HRD1 and UBE2J1 target misfolded MHC class I heavy chains for endoplasmic reticulum-associated degradation

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The assembly of MHC class I molecules is governed by stringent endoplasmic reticulum (ER) quality control mechanisms. MHC class I heavy chains that fail to achieve their native conformation in complex with β2-microglobulin (β2m) and peptide are targeted for ER-associated degradation. This requires ubiquitination of the MHC class I heavy chain and its dislocation from the ER to the cytosol for proteasome-mediated degradation, although the cellular machinery involved in this process is unknown. Using an siRNA functional screen in β2m-depleted cells, we identify an essential role for the E3 ligase HRD1 (Synoviolin) together with the E2 ubiquitin-conjugating enzyme UBE2J1 in the ubiquitination and dislocation of misfolded MHC class I heavy chains. HRD1 is also required for the ubiquitination and degradation of the naturally occurring hemochromatosis-associated HFE-C282Y mutant, which is unable to bind β2m. In the absence of HRD1, misfolded HLA-B27 accumulated in cells with a normal MHC class I assembly pathway, and HRD1 depletion prevented the appearance of low levels of cytosolic unfolded MHC I heavy chains. HRD1 and UBE2J1 associate in a complex together with non-β2m bound MHC class I heavy chains, Derlin 1, and p97 and discriminate misfolded MHC class I from conformational MHC I-β2m-peptide heterotrimers. Together these data support a physiological role for HRD1 and UBE2J1 in the homeostatic regulation of MHC class I assembly and expression.

The MHC class I (MHC I) heterotrimer, consisting of heavy chain (HC), β2-microglobulin (β2m), and an 8- to 10-residue peptide ligand, plays a vital role in immune surveillance of intracellular pathogens. Its assembly is governed by a stringent endoplasmic reticulum (ER) quality control process (1). The newly synthesized MHC I HC is cotranslationally inserted into the ER and rapidly associates with BiP, calnexin, and ERp57 (2). Subsequent binding of the HC to β2m is an early regulatory step that assists HC folding and is essential for incorporation of MHC I into the peptide-loading complex (PLC) (2), consisting of the transporter associated with antigen processing (TAP), tapasin, calreticulin, and ERp57. The PLC facilitates loading of high-affinity peptides onto the HC–β2m heterodimer. This process is tightly regulated, and only correctly assembled MHC I complexes are released from the PLC for export to the cell surface. MHC I HCs failing to achieve their native conformation, for example in the absence of β2m or peptide, are retained intracellularly and degraded (3).

Removal of misfolded proteins from the lumen of the ER is essential for maintenance of cellular homeostasis. The accumulation of misfolded proteins induces ER stress and activation of the unfolded protein response (4). ER-associated degradation (ERAD) involves protein substrate recognition, ubiquitination, and dislocation from the ER to the cytosol for proteasome-mediated degradation. The ubiquitination reaction requires activation of ubiquitin by an E1 enzyme, transfer to an E2 ubiquitin-conjugating enzyme, and targeting of ubiquitin to the substrate protein by the E3 ligase. The E3 confers substrate specificity and is therefore the pivotal enzyme (5). In contrast to yeast, in which Hrd1p and Doa10p are the only two known ERAD E3s, at least five mammalian ERAD E3 ligases are known (HRD1, gp78, RMA1, TEB4, and TRC8) (6–9) and further ERAD E3s may still be identified. The two mammalian orthologs of yeast Hrd1p, HRD1 (Synoviolin) and gp78 (AMFR), have distinct substrate specificities, exemplifying the increased complexity of mammalian ERAD (6, 7). HRD1 is implicated in the pathogenesis of rheumatoid arthritis (10), and reported substrates include α1-antitrypsin variants (11) and amyloid precursor protein (12). Three E2 ubiquitin-conjugating enzymes function in mammalian ERAD: UBE2J1 and UBE2J2 (yeast Ubc6p orthologs) and UBE2G2 (yeast Ubc7p ortholog) (7, 8, 13).

Many viruses down-regulate cell-surface MHC I to evade immune detection (2). The human cytomegalovirus US2 and US11 gene products bind newly synthesized MHC I HCs and initiate their rapid dislocation to the cytosol for proteasomal degradation and helped define mammalian ERAD (14, 15). US2-mediated MHC I degradation requires signal peptide peptidase and the E3 ligase TRC8 (9, 16), whereas US11 utilizes a distinct Derlin 1, p97, and SEL1L-dependent pathway (17–19). HRD1 and gp78 are found in complex with Derlin I, although no functional role for these ligases has been demonstrated in US11-mediated MHC I ERAD (6, 19). Despite the crucial role of US2 and US11 in identifying several ERAD components, whether MHC I degradation normally uses the same cellular machinery is unknown. We found no role for TRC8 in endogenous MHC I degradation (9), so we performed a targeted siRNA screen to identify the E3 ligase(s) responsible.

In the absence of β2m, MHC I fails to traffic to the cell surface and is targeted to the cytosol for proteasomal degradation, where an intermediate form of soluble, deglycosylated MHC I HC can be detected (3). Here we identify an absolute requirement for the E3 ligase HRD1 and E2-conjugating enzyme UBE2J1 in the ubiquitination and degradation of misfolded MHC I, suggesting an important role for these proteins in MHC I regulation.

Results

siRNA Screen Identifies HRD1 as the E3 Ligase Required for the Dislocation of Free MHC I HC. To identify the cellular machinery required for the ubiquitination of misfolded, β2m-deficient MHC I HCs, we performed a flow cytometry-based siRNA optical screen. HeLa cells expressing a luminal GFP-tagged MHC I HC (GFP-HLA-A2) (9) were stably depleted of β2m (HeLa GFP-HLA-A2 shβ2m cells), resulting in low cell-surface MHC I (Fig. S1). The rationale for the screen is that in the absence of β2m, the misfolded MHC I HC is dislocated from the ER to the cytosol and degraded by the proteasome (3). Depletion of any essential component of the MHC I ERAD pathway should rescue the GFP-HLA-A2 HC from proteasome-mediated degradation, with a consequent gain in GFP signal.

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A targeted siRNA screen of 18 putative ER membrane E3 ligases (Table S1) was performed in the HeLa GFP-HLA-A2 shβ2m cells. Depletion of a single ligase, HRD1, showed a significant gain in fluorescence (*Z* score 3.72) (Fig. 1 *A* and *B*), whereas depletion of gp78, the other mammalian ortholog of yeast Hrd1p (7), had no effect on GFP-HLA-A2 levels (Fig. 1*B*). In HeLa GFP-HLA-A2 US11-expressing cells, depletion of HRD1 and gp78 had no effect on GFP-HLA-A2 levels (Fig. S2). Deconvolution of the screening pool of four oligos (Fig. S3) showed effective HRD1 depletion with oligo #1 (Fig. 1*C*), which was used in subsequent experiments.

We validated our findings by showing that HRD1 regulates the degradation of endogenous misfolded MHC I. The absence of β2m is associated with a marked loss of MHC I HC, consistent with targeting for degradation (Fig. 1*C*) (3). Depletion of HRD1 effectively rescues MHC I from degradation (Fig. 1*C*), with little effect on MHC I levels in wild-type cells where the majority of MHC I HCs are correctly folded (Fig. S4).

HRD1 RING Mutant Exerts a Dominant Negative Effect and Protects β2m-Deficient MHC I from Degradation. HRD1 is a polytopic ER-resident E3 ligase with a C-terminal RING-H2 domain (6). Disruption of this RING, by substitution of a zinc-binding cysteine residue (HRD1-C1A), inactivates HRD1 and acts as a dominant-negative mutant (6). We tested the requirement for a functional RING domain in HRD1-mediated degradation of MHC I HCs by expressing wild-type HRD1 (HRD1wt) and the HRD1 RING mutant (HRD1-C1A) in the HeLa GFP-HLA-A2 shβ2m cells (Fig. 1*D*), with cotransfection of red fluorescent protein as an indicator of HRD1 expression. The HRD1-C1A RING mutant increased GFP-HLA-A2 fluorescence, as seen after HRD1 depletion (Fig. 1*D* vs. 1*B*), with a corresponding recovery in endogenous MHC I HCs after HRD1-C1A transfection (Fig. 1 *E* and *F*). The rescue of misfolded MHC I HCs by the RING mutant was more marked in β2m-depleted 293T cells owing to higher transfection levels (Fig. 1*F*). The HRD1 RING mutant is therefore unable to degrade β2m-deficient MHC I HCs and exerts a dominant-negative effect, protecting misfolded MHC I HCs from degradation.

HRD1 Associates with β2m-Deficient MHC I HCs. Because MHC I HCs are potential HRD1 substrates, do they interact? E3–substrate interactions are transient and often difficult to detect (5), so cells were treated with IFN-γ and proteasome inhibitor to increase endogenous MHC I expression and preserve any HRD1–MHC I interaction. Endogenous HRD1 was readily visualized in association with misfolded MHC I HCs from β2m-depleted but not wild-type HeLa cells (Fig. 2*A*). The increased levels of coprecipitated HRD1 seen after IFN-γ treatment likely reflect increased synthesis of MHC I, although the low levels of HRD1 under these conditions is as yet unexplained.

Misfolded MHC I HCs Are Retained in the ER After HRD1 Depletion. After proteasome inhibition, dislocated MHC I HCs are detected as non–membrane-bound, soluble, deglycosylated species (3, 15). To determine whether the accumulation of soluble MHC I HCs is HRD1 dependent, cells were fractionated into membrane and soluble (cytosolic) forms, detergent solubilized, and misfolded MHC I HCs immunoprecipitated with HC10 antibody. Dislocated MHC I HCs are detected as faster migrating (deglycosylated) species in the soluble fraction of β2m-negative cells (Fig. 2*B*, lane 2). Depletion of HRD1 elicits a striking accumulation of MHC I in the membrane fraction and a reciprocal loss of HCs from the soluble fraction. HRD1 is therefore an essential component of the cellular machinery required for dislocation of misfolded MHC I HCs from the ER to the cytosol. Furthermore, dislocation of MHC I HCs in normal, β2m-replete cells is also HRD1 dependent. Low levels of MHC I HCs are just detectable in long exposures of immunoblots from the soluble fraction of HeLa cells (Fig. 2*C*), and their appearance is prevented by HRD1 depletion.

The effect of HRD1 on newly synthesized MHC class I HCs was further examined by ^{35}S-methionine radiolabeling and pulse-chase analysis of β2m-depleted cells (Fig. 2*D*). In the absence of β2m, 90% of both the HLA-A and -B MHC I HCs were degraded within 3 h of synthesis, with a half-life of ≈80 min (Fig. 2*D*). Depletion of HRD1 inhibited degradation, such that 70% of the labeled MHC I HCs remained by the end of the chase (Fig. 2*D*, *Lower*).

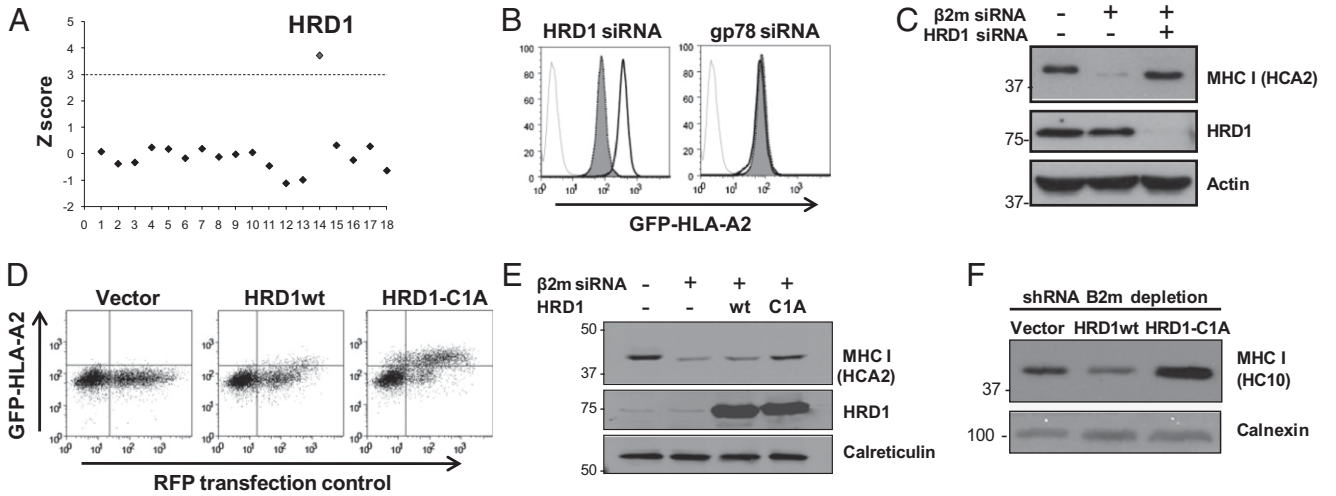

Fig. 1. Ubiquitin E3 ligase HRD1 is required for the degradation of β2m-deficient MHC I. (*A* and *B*) Cytofluorometric analysis of GFP levels in HeLa GFP-HLA-A2 shβ2m cells on siRNA depletion of ER membrane E3 ligases. (*A*) HRD1 depletion significantly increases GFP signal. *Z* scores calculated from two independent experiments. *Z* score of 3.0 (dashed line) equates to $P = 0.05$ (Bonferroni corrected). Numbers on *y* axis refer to E3s tested (Table S1). (*B*) GFP signal in siRNA treated (black line) vs. mock (shaded) HeLa GFP-HLA-A2 shβ2m cells. (*C*) HRD1 depletion rescues endogenous MHC I in β2m-depleted HeLa cells. Immunoblot for HRD1 and MHC I on siRNA depletion of β2m with or without HRD1. (*D–F*) HRD1-C1A RING mutant has a dominant negative effect rescuing misfolded MHC I from degradation. (*D*) GFP levels in HeLa GFP-HLA-A2 shβ2m cells transfected with wild-type HRD1 (HRD1wt), HRD1-C1A, or vector control. Tomato red fluorescent protein (RFP) was cotransfected (10%) as a marker of HRD1 expression. (*E* and *F*) HRD1-C1A rescues endogenous MHC I in β2m-depleted cells. (*E*) Immunoblot analysis of MHC I, HRD1, and control calreticulin in siRNA β2m-depleted HeLa cells after transfection with HRD1wt, HRD1-C1A, or vector control. (*F*) Immunoblot for MHC I and control calnexin in 293T cells cotransfected with β2m shRNA and either HRD1wt, HRD1-C1A, or vector control. Molecular mass in kilodaltons is indicated on the left for immunoblots throughout.

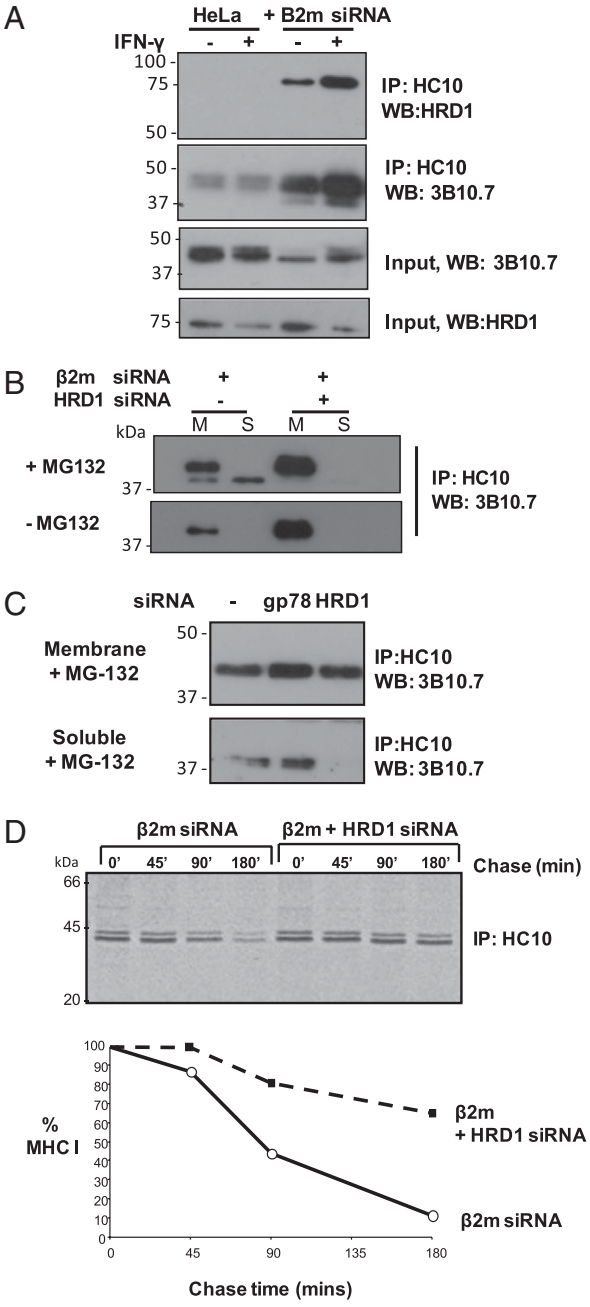

Fig. 2. Misfolded MHC I HCs interact with HRD1 and are retained in the ER in its absence. (*A*) HRD1 associates with misfolded MHC I. β2m or mock siRNA-depleted HeLa cells were treated with or without IFN-γ 200 U/mL for 12 h and MG132 50 μM for 5 h, before lysis in 1% Digitonin, MHC I immunoprecipitation with HC10, and immunoblot for HRD1 and MHC I (3B10.7). (*B*) HRD1 depletion prevents dislocation of misfolded MHC I from the membrane to the cytosol. β2m-depleted HeLa cells were fractionated into membrane (M) and soluble (S) fractions. MHC I HCs were immunoprecipitated (HC10) from each fraction and visualized by immunoblot (3B10.7). In the presence of MG132 (40 μM for 4 h), MHC I HC is visible in the soluble fraction on β2m depletion but is retained in the membrane on additional HRD1 depletion. (*C*) HRD1 depletion prevents dislocation of MHC I HC in wild-type HeLa cells. HeLa cells depleted of HRD1 or gp78 were treated as in *B*. Low levels of MHC I HC are visible in the soluble fraction after long exposure of immunoblots but are not detected on HRD1 depletion. (*D*) HeLa cells depleted of β2m alone or codepleted of β2m and HRD1 were radiolabeled with ^{35}S and MHC I HCs immunoprecipitated (HC10) from detergent lysates at the indicated chase times.

siRNA Screen Identifies UBE2J1 as the E2 Ubiquitin-Conjugating Enzyme Required for the Degradation of β2m-Deficient MHC I. No functional HRD1–E2–substrate in vivo partnership has been reported, and we therefore screened an siRNA library targeting known E2 enzymes using the HeLa GFP-HLA-A2 shβ2m cells to identify the E2 recruited by HRD1 for MHC I HC degradation. Depletion of only one of the 40 identified E2 enzymes (UBE2J1) gave a significant rescue in GFP-fluorescence (*Z* score 3.53; Fig. 3*A*). UBE2J1 is a mammalian ortholog of yeast Ubc6p, one of the two principal yeast ERAD E2s. Depletion of neither UBE2J2, the other mammalian ortholog of Ubc6p, UBE2G1, nor UBE2G2 (mammalian Ubc7p orthologs) significantly increased GFP-HLA-A2 levels (Fig. 3*B*). Effective UBE2J1 depletion of both endogenous and overexpressed UBE2J1 was confirmed (Fig. 3*C* and Fig. S5) and rescued endogenous MHC I HCs to levels similar to those seen after HRD1 depletion (Fig. 3*C*).

HRD1 and UBE2J1 Are Required for Ubiquitination of Misfolded MHC I HCs. To determine whether HRD1 and UBE2J1 are required for MHC I ubiquitination, HCs were immunoprecipitated and probed with ubiquitin-specific antibodies. Ubiquitinated MHC I HCs were visualized as high-molecular-weight species in β2m-depleted cells (Fig. 3*D* and Fig. S6) and were substantially reduced after depletion of either HRD1 or UBE2J1 alone and almost completely lost after combined HRD1/UBE2J1 depletion (Fig. 3*D*). The marked accumulation of MHC I together with the loss of ubiquitinated MHC I species indicates that, although we cannot exclude involvement of other E3 or E2 enzymes, they cannot compensate for HRD1 or UBE2J1, which seem to be critical for MHC I HC ubiquitination and dislocation.

Misfolded MHC I HCs Are Found in Complex with HRD1, UBE2J1, p97, and Derlin 1. To characterize additional components of the MHC I dislocation complex we showed that FLAG-tagged UBE2J1, but not UBE2J2, coprecipitated endogenous HRD1 and calnexin (Fig. 4*A*). The proportion of HRD1 coprecipitating with UBE2J1 was similar in wild-type and β2m-depleted cells, indicating that HRD1 readily associates with UBE2J1 irrespective of the MHC I status.

Mammalian HRD1 binds to SEL1L, which forms the core of an ER multiprotein complex containing Derlin 1 and the p97 ATPase (17–19), all of which apart from HRD1 are involved in US11-mediated MHC I dislocation (19, 20). We therefore looked for evidence of association with these proteins, or their functional role in MHC I dislocation. Free (HC10 reactive) but not conformational (W6/32 reactive) MHC I HCs associated with endogenous HRD1, UBE2J1, Derlin 1, and p97 (Fig. 4*B*), consistent with the premise that US11 hijacks an endogenous MHC I degradation pathway. Furthermore, depletion of SEL1L rescued both the fluorescent signal in β2m-depleted GFP-HLA-A2 cells and endogenous MHC I levels (Fig. 4 *C* and *D*), whereas depletion of gp78 and TRC8 had no effect. These results imply a role for SELIL, in addition to HRD1 and UBE2J1, in the degradation of β2m-free MHC I HCs and suggest that Derlin 1 and p97 are also involved in their dislocation.

HRD1 Is Required for the Degradation of HFE-C282Y. Having shown that HRD1 is required for the degradation of β2m-deficient MHC I HCs, we wanted to validate its role in MHC I misfolding. Hereditary hemochromatosis, the commonest autosomal recessive disorder in Caucasians, is caused by mutations in the MHC-I-like gene HFE (21). The HFE protein resembles classical MHC I, and 85% of individuals with hereditary hemochromatosis are homozygous for a mutation at position 282 of the HFE α3 chain (C282Y), which prevents HFE binding β2m and results in ER retention of misfolded HFE-C282Y and reduced cell-surface HFE expression (22). HFE-C282Y is less stable than wild-type HFE, being degraded with a half-life of 3 to 4 h (22, 23). We therefore tested whether HRD1 affects HFE-C282Y degradation (Fig. 5*A*). Pulse-chase analysis showed that FLAG-HFE-C282Y is retained in the ER with a half-life of 3 h, and its degradation is

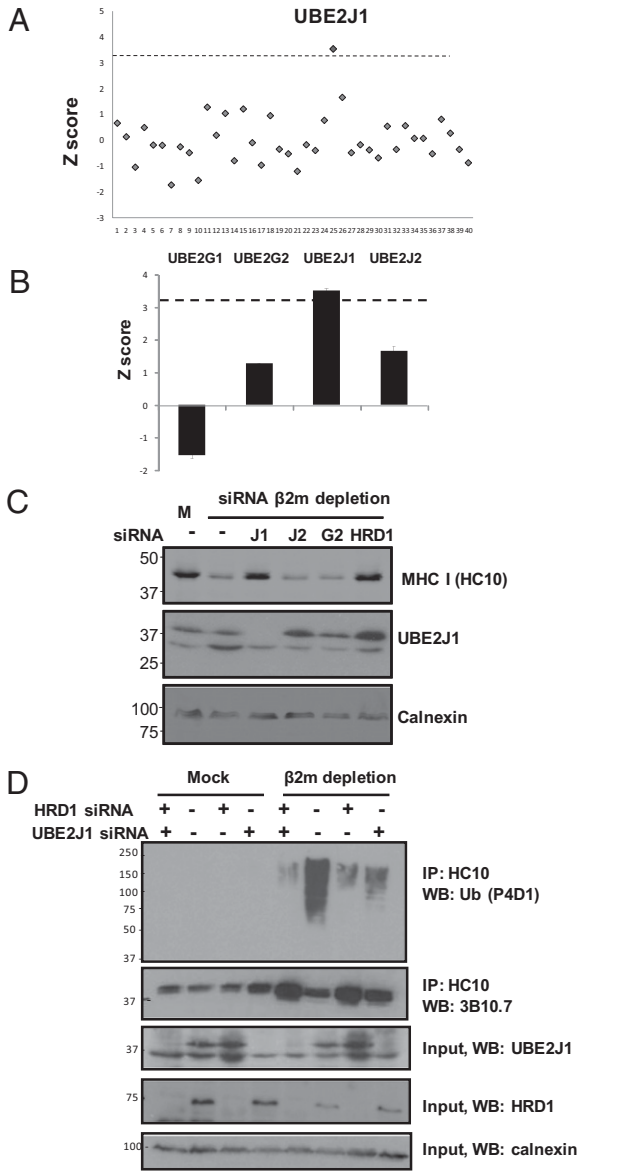

Fig. 3. E2 ubiquitin-conjugating enzyme UBE2J1 is required for ubiquitination and degradation of β2m-deficient MHC I. (*A* and *B*) Cytofluorometric analysis of GFP levels in HeLa GFP-HLA-A2 shβ2m cells on siRNA depletion of E2 ubiquitin-conjugating enzymes. *Z* scores calculated from two independent experiments. A *Z* score of 3.2 (dashed line) equates to $P = 0.05$ (Bonferroni corrected). Numbers on *y* axis refer to E2s tested (Table S2). (*B*) *Z* scores of ER membrane-associated E2 enzymes. Only UBE2J1 depletion significantly rescues GFP signal. (*C*) UBE2J1 depletion rescues endogenous MHC I in β2m-depleted HeLa cells. Immunoblot of MHC I, UBE2J1, and control calnexin on siRNA depletion of β2m and E2 enzymes UBE2J1(J1), UBE2J2(J2), and UBE2G2 (G2). (*D*) HRD1 and UBE2J1 depletion prevents ubiquitination of MHC I. β2m siRNA and mock-depleted HeLa cells were depleted of HRD1 with or without UBE2J1. After incubation with MG132 50 μM, lysates were immunoprecipitated with HC10 (MHC I HC) and probed for polyubiquitin (P4D1) and MHC I (3B10.7). Lysates (10%) were directly probed for HRD1, UBE2J1, and calnexin.

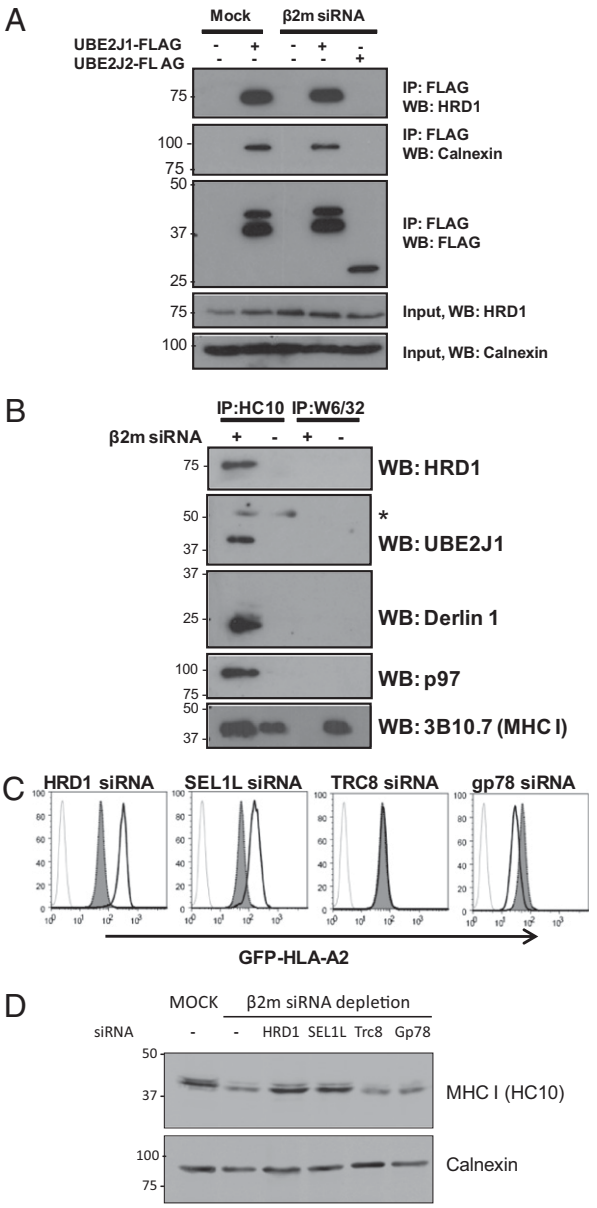

Fig. 4. HRD1 and UBE2J1 are found in a complex with unfolded MHC I, p97, and Derlin 1. (*A*) UBE2J1 associates with HRD1 and calnexin. β2m or mock siRNA-depleted HeLa cells were transfected with UBE2J1-FLAG or UBE2J2-FLAG. After incubation with MG132 50 μM, 1% Digitonin lysates were immunoprecipitated with anti-FLAG antibody (M2) and probed for HRD1 and calnexin. (*B*) β2m or mock siRNA-depleted HeLa cells were pretreated with IFN-γ for 12 h and MG132 50 μM for 5 h, before lysis in 1% Digitonin. Each sample was split and immunoprecipitated with either HC10 or W6/32 before immunoblot for HRD1, UBE2J1, Derlin 1, p97, and 3B10.7. *Secondary antibody cross-reacting with antibody HC. (*C* and *D*) Depletion of SEL1L protects misfolded MHC I from degradation. (*C*) GFP levels in siRNA β2m-depleted HeLa GFP-HLA-A2 cells on siRNA depletion (black line) of SEL1L, HRD1, gp78, or TRC8, vs. mock (shaded). (*D*) Immunoblot analysis of endogenous MHC I in samples treated as in *C*.

effectively inhibited by HRD1 depletion (Fig. 5*A*). Furthermore, ubiquitination of HFE-C282Y is prevented by HRD1 depletion (Fig. 5*B*). FLAG-HFE-C282Y coprecipitated endogenous HRD1 (Fig. 5*C*), although an interaction was detected only in the presence of proteasome inhibitors, presumably due to protection of HFE-C282Y from degradation and preservation of the E3-substrate interaction. Therefore, HRD1 is also required for the ubiquitination and degradation of the naturally occurring HFE-C282Y mutant, suggesting a role for HRD1 in the pathogenesis of hemochromatosis.

HRD1 Depletion Leads to Accumulation of HC10-Reactive, Disulphide-Linked HC Dimers in HLA-B27–Expressing Cells. Ninety-five percent of Caucasians with ankylosing spondylitis carry HLA-B27, one of

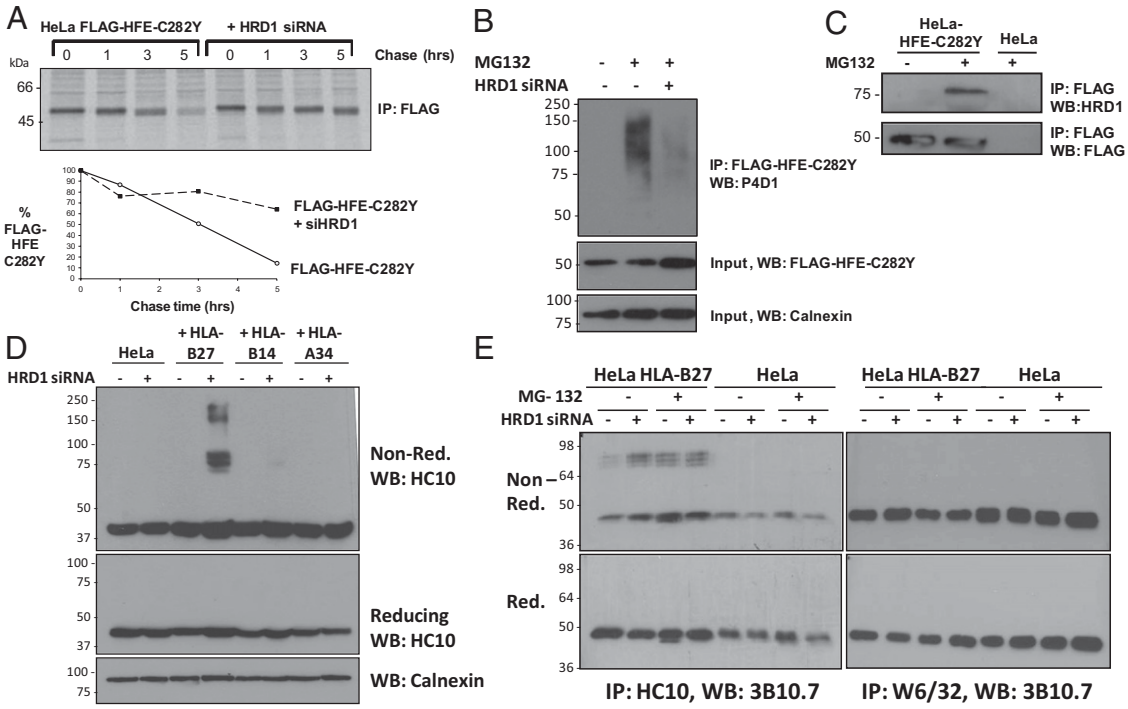

Fig. 5. HRD1 is required for the degradation of HFE-C282Y and HLA-B27 HC. (*A* and *B*) HRD1 depletion prevents ubiquitination of FLAG-HFE-C282Y and inhibits its degradation. (*A*) HRD1 or mock siRNA-depleted FLAG-HFE-C282Y–expressing HeLa cells were ^{35}S radiolabeled and FLAG-HFE-C282Y immunoprecipitated (anti-FLAG) from lysates taken at the indicated chase times. (*B*) HRD1 or mock siRNA-depleted HeLa FLAG-HFE-C282Y cells were treated with or without MG132 50 μM before Triton X-100 lysis, immunoprecipitation of FLAG-HFE-C282Y (anti-FLAG), and immunoblot for polyubiquitin (P4D1). Lysates (10%) were directly immunoblotted for FLAG-HFE-C282Y and calnexin control. (*C*) HRD1 interacts with HFE-C282Y. HeLa FLAG-HFE-C282Y cells and control HeLa cells were treated with or without MG-132 50 μM before lysis in 1% Digitonin, immunoprecipation of FLAG-HFE-C282Y, and immunoblot for HRD1. (*D* and *E*) HRD1 depletion leads to accumulation of HLA-B27 HC dimers. (*D*) Lysates from HRD1 or mock siRNA-depleted HeLa cells expressing HLA-B27, HLA-B14, or HLA-A34 were separated by nonreducing or reducing SDS/PAGE and immunoblotted with HC10. (*E*) HRD1 or mock siRNA-depleted HeLa and HeLa HLA-B27 cells were treated with or without MG-132 50 μM. Triton X-100 lysates were immunoprecipitated sequentially with HC10 then W6/32, separated by nonreducing or reducing SDS/PAGE, and immunoblotted with 3B10.7.

the strongest HLA disease associations. HLA-B27 HCs are unusual in their propensity to misfold, despite a normal supply of β2m and peptide, and a proportion of newly synthesized HLA-B27 HCs are targeted for ERAD (24). HLA-B27 forms β2m-free, disulphide-linked HC homodimers and oligomers in the ER (25, 26), which accumulate and induce ER stress and unfolded protein response activation (27). On HRD1 depletion, under nonreducing conditions, lysates and HC10 immunoprecipitates from HLA-B2705–expressing HeLa cells revealed accumulation of bands (70–95 kDa) consistent with HLA-B27 HC dimers (Fig. 5 *D* and *E*) (25, 26). This effect is not seen in HLA-B14– or HLA-A34–expressing cells, consistent with the increased propensity of HLA-B27 to misfold. These HC dimers also accumulate on proteasome inhibition (Fig. 5*E*, *Upper Left*) and are lost upon reduction but are associated with the appearance of a faster-migrating HC10-reactive species, which likely represents deglycosylated HLA-B27 (Fig. 5*E*, *Lower Left*, lane 3). The appearance of this deglycosylated species is prevented by additional HRD1 depletion (Fig. 5*E*, *Lower Left*, lane 4), suggesting inhibition of HC dislocation to the cytosol. Therefore, the degradation of misfolded HLA-B27 HCs is HRD1 dependent even in the presence of β2m and an intact MHC I assembly pathway.

Discussion

The coordinated multistep assembly of MHC I provides an excellent model for studying ER quality control. MHC I HCs are cotranslationally glycosylated, and after trimming to the monoglucosylated form interact with BiP, calnexin, and ERp57, facilitating intrachain disulfide bond formation (2). However, the absence of β2m precludes subsequent folding and assembly steps, resulting in an MHC I HC that can no longer be monoglucosylated, and then enters the degradative pathway. By focusing on this extreme situation—the absence of β2m—we identified a role for HRD1 and UBE2J1 in MHC I ER quality control. We also find HRD1 to be essential for the degradation of the naturally occurring HFE-C282Y mutant found in hemochromatosis. Furthermore, HRD1 depletion leads to (*i*) accumulation of misfolded HLA-B27 in cells with a normal MHC I assembly pathway, as well as (*ii*) loss in detection of the low levels of soluble, unfolded HCs identified in the cytosol of normal cells. Together these data support a physiological role for HRD1 and likely UBE2J1 in the homeostatic regulation of cell-surface MHC I expression, thus providing physiological "quantity" as well as "quality" control (28).

HRD1 may recognize misfolded MHC I HCs directly or indirectly via luminal acceptors, such as OS-9 and XTP3-B (11). Yeast Hrd1p requires Yos9p to degrade membrane-tethered glycoproteins with luminal lesions (29) but may directly recognize substrates with structural abnormalities in the transmembrane region (30). The determinants of substrate specificity for mammalian E3 ligases are largely unknown (5), although our findings clearly place HRD1 early in the MHC I assembly pathway.

Identifying the cellular machinery required for MHC I dislocation has been led by studies using US2/11 viral inhibitors of MHC I, with little information on the machinery required for physiological MHC I degradation, in the absence of viral inhibitors. The prediction that viral proteins recruit MHC I to components of the cellular ERAD machinery is borne out by our studies. Misfolded β2m-deficient MHC I HCs use Derlin 1, SEL1L, and p97, previously identified in the US11 pathway (17–19). However, depletion of HRD1 does not rescue MHC I in US11-expressing cells, suggesting that US11 either recruits other E3 ligases or circumvents the need for a cellular ligase in MHC I

dislocation. In contrast, US2-mediated MHC I dislocation uses a less-well characterized pathway—recruiting the TRC8 E3 ligase and signal peptide peptidase (9, 16). Although endogenous UBE2J1, HRD1, Derlin 1, and p97 coprecipitate free HC10-reactive MHC I HCs, they did not bind conformational, W6/32-reactive MHC I. The cellular ERAD machinery therefore distinguishes non–β2m-bound MHC I HCs from folded MHC I-β2m-peptide heterotrimers destined for export to the cell surface.

UBE2J1 was identified as the E2 conjugating enzyme required for HRD1-mediated ubiquitination of free MHC I HCs. UBE2J1 and J2, the mammalian homologs of yeast Ubc6p, share 90% homology and are tail-anchored ER membrane proteins (13). Depletion of UBE2J2 did not rescue MHC I from degradation, emphasizing distinct roles for these enzymes. UBE2J1 associates with MHC I HCs, and MHC I ubiquitination was dramatically reduced on depletion of HRD1 and UBE2J1, suggesting that HRD1 specifically co-operates with UBE2J1 to ubiquitinate and dislocate MHC I HCs. This in vivo demonstration of a functional E2 partner for mammalian HRD1 (4, 8) contrasts with yeast Hrd1p, which predominantly functions with Ubc7p (UBE2G2) (8).

Interactions between an E2 and its cognate E3 are usually transient, of low affinity, and difficult to detect (5). Surprisingly, UBE2J1 readily coprecipitated HRD1, an interaction that was relatively stable and not dependent on misfolded MHC I HCs. This may reflect UBE2J1 binding HRD1 at a site distinct from its RING, or to another component of the HRD1 complex. This situation is reminiscent of UBE2G2, which binds gp78 with high affinity through its UBE2G2-binding domain (G2BR) (7), although HRD1 lacks any region homologous to the G2BR. We predict that HRD1 and UBE2J1 form a functional E3–E2 pair for the turnover of other ERAD cellular substrates.

In summary, we have identified a role for HRD1 and UBE2J1 in the regulation of endogenous MHC I. Together with TRC8 and the virally encoded K3 and K5, four E3 ligases are now known to ubiquitinate and regulate human MHC I, emphasizing the versatility of the ubiquitin system in coordinating MHC I assembly and degradation. Although HRD1 is essential for the degradation of non–β2m-bound MHC I HCs, other E3 ligases could target MHC I for degradation at later stages in its assembly as well as at the plasma membrane. HRD1 and UBE2J1 fail to associate with β2m-bound MHC I HCs, implying that a second ER quality-control checkpoint may be required for the recognition of "empty" β2m–HC heterodimers (3).

Materials and Methods

More details on cell lines, antibodies, immunoprecipitation and immunoblotting, cell fractionation, and metabolic labelling and pulse-chase are provided in *SI Materials and Methods*.

RNAi Screen. RNAi screening was performed as previously described (9) and was targeted to 18 candidate E3 ligases, consisting of known ER membrane E3s and uncharacterized E3s with predicted transmembrane domains (31), as well as a library of 40 E2 ubiquitin-conjugating enzymes. Each gene was targeted with an siRNA pool of four oligonucleotides (final concentration 80 nM). Cytofluorometric analysis of GFP levels was performed at 60 h and measurements normalized to mock-transfected controls. *Z* scores were calculated from two independent experiments. A Bonferroni correction was applied to determine the threshold *Z* score for statistical significance ($P < 0.05$).

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