin ligases described, at least 15 contain an RNA-binding domain\(^4\) in addition to the RING domain, which defines the largest family of E3 ligases and is required for the ubiquitination reaction. In a small interfering RNA (siRNA) ubiquitome screen, we identified MEX-3C, a canonical member of this novel family of RNA-binding ubiquitin E3-likes, which regulates the cell surface expression of major histocompatibility complex (MHC) class I proteins, via the post-transcriptional regulation of MHC-I mRNA. MEX-3C therefore provides a direct link between ubiquitination and mRNA degradation\(^3\).

The four members of the mammalian MEX-3 gene family (MEX-3A–D) each contain two RNA-binding KH domains and a ubiquitin E3-ligase RING domain\(^5\). This family has arisen by gene duplication from the MEX-3 orthologue in Caenorhabditis elegans, which also has two RNA-binding KH domains but lacks the RING domain. MEX-3C binds the 3' untranslated region (UTR) of its target mRNA HLA-A2 (an MHC-I allotype) through its KH domains and together with its cargo mRNA shuttles from the nucleus to the cytosol\(^1,3\). This HLA-A2 mRNA substrate bound to MEX-3C cannot be translated into protein, a function conserved with its C. elegans MEX-3 ancestor that also binds mRNA but lacks the RING domain\(^6\). However, although the C. elegans MEX-3 inhibits translation of its substrate mRNA\(^6\), MEX-3C not only inhibits translation but also induces the degradation of its target mRNA in a ubiquitin-dependent manner\(^3\). In the absence of a RING domain, MEX-3C is still able to inhibit substrate translation, but there is an absolute requirement for the RING domain, and therefore E3-ubiquitin ligase activity, for HLA-A2 mRNA degradation. RINGless MEX-3C therefore behaves more like its C. elegans ancestor, in that its substrate HLA-A2 mRNA is effectively sequestered and not translated, but is no longer degraded.

Eukaryotic mRNAs are protected from decay at their 5' and 3' ends by the cap and poly(A) tail, respectively. The degradation of mRNAs starts with the removal of the poly(A) tail by a process called deadenylation. This process is mediated by the concerted action of two complexes, namely CCR4-NOT and PAN2-PAN3. Studies in several model organisms show that deadenylation is a rate-limiting step for mRNA degradation\(^7\), and its impaired regulation is associated with a variety of cellular conditions in mammalian cells\(^8\). However, how mammalian deadenylation is regulated remains poorly understood.

Our characterization of MEX-3C's E3 ligase activity in the regulation of mRNAs decay now establishes a new role for ubiquitin in the regulation of deadenylation. Here, we show that MEX-3C associates with different members of the cytoplasmic deadenylation complexes and ubiquitinates CNOT7, the main catalytic subunit of the CCR4-NOT deadenylation machinery. Ubiquitination of this subunit (CNOT7) by MEX-3C regulates its deadenylation activity and is required for HLA-A2 mRNA degradation. Moreover, since neither proteasome nor lysosome inhibitors, nor the use of ubiquitin mutants that prevent the formation of protein degradation signalling K11- and K48-linked chains, rescued MEX-3C-mediated mRNA degradation, our findings point to a new non-proteolytic function for ubiquitin in the regulation of mRNA decay.

**Results**

**MEX-3C interacts with the major deadenylation complexes.** To establish the ubiquitin-related mechanism responsible for MEX-3C-mediated degradation of mRNAs, we first sought to identify MEX-3C-binding partners involved in mRNA degradation, or potential ubiquitination substrates. We performed a pull-down with RINGless MEX-3C expressed in HEK293T cells in the presence of RNase-I, followed by mass spectrometry analysis (Supplementary Table 1). The rationale behind this experiment was that the RINGless mutant form of MEX-3C, which is unable to ubiquitinate, should act as a substrate trap and remains bound to its ubiquitination substrates. Analysis of the RINGless MEX-3C interactome reflected MEX-3C's involvement in different stages of RNA metabolism, primarily mRNA processing, with an emphasis on mRNA degradation (Fig. 1a).

The cytosolic degradation of eukaryotic mRNAs requires the initial shortening of the 3'-poly(A) tail (deadenylation) and subsequent removal of the 5'-cap (decapping). Deadenylation is mediated by the concerted action of two complexes, CCR4-NOT and PAN2-PAN3, both of which were found in association with MEX-3C and subsequently confirmed in immunoprecipitated (IP) blots following RNase-I treatment (Supplementary Table 1, Fig. 1b and Supplementary Fig. 1a for control IP). PolyA-binding protein interacts with MEX-3C through its RNA cargo\(^9\). To control for effective RNAse-I treatment, we showed that under the experimental conditions used, our mass spectrometry analysis did not identify PolyA-binding protein bound to MEX-3C (Supplementary Table 1).

The identification of MEX-3C bound to the cytosolic deadenylation complexes is especially relevant as, despite deadenylation of mRNAs being the initial and often rate-limiting step in mRNA degradation\(^9\), little is known about how these deadenylases are regulated in mammalian cells. To determine whether MEX-3C promotes the deadenylation of its endogenous model substrate, HLA-A2 mRNA in-vivo, we used an reverse transcription–PCR-based assay to measure the length of HLA-A2 mRNA poly(A) tail in MEX-3C-expressing cells (Fig. 1c) following fluorescence-activated cell sorting (FACS) (Supplementary Fig. 1b). MEX-3C promotes the shortening of the poly(A) tail of HLA-A2 mRNA (Fig. 1c right panel), but not of the ACTIN control mRNA (Fig. 1c left panel); an activity that requires its ubiquitin-ligase activity as it is not seen with its RINGless mutant form (Fig. 1c). Similar results were obtained for FF-Luc-HLA-A2 3'UTR reporter mRNA\(^3\). Taken together, these results led us to hypothesize that MEX-3C's E3 ligase activity controls mRNA decay through the regulation of deadenylation (Fig. 1d).

We wanted to determine which deadenylation subunit was responsible for the mRNA degradation, and therefore used siRNAs to deplete cells of deadenylase components. MEX-3C bound both the CCR4-NOT and PAN2-PAN3 deadenylase complexes in HEK293T cells. Despite effective depletion of all deadenylase components (Supplementary Fig. 2c), only the siRNA-mediated depletion of CNOT7/8(Caf1), a component of the CCR4-NOT complex, significantly rescued MEX-3C-mediated degradation of endogenous HLA-A2 mRNA (Supplementary Fig. 2b) and of the reporter FF-Luc-HLA-A2 3'UTR mRNA\(^3\) (Fig. 2b). By analogy with RINGless MEX-3C, depletion of the CNOT7/8(Caf1) deadenylase subunit prevented mRNA degradation, but importantly did not affect MEX-3C's ability to repress HLA-A2 translation (Fig. 2a and Supplementary Fig. 2a). The FF-Luc-HLA-A2 3'UTR reporter system\(^5\) was used in this experiment as we previously showed it reduces the bias seen with endogenous HLA-A2 at the transcriptional level\(^3\) and following mRNA maturation. Although depletion of CNOT7/
Identity of interacting proteins is shown in Supplementary Table 1. (labelled specific RNA substrate (Flc-5 MEX-3C-expressing cells were incubated with a 5' strep-MEX-3C pull-downs from wild-type (WT) and RINGless specific (fluorescein labelled) RNA substrate for CNOT7 (ref. 12).) Deadenylation. We therefore set up an in-vitro deadenylation assay using a specific (fluorescein labelled) RNA substrate for CNOT7 (ref. 12). Strep-MEX-3C pull-downs from wild-type (WT) and RINGless MEX-3C-expressing cells were incubated with a 5' fluorescent labelled specific RNA substrate (Flc-5'-UCUAAAUA20) to assay the deadenylation activity of CNOT7 over time. Degradation of 5'-fluorescein-labelled RNA deadenylation substrate was visualized by denaturing polyacrylamide gel electrophoresis. The deadenylation substrate was readily degraded following WT MEX-3C IP, an effect not seen with RINGless MEX-3C (Fig. 3b,c). These results confirm that MEX-3C's E3-ligase activity is required for the deadenylation of its substrate. Ubiquitination of CNOT7 regulates its deadenylation activity. Since Caf1 (CNOT7/8) is the major catalytic component of the CCR4-NOT deadenylation complex, and was the only deadenylation subunit whose depletion prevented MEX-3C-mediated degradation of FF-Luc-HLA-A2 3'UTR mRNA, we determined whether CNOT7 was a ubiquitination target of MEX-3C. Endogenous or overexpressed (HA-tagged) CNOT7 was IP under denaturing conditions to prevent interaction with other proteins. Ubiquitin immunoblot analysis showed CNOT7 ubiquitination with WT but not RINGless MEX-3C (Fig. 4a,b), confirming that CNOT7 is indeed ubiquitinated in a MEX-3C-dependent manner. This ubiquitination did not promote CNOT7 protein degradation (Fig. 1b and Supplementary Fig. 3a,b) as CNOT7’s.
A new non-proteolytic function for ubiquitin in mRNA decay. In addition to its role in proteolysis, ubiquitin provides a signal for a range of non-proteolytic functions, by virtue of forming chains of distinct topologies depending on whether they are linked through one of its seven Lysine (K) residues or at the N-terminus. Substrates modified by K48-linked polyubiquitin chains are targeted to proteasomes for degradation. In contrast, K63-linked chains provide non-proteolytic signals, as characterized in DNA damage and repair pathways, kinase signalling pathways and endocytosis. Since neither proteasome nor lysosome inhibitors rescued MEX-3C-mediated degradation of the FF-Luc-HLA-A2 3′ UTR mRNA, a new non-proteolytic function for ubiquitin in the regulation of mRNA decay was suggested.

To determine the ubiquitin chain linkage required for MEX-3C-mediated degradation of HLA-A2 mRNA, we used a range of Lysine-to-Arginine Ubiquitin-Green Fluorescent Protein (UB-GFP) mutants. These ubiquitin mutants are particularly useful as the co-translational cleavage of GFP from ubiquitin provides a quantitative surrogate marker for mutant ubiquitin expression, as well as providing the CNOT7 4K-mutant, which is no longer ubiquitinated by MEX-3C (Supplementary Fig. 4c) and thus provides a new non-proteolytic function for ubiquitin in the regulation of mRNA decay.

We therefore determined the effect of the ubiquitin mutants on MEX-3C-mediated mRNA degradation. Degradation of the FF-Luc-HLA-A2 3′ UTR reporter mRNA was rescued by ubiquitin mutants that cannot form K6- and K63-linked chains (Fig. 5d) suggesting an important role for these lysine residues in degradation. Furthermore, CNOT7 ubiquitination by MEX-3C was significantly reduced with the K6R and K63R ubiquitin mutants (Supplementary Fig. 4d). Conversely, neither MEX-3C-mediated ubiquitination of CNOT7 (Supplementary Fig. 4d) nor the degradation of FF-Luc-HLA-A2 3′ UTR target mRNA was impaired in the presence of K11- or K48-linkage ubiquitin mutants, which are traditionally associated with protein degradation signals (Fig. 5d). Taken together, these results suggest a non-proteolytic function for ubiquitin in the regulation of mRNA decay by MEX-3C.

protein half-life was unaffected following cycloheximide treatment either in the absence (shMEX-3C) or in the presence of exogenously expressed WT or RINGless MEX-3C (Supplementary Fig. 3c).

We then wanted to determine which CNOT7 lysine residue is ubiquitinated by MEX-3C and use these findings to ask how MEX-3C-mediated ubiquitination of CNOT7 affects its deadenylation activity in vitro, and the stability of the FF-Luc-HLA-A2 3′ UTR reporter mRNA in vivo. The choice of lysines to be mutated by site-directed mutagenesis was based on the structure of CNOT7 and the lysine residues (K200 and K206) reported to be ubiquitinated on mass spectrometry data sets. A representation of CNOT7's structure highlights (in blue) the exposed lysine residues mutated (Supplementary Fig. 4a). (The CNOT7 K203R variant was toxic to cells and therefore excluded from this and further experiments). In comparison to WT CNOT7, ubiquitination of the K206R mutant was mildly impaired, whereas ubiquitination was completely lost with the 4K (K196R,K200R,K203R,K206R) CNOT7 mutant (Fig. 4c).

Using the in-vitro CNOT7 deadenylation assay we found that the CNOT7 4K-mutant, which is no longer ubiquitinated by MEX-3C (Fig. 4c), failed to efficiently degrade CNOT7's deadenylation substrate in vitro (Fig. 4d). Furthermore, overexpression of this mutant form of CNOT7 (4K-mutant) inhibited MEX-3C-mediated degradation of the FF-Luc-HLA-A2 3′ UTR reporter mRNA in vivo (Fig. 4e).

To control for the structural integrity of CNOT7 4K-mutant, we performed the in-vitro CNOT7 deadenylation assay following CNOT7 pull-down in the absence of exogenous MEX-3C as previously described (Suzuki et al.). This CNOT7 4K-mutant remains functionally active in the absence of MEX-3C (Supplementary Fig. 4b) suggesting that the folding of CNOT7 4K-mutant and its ability to form a functional deadenylation complex (Fig. 4d) remained intact. Although ubiquitination is not required for CNOT7's basal deadenylation, these results highlight that this deadenylation activity can be modulated in a novel ubiquitin-dependent manner in mammalian cells for specific mRNAs.

![Figure 2](image_url)
Discussion

We have identified a new role for ubiquitin in the regulation of deadenylation, the initial and rate-limiting step in mRNA degradation. MEX-3C, a member of the recently described family of RNA-binding ubiquitin E3-ligases, associates with the cytoplasmic deadenylation complexes, and ubiquitinates CNOT7, the main deadenylase subunit of the CCR4-NOT machinery. Ubiquitination of CNOT7 by MEX-3C promotes its deadenylation activity and therefore MHC-I mRNA degradation.

In addition to its established role in protein regulation/degradation, ubiquitination provides a critical signal for many other cellular regulatory functions and here, we have uncovered
Figure 5 | A non-proteolytic function for ubiquitin in the regulation of mRNA decay. (a) Neither proteasome nor lysosomal inhibitors rescue MEX-3C-mediated degradation of FF-Luc-HLA-A2-3’UTR mRNA in vivo. CcmA, Concanamycin A. FF-Luc-HLA-A2-3’UTR mRNA levels were analysed as in Fig. 2. Results are expressed as mean ± s.d. of three independent experiments and relative to empty vector control. (b, c) Ubiquitin lysine mutants are unable to rescue MEX-3C-mediated downregulation of HLA-A2 protein levels (b, as determined by flow cytometry analysis) or luciferase protein levels from FF-Luc-HLA-A2-3’UTR reporter (c, as determined by relative luciferase activity). (d) also serves as a control for the ubiquitin mutant (GFP) expression levels for the following experiments. (d) MEX-3C-mediated degradation of FF-Luc-HLA-A2-3’UTR reporter mRNA is rescued by K6R and K63R ubiquitin mutants. *P<0.05 and **P<0.005, both versus +MEX-3C + WT Ubiquitin treatment. NS, not significant; unpaired Student’s t-test. Results for this figure are expressed as mean ± s.d. of three independent experiments and relative to empty vector control/GFP.

A number of potential mechanisms may account for MEX-3C’s ability to regulate mRNA decay through ubiquitination. Ubiquitin may induce conformational changes in CNOT7 that activate deadenylation. Alternatively, ubiquitinated CNOT7 provides a scaffold to recruit accessory proteins for activation of the degradation machinery. A similar ubiquitin-mediated regulation has been observed in the activation and disassembly of the spliceosome at distinct steps of the splicing reaction.

MEX-3C is not the only ubiquitin E3-ligase to bind and regulate RNA, but belongs to a family of at least 15 RNA-binding proteins with ubiquitin ligase activity. Another prominent member of this family is CNOT4, itself a component of the CCR4-NOT deadenylation complex, and has been best studied in yeast where its orthologue (Not4) has multiple functions. These include nuclear transcriptional regulation, mRNA maturation and quality control, co-translation protein quality control and mechanisms that regulate deadenylation in mammalian cells are highly regulated but poorly understood. Deadenylation and RNA turnover play an important role in a broad range of cellular conditions including development, mRNA surveillance, DNA damage, cell differentiation and cancer. Understanding how ubiquitin regulates mRNA abundance and protein production will provide a better mechanistic understanding of different disease states.
proteasome assembly.24 Not4’s contribution to mRNA degradation by Ccr4 (yeast homologue of CNOT6) and Caf1 (yeast homologue of CNOT7) is unclear. By analogy to MEX-3C, CNOT4 may also play a role in activation of the deadenylases by Ccr4 (yeast homologue of CNOT6) and Caf1 family members. Prolongation of the half-life of specific ARE-mRNAs25.

Methods

Cells, plasmids and transfections. HEK293T cells were grown in RPMI-1640 medium supplemented with 10% FCS. Cells were transfected using 293-Transit Reagent (Mirus Bio) and analysed by flow cytometry or immunoblotting at 48 or 72 h following transfection. The Streptag-His-MEX-3C and myc-MEX-3C proteins and the FF-Luc-HLA-A2.3’UTR reporter are the previously described26. The UB-GFP mutants are described by Bonneau J.M. et al.27. The pCMV5-CHA-CNOT7 construct is previously described28. The Lysine mutant forms of CNOT7 were made by site-directed mutagenesis as described in Mittal S. et al.29. For the luciferase quantitative PCR assays, an TK Renilla luciferase reporter (pRL-TK) gene was co-transfected at a 1:20 ratio to provide an internal control. All assays were performed in triplicate, with the Renilla-luciferase control used to standardize transfection efficiency. Results are relative to control levels (set at 1), and expressed as the mean ± S.E. of at least three independent experiments. siRNA-mediated depletion in HEK293T cells was performed using Oligofectamine (Invitrogen) at 75 nM final concentration and following the manufacturer’s guidelines. The siRNAs used were ON-TARGET plus pools of four from Dharmacon: MEX-3C (RKHD2; LU-006897-00-0002), CNOT7 (C7AFI; LU-012897-00-0002), CNOT6 (CCRA; LU-016411-00-0002), CNOT6 (LU-019101-00-0002), CNOT7 (LU-018791-00-0002), PAN2 (LU-012192-00-0002), DCP1A (LU-021192-00-0002). MEX-3C depletion using shRNA against MEX-3C (shMEX-3C) was previously described26. Mock knock-downs (siCONTROL) were performed using RISC-free Universal Control (Sigma). Cells were cultured for 60 h and then assayed by FACScalibur (BD) or quantitative reverse transcription (qRT–PCR).

IP and immunoblotting. For IPs, cells were lysed 72 h post transfection in 1% NP-40 in Tris-Buffered Saline (TBS) with 1 μM ZnCl2, 0.5 mM phenylmethyl sulphonyl fluoride (PMSF), 10 μM iodoacetamide (IAA) and Roche complete protease inhibitor for 30 min on ice. Strep-His-tagged proteins were IP with Streptactin sepharose beads (IBA GmbH) for 2 h. After three washes in lysis buffer, samples were eluted in SDS sample buffer (10 min at 98°C). (For myc-tagged MEX-3C proteins, IPs were done as previously described in Cano et al.) IP samples were then separated by SDS-PAGE, transferred to PVDF membranes, and probed for CNOT7, CNOT6, and HA-CNOT7 constructs. After 72 h, cells were lysed (1% NP-40 in TBS with 5% FCS and visualized with goat anti-mouse Cy5-conjugated secondary antibody (Jackson Immunoresearch Laboratories). Cells were fixed in PBS with 1% paraformaldehyde (PFA), read on a FACScalibur (BD) and analysed in Flowjo.

Flow cytometry. Cells were stained with mAb B87.2 (anti-HLA-A2) primary antibody in PBS + 5% FCS and visualized with goat anti-mouse Cy5-conjugated secondary antibody (Jackson Immunoresearch Laboratories). Cells were fixed in PBS with 1% paraformaldehyde (PFA), read on a FACScalibur (BD) and analysed in Flowjo.
Proteasome and lysosome Inhibition. HEK293T cells were transfected with either pQE empty vector (EV) or WT and Strep-His-MEX-3C, together with the FF-Luc-HLA-A2 3’ UTR and Renilla luciferase reporter (pRL-TK, at a 1:20 ratio). After 48 h transfection, cells were incubated for 4 h before lysis with 40 μM MG-132, 10 μM Lactacystin, 100 nM Concanamycin A or 200 nM Bafilomycin. FF-Luc-HLA-A2-3’ UTR reporter mRNA levels were analysed by qRT–PCR and normalized to Renilla-Luc, to standardize for transfection efficiency.

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Author contributions

F.C. designed and performed the experiments and wrote the manuscript. R.R. designed and performed the experiments. G.S.W. contributed new reagents. P.J.L. helped design the experiments and write the manuscript.

Additional information

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