Ubiquitin charging of human class III ubiquitin-conjugating enzymes triggers their nuclear import

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Ubiquitin is a small polypeptide that is conjugated to proteins and commonly serves as a degradation signal. The attachment of ubiquitin (Ub) to a substrate proceeds through a multi-enzyme cascade involving an activating enzyme (E1), a conjugating enzyme (E2), and a protein ligase (E3). We previously demonstrated that a murine E2, UbcM2, is imported into nuclei by the transport receptor importin-11. We now show that the import mechanism for UbcM2 and two other human class III E2s (UbcH6 and UBE2E2) uniquely requires the covalent attachment of Ub to the active site cysteine of these enzymes. This coupling of E2 activation and transport arises from the selective interaction of importin-11 with the Ub-loaded forms of these enzymes. Together, these findings reveal that Ub charging can function as a nuclear import trigger, and identify a novel link between E2 regulation and karyopherin-mediated transport.

Introduction

The ubiquitin (Ub) system plays a fundamental role in regulating many cellular processes. Ub is a 76-aa polypeptide that is conjugated onto the ε-amino group of lysine residues. Ubiquitylation of a protein commonly serves to mark the modified protein for proteasome-mediated degradation, but can also signal a multitude of other responses including receptor internalization and endocytic trafficking (Levkowitz et al., 1998; Lucero et al., 2000), histone modification (Robzyk et al., 2000), vesicular trafficking (Katzmann et al., 2001), DNA repair (Spence et al., 1995; Hofmann and Pickart, 1999), viral budding (Patnaik et al., 2000; Garrus et al., 2001), and transcriptional regulation (Kaiser et al., 2000; Salghetti et al., 2001).

The covalent attachment of Ub to a target protein proceeds through a multi-enzyme cascade. Ub is first activated in an ATP-dependent manner by the Ub-activating enzyme (E1). Subsequent to its activation, Ub is transferred to the active site cysteine of a Ub-conjugating enzyme (E2) in a transesterification reaction. E2s are broadly grouped into four classes: class I E2s consist of an ∼150-aa catalytic core domain (UBC); class II enzymes possess the UBC plus a COOH-terminal extension;

class III E2s are comprised of the UBC and an NH₂-terminal extension; and class IV E2s have both an NH₂- and COOH-terminal extension appended to the UBC domain. A third enzymatic component, a Ub protein ligase (E3), cooperates with the E2 to transfer Ub to substrates. One class of E3 ligases (RING finger proteins) functions to direct E2s to substrates, and the Ub moiety is transferred directly from the E2 to the substrate. A second class (homologous to E6-AP COOH terminus [HECT] domain proteins) contains an active site cysteine and accepts Ub from the E2 and transfers it to the substrate. After transfer of the first Ub to a target lysine, subsequent Ubs are attached sequentially to a lysine of the previously added Ub. When lysine 48 of Ub is used for poly-Ub chain assembly, the resulting poly-Ub structure signals delivery of the modified target to the 26S proteasome for destruction. In contrast, poly-Ub chains constructed through other lysines (e.g., K63) of Ub typically result in nonproteolytic outcomes. Protein targets can also be regulated in nonproteolytic ways by mono-ubiquitylation. Balance in the Ub system is achieved by a set of deubiquitylating isopeptidases that cleave Ub off of substrates (for review see Glickman and Ciechanover, 2002).

The enzymatic cascade responsible for ubiquitylating target proteins is complex, and its regulation is only beginning to be understood. The complexity stems from the large number of E2 and E3 enzymes that exist in eukaryotes; in humans, more than 30 E2s and hundreds of putative E3 ligases have been identified. In addition, multiple E2s can interact with a common E3 partner, and a single E2 can function with a vari-

ety of E3 ligases, of both the RING finger and HECT domain types (Kumar et al., 1997; Lorick et al., 1999; Nuber and Scheffner, 1999). This promiscuity suggests that additional levels of regulation, such as compartmentalization, chaperones, or scaffold proteins, may restrict E2–E3 interactions within living cells.

Although some Ub cascade enzymes have been well studied (e.g., Cdc34; Deffenbaugh et al., 2003), most remain poorly understood. And, in general, there is a paucity of information about the molecular mechanisms governing the specificity, localization, and overall control of these enzymes. Our efforts to characterize the localization properties of a murine class III E2, called UbcM2, revealed that this enzyme can localize to the nucleus and can constitutively shuttle in and out of the nucleus. Importantly, we also found that UbcM2 is imported into the nucleus by a nuclear transport receptor, called importin-11 (S.M. Plafker and Macara, 2000).

Importin-11 is a 116-kD protein that belongs to the family of nuclear transport receptors commonly referred to as karyopherins. Karyopherins are soluble proteins that mediate the translocation of nucleic acids and proteins through nuclear pore complexes in a Ran-dependent fashion. They can be classified into two main groups, with importins facilitating nuclear import, and exportins facilitating nuclear export. Ran is a small GTPase that is predominantly GTP-bound in the nucleus and GDP-bound in the cytoplasm, and this gradient of RanGTP across the nuclear envelope directs the vectoriality of cargo transport. Importins bind their cargo only in the cytoplasm, where RanGTP is absent, and release it in the nucleus, where RanGTP binds the importin. Conversely, exportins bind their cargo only in the presence of RanGTP, and the exportin/cargo/RanGTP complex disassembles in the cytoplasm upon the hydrolytic conversion of Ran:GTP to Ran:GDP (for review see Macara, 2001).

During our initial analyses of UbcM2 import by importin-11, we had met considerable difficulties in detecting a robust interaction between recombinant UbcM2 and importin-11 in the absence of cell lysates (S.M. Plafker and Macara, 2000). This problem raised the possibility that the interaction might be promoted or stabilized by some type of post-translational modification of either the karyopherin or the cargo. We have now found that only the Ub-loaded form of UbcM2 efficiently binds importin-11. Furthermore, we show that other class III E2s can localize to the nucleus by accessing the importin-11 pathway and do so in an Ub activation-dependent fashion. Thus, as a consequence of being transported by importin-11, the catalytic activation and nuclear transport of these enzymes are coupled. In addition, our data indicate that Ub charging can function as a trigger for nuclear import.

Results

In previous work, we found that UbcM2, a murine class III E2, is imported into the nucleus by the importin-11 transport receptor (S.M. Plafker and Macara, 2000). One implication of these findings was that importin-11 might influence both the localization and the enzymatic activity of UbcM2.
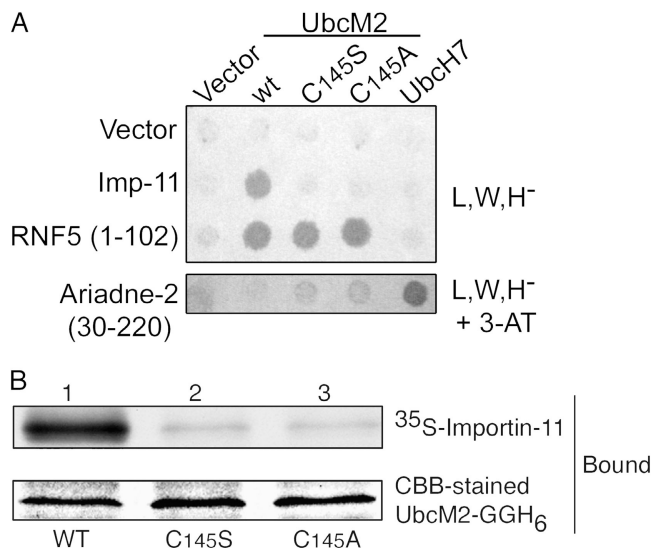
Figure 1. **The active site cysteine of UbcM2 is required for the interaction with importin-11.** (A) HF7c (MATa) yeast expressing the indicated bait proteins (on left) as GAL4-DBD fusions were mated with W303α (MATα) strains expressing VP16 TA domain fusions (across top). Diploid yeast were selected on Leu⁻/Trp⁻ plates and replica-plated onto Leu⁻/Trp⁻/His⁻ plates with (L,W,H⁻ + 3-AT) or without (L,W,H⁻) 3-amino triazole (3-AT). (B) Wt (lane 1), C145S (lane 2), or C145A (lane 3) UbcM2 fused to two GFPs and 6× His tag (UbcM2-GGH$_6$) were mixed with Ni^{2+}-agarose beads and a reticuloctye lysate containing ^{35}S-labeled importin-11. Bound proteins (50% of bound) were separated by SDS-PAGE and detected by CBB staining (UbcM2-GGH$_6$) or fluorography (^{35}S-importin-11).

The active site cysteine of UbcM2 is required for importin-11 binding

The difficulty in detecting a robust interaction of UbcM2 with importin-11 in the absence of cell lysates suggested that UbcM2 might require a post-translational modification in order to be recognized as a transport cargo. One obvious modification is the attachment of Ub to the active site cysteine of the enzyme. As for all E2s, this cysteine receives activated Ub from E1 by forming a thiolester linkage with the COOH-terminal glycine of Ub (for review see Glickman and Ciechanover, 2002). To examine whether the activation state and nuclear transport of UbcM2 are coupled, we first tested the sensitivity of the UbcM2/importin-11 interaction to the integrity of the active site residue (Cys145). Active site mutants of UbcM2, bearing either a serine (C145S) or alanine (C145A) substitution, were expressed as fusions with the transactivation domain of VP16 and assayed for their ability to interact with a GAL4 DNA-binding domain fusion of human importin-11 in a yeast two-hybrid assay. Wild-type (wt) UbcM2 interacted with importin-11 in this assay, whereas the active site mutants did not (Fig. 1 A). In addition, importin-11 showed selectivity among E2s, as it bound the class III E2, UbcM2, but not the human class I E2, UbcH7. Expression of all E2s in this assay was confirmed by interactions with the RING finger domains of the putative E3 ligases, RNF5 or Ariadne-2.

To corroborate the two-hybrid results, we performed in vitro binding assays using bacterially expressed UbcM2 proteins and rabbit reticulocyte lysates (RRLs) containing ^{35}S-labeled importin-11. RRLs contain many of the components of the Ub
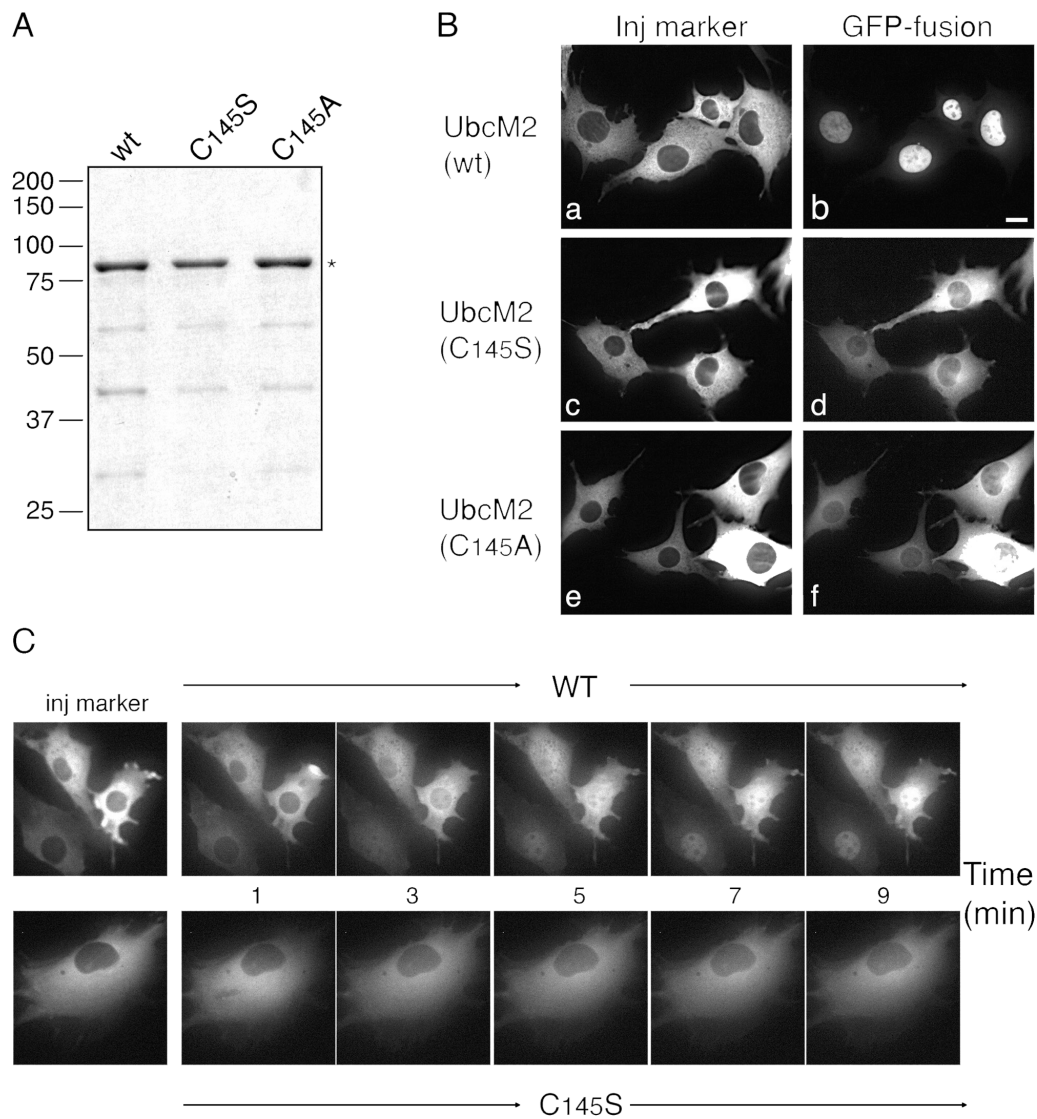
Figure 2. **The active site cysteine of UbcM2 is required for the enzyme to be efficiently imported in the nucleus.** BHK cells were micro-injected into the cytoplasm with a mixture of TRITC-labeled dextran (Inj marker; 1 mg/ml) plus either wt, C145S, or C145A UbcM2-GGH$_6$ (9 μM). (A) Aliquots of the injection mixtures were solubilized, separated by SDS-PAGE, and the proteins were detected by CBB staining. Full-length proteins are denoted with an asterisk, and the migration of molecular size markers is indicated on the left. (B) After injection, cells were incubated for 20 min at 37°C and then imaged live by fluorescence microscopy. 50–75 cells were injected with each protein and representative cells are shown for wt (a and b), C145S (c and d), and C145A (e and f). Bar, 10 μm. (C) WT and C145S UbcM2-GGH$_6$ were injected into cells maintained at 37°C on a heated stage and were imaged by time-lapse fluorescence microscopy for the time course indicated.

proteolytic system including Ub and E1 (Ciechanover et al., 1981, 1982; Wilkinson et al., 1980). Therefore, these programmable lysates fulfill the dual purpose of expressing ^{35}S-labeled importin-11, and charging exogenously added UbcM2 with Ub. Wt, C145S, and C145A fusion proteins of UbcM2 bearing two GFP moieties and a 6× His tag (UbcM2-GGH$_6$) were immobilized on Ni^{2+}-agarose beads and assayed for binding of ^{35}S-importin-11. Bead-associated proteins were analyzed by SDS-PAGE, Coomassie brilliant blue (CBB) staining, and fluorography. In contrast to wt UbcM2 (Fig. 1 B, lane 1), neither C145S UbcM2 nor C145A UbcM2 efficiently precipitated ^{35}S-importin-11 (Fig. 1 B, lanes 2 and 3). These binding data are in agreement with those in Fig. 1 A and demonstrate that an intact active site cysteine is necessary for UbcM2 to bind importin-11.

Enzymatically inactive UbcM2 is not transported into the nucleus

The data from Fig. 1 suggested that the C145S and C145A mutants would not be imported into the nuclei of cells by importin-11. We tested this prediction using a micro-injection assay. BHK cells were injected in the cytoplasm with equal amounts (Fig. 2 A) of either wt, C145S, or C145A UbcM2-GGH$_6$, and after a 20-min incubation at 37°C the intracellular localization of the reporter proteins was examined in live cells by GFP fluorescence microscopy. Wt UbcM2 localized efficiently to the nuclei of injected cells (Fig. 2 B, a and b), whereas the mutants remained largely in the cytoplasm (Fig. 2 B, c–f). These findings were further confirmed in a parallel experiment using time-lapse fluorescence microscopy to com-
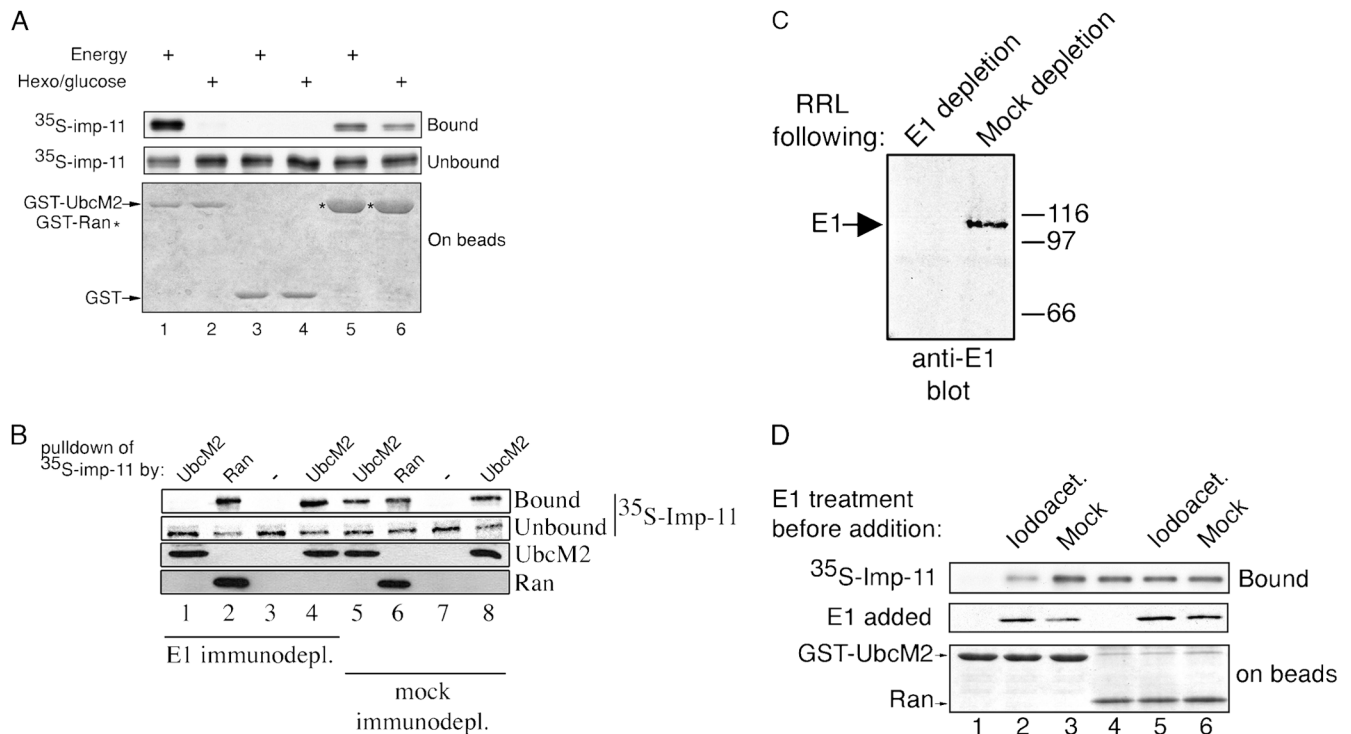
Figure 3. **The enzymatic activity of E1 is required for the interaction of UbcM2 with importin-11.** (A) ^{35}S-importin-11–expressing reticulocyte lysates were pretreated with buffer (+ energy) or an ATP depletion mixture (hexo/glucose) for 30 min before being mixed with glutathione Sepharose beads and either GST-UbcM2 (lanes 1 and 2), GST (lanes 3 and 4), or GST-Ran (Q69L) (lanes 5 and 6). GST-Ran is marked with an asterisk. Bound and unbound proteins were resolved by SDS-PAGE and detected by CBB staining (GST fusions) or fluorography (^{35}S-importin-11). (B) ^{35}S-importin-11–expressing reticulocyte lysates were immunodepleted (lanes 1–4) or mock depleted (lanes 5–8) of E1 before being mixed with myc-UbcM2-H$_6$ (UbcM2; lanes 1, 4, 5, and 8), Ran (Q69L)-H$_6$ (Ran; lanes 2 and 6), or no protein (lanes 3 and 7) and Ni^{2+}-agarose beads. Purified E1 was added back (lanes 4 and 8) to restore the binding interaction. Bead-associated proteins and unbound ^{35}S-importin-11 were analyzed by SDS-PAGE and detected by CBB staining (UbcM2, Ran) or fluorography (^{35}S-importin-11). (C) Anti-E1 Western blot of the E1 remaining (3% of lysate) in ^{35}S-importin-11–expressing reticulocyte lysates after immunodepletion (E1 depletion) or mock depletion (Mock depletion). Bands corresponding to E1 is indicated with an arrow, and molecular size markers are denoted to the right of the blot. (D) ^{35}S-importin-11–expressing reticulocyte lysates were immunodepleted of E1 and then supplemented with either buffer (lanes 1 and 4), enzymatically inactivated E1 (Iodoacet.; lanes 2 and 5), or mock-inactivated E1 (Mock; lanes 3 and 6). GST-UbcM2 is immobilized on glutathione Sepharose beads (lanes 1–3) and Ran (Q69L)-H$_6$ is on Ni^{2+}-agarose beads (lanes 4–6). Bound proteins were detected as in B and a Western blot of the E1 added to each lysate is also shown. For A, B, and D, bound represents 50% of bead-associated proteins and unbound represents 10% of proteins remaining in lysate.

pare the transport kinetics of wt UbcM2 and the C145S active site mutant (Fig. 2 C).

The interaction of importin-11 with UbcM2 requires the enzymatic activity of E1

Although the data from Figs. 1 and 2 demonstrate that an intact active site is necessary for UbcM2 to bind importin-11 and to be imported into the nucleus, they do not distinguish between whether Ub charging of the active site promotes importin-11 binding or if the unloaded active site cysteine comprises part of the NLS. To address this issue, we performed a series of in vitro binding experiments using the RRL expression system.

First, we tested the ATP dependence of complex formation. The initial step in the enzymatic activation of Ub by E1 involves ATP hydrolysis and the subsequent adenylation of Ub (Ciechanover et al., 1981, 1982; Haas and Rose, 1982; Haas et al., 1982, 1983). ATP depletion will inhibit Ub activation by E1 and thereby preclude the charging of UbcM2 with Ub. ^{35}S-importin-11–containing reticulocyte lysates were pretreated either with a hexokinase/glucose ATP depletion mixture or were

mock treated before being mixed with glutathione Sepharose beads and either GST-UbcM2, GST, or GST-Ran. We previously demonstrated that importin-11 binds the Ran GTPase (S.M. Plafker and Macara, 2000), so the binding of importin-11 to GST-Ran was included as a control to ensure that treatments of the lysate did not adversely affect the function of importin-11. Bound and unbound proteins were resolved by SDS-PAGE and detected by CBB staining or fluorography. We found that complex formation between UbcM2 and importin-11 was ATP dependent (Fig. 3 A, compare lane 1 with lane 2), whereas Ran binding to importin-11 was not affected by ATP depletion (Fig. 3 A, compare lane 5 with lane 6). As expected, ^{35}S-importin-11 was not precipitated by GST alone (Fig. 3 A, lanes 3 and 4). These results establish that ATP is required for the UbcM2/importin-11 interaction and are consistent with a requirement for E1 activity.

To directly evaluate the requirement for E1, we compared the amount of ^{35}S-importin-11 precipitated by bacterially expressed myc-UbcM2-H$_6$ from an E1-immunodepleted RRL versus a mock-depleted RRL (Fig. 3 B). The quantitative immunodepletion of E1 from the RRL using an anti-E1 pAb was
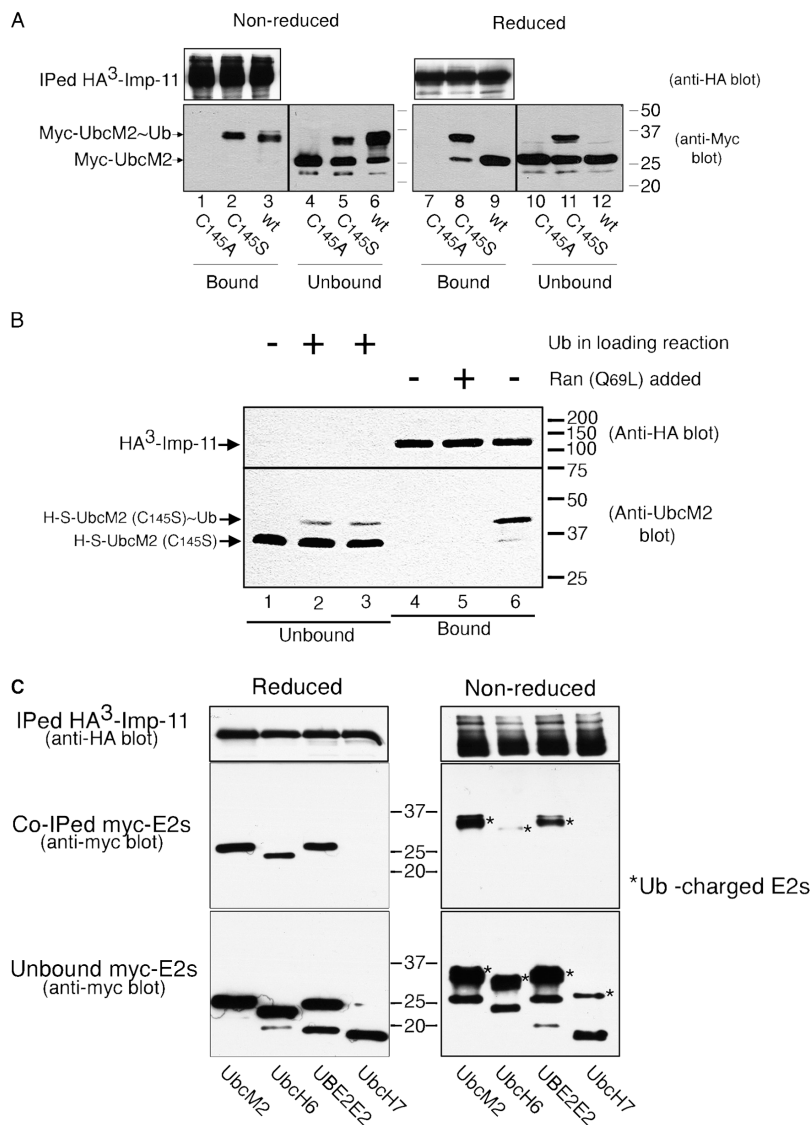
Figure 4. Importin-11 interacts in vivo with the Ub-charged forms of class III E2 enzymes. (A) Transfected HEK293T cells expressing HA^3-importin-11 and myc-tagged UbcM2 (wt, C145A, or C145S) were harvested under nonreducing conditions and exposed to 12CA5 antibody and protein A–Sepharose beads to precipitate HA^3-importin-11 and any associated myc-tagged UbcM2. Bead-associated and unbound proteins were separated by both nonreducing and reducing SDS-PAGE and detected by Western blotting with 12CA5-HRP (anti-HA blot) or anti-myc-HRP (anti-Myc blot) conjugates and ECL. Ub-charged UbcM2 migrates more slowly than its uncharged counterpart in nonreducing SDS-PAGE (lane 6). Under reducing conditions, Ub is readily removed from wt UbcM2, but not from the C145S mutant (wt, lanes 6 and 12 vs. C145S, lanes 5 and 11). The migration of molecular size markers is indicated to the right of the blots. (B) Lysates from transfected HEK cells expressing HA^3-importin-11 were mixed with recombinant (C145S) UbcM2 not loaded (lanes 1 and 4) or preloaded with Ub (lanes 2, 3, 5, and 6). Precipitation of the HA^3-importin-11 and any bound, recombinant (C145S) UbcM2 was then done as described in A, except that one lysate (lane 5) was spiked with (Q69L) Ran before 12CA5 addition. Samples were resolved by reducing SDS-PAGE, and HA^3-importin-11 and (C145S) UbcM2 were detected with a 12CA5 antibody (Anti-HA blot) or an anti-UbcM2 antibody (Anti-UbcM2 blot), respectively. 75% of bound (lanes 4–6) and 5% of unbound (lanes 1–3) are shown. The migration of molecular size markers are indicated to the right. Ub-charged (C145S) UbcM2 (H-S-UbcM2 (C145S)~Ub) migrates more slowly than the uncharged enzyme (H-S-UbcM2 (C145S)). (C) Same experiment as in A, except that in place of the UbcM2 mutants, myc-tagged forms of UbcH6, UBE2E2, and UbcH7 were each coexpressed with HA^3-importin-11. The Ub-charged form of each E2 is marked with an asterisk. For experiments A and C, bound represents 50% of total and unbound represents 10% of total.

confirmed by Western blotting (Fig. 3 C). Mock-depleted lysates were treated with rabbit IgG. Again, the levels of ^{35}S-importin-11 precipitated from each lysate by Ran were also examined to ensure that the function of ^{35}S-importin-11 was not compromised by the immunodepletion. As shown in Fig. 3 B, immunodepletion of E1 prevented the binding of importin-11 to UbcM2 (Fig. 3 B, lane 1), but not to Ran (Fig. 3 B, lane 2). Furthermore, readdition of purified E1 to the E1-depleted lysate restored the importin-11/UbcM2 interaction (Fig. 3 B, lane 4), thus confirming the requirement for E1.

Together, these immunodepletion data indicate that energy and E1 are required for the UbcM2/importin-11 interaction. To directly demonstrate the requirement for active E1, an experiment was performed in which E1-immunodepleted lysates were supplemented with either buffer, enzymatically inactivated E1 (i.e., iodoacetamide treated), or mock-treated E1. The amount of ^{35}S-importin-11 precipitated from each lysate by GST-UbcM2 or Ran was then assessed as in Fig. 3 B. The results show again that depletion of E1 from the RRL prevented the interaction of ^{35}S-importin-11 with UbcM2 (Fig. 3 D, lane

1), but not with Ran (Fig. 3 D, lane 4). Importantly, the interaction was restored when active E1 was reintroduced to the lysate (Fig. 3 D, lane 3), but was not efficiently restored by E1 that had been catalytically incapacitated by the alkylating agent iodoacetamide (Fig. 3 D, lane 2). The failure of inactive E1 to promote complex formation argues that this protein does not bridge the UbcM2/importin-11 interaction and is consistent with our finding that the E1 protein could not be detected by Western blot analysis of UbcM2/importin-11 complexes (unpublished data). Together, these experiments support the hypothesis that importin-11 interacts selectively with Ub-charged UbcM2.

Importin-11 recognizes the Ub-loaded form of class III Ubcs

We used coimmunoprecipitation to examine if importin-11 has a binding preference for Ub-loaded UbcM2 versus the unloaded enzyme. Human embryonic kidney (HEK) 293T cells were cotransfected with plasmids expressing HA^3-tagged importin-11 and myc-tagged E2s (i.e., wt UbcM2, C145S UbcM2, and C145A UbcM2). Transfected cells were collected and lysed in

a detergent-containing buffer lacking reducing agents. The resulting lysates were sequentially exposed to 12CA5 mAbs and protein A–Sepharose beads to precipitate HA[3]-importin-11 and any associated myc-tagged UbcM2. Fractions of the bead-bound and unbound tagged proteins were then analyzed in parallel by nonreducing and reducing SDS-PAGE followed by Western blotting. Nonreducing conditions were used to maintain the thiolester linkage between the active site cysteine of UbcM2 and the COOH-terminal glycine of Ub. Addition of β-mercaptoethanol (β-ME) reduces the thiolester bond and results in removal of Ub from the active site, as illustrated by collapse of the slower migrating 33-kD myc-UbcM2 band to the faster migrating 25-kD band (Fig. 4 A, compare lane 6 with lane 12).

HA[3]-tagged importin-11 specifically coprecipitated Ub-loaded, wt UbcM2 (Fig. 4 A, lane 3). The coprecipitated band corresponding to charged enzyme displayed the characteristic 8-kD shift indicative of the covalent attachment of Ub. Surprisingly, we observed that when expressed in HEK293T cells, a fraction of the C145S UbcM2 was loaded with Ub in a stable, primarily nonreducible form, indicative of oxy-ester bond formation (Fig. 4 A, lane 5). Strikingly, this Ub-loaded C145S UbcM2 was bound by importin-11 (Fig. 4 A, lanes 2 and 8). The C145A mutant was neither charged with Ub nor coprecipitated by the transport receptor (Fig. 4 A, lanes 1 and 4), and none of the UbcM2 proteins were detectably precipitated from lysates lacking HA[3]-importin-11 (unpublished data). We attribute accumulation of the loaded form of C145S UbcM2 to the extended period of time (48–72 h) that the protein was exposed to Ub and E1 at 37°C and to the fact that the oxy-ester is a dead-end complex that cannot be efficiently processed by the cell (Sung et al., 1991). In contrast, our in vitro binding assays using this mutant were performed at 4°C and the micro-injection experiments were done over the course of 1–2 h. In support of this explanation, we have found that 1–10% of recombinant C145S UbcM2 can be loaded with Ub in vitro, using purified E1 and recombinant Ub, if the reactions are incubated at 37°C for at least 3 h, and that the oxy-ester bond generated is largely resistant to reduction by β-ME and heat (Fig. 4 B, lanes 2 and 3).

To demonstrate that the UbcM2 recognized by importin-11 is loaded with Ub at the active site, we took advantage of the finding that recombinant C145S UbcM2 can be stably charged with Ub at 37°C, but not at 4°C. Lysates from HEK293Ts expressing HA[3]-tagged importin-11 were combined with recombinant (C145S) UbcM2 that had been preincubated at 37°C in a loading mixture with purified E1 plus an energy-regenerating system and Ub. As a negative control, a loading reaction was performed in the absence of Ub. The lysates and loading reactions were then combined with 12CA5 mAbs and protein A–Sepharose beads and were incubated at 4°C. Because (C145S) UbcM2 is not detectably loaded with Ub at 4°C, no charging of the mutant enzyme occurs during the lysate incubation and pulldown phase of the experiment (Fig. 4 B, lane 1). Bound and unbound HA[3]-importin-11 and (C145S) UbcM2 were then detected by Western blotting. From this experiment, we found that (1) a fraction of (C145S) UbcM2 was charged with Ub in vitro (Fig. 4 B, lanes 2 and 3) and was selectively bound by HA[3]-importin-11 (Fig. 4 B, lane 6); (2) this binding could be
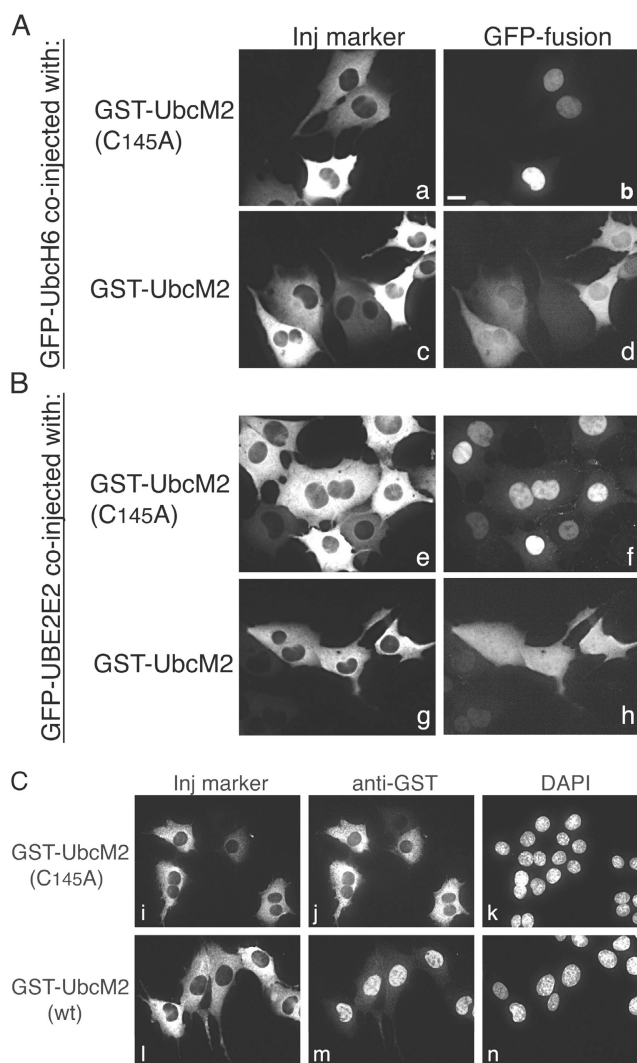

Figure 5. **Class III E2s can access the nucleus by the same import pathway.** BHK cells were microinjected in the cytoplasm with TRITC-labeled dextran (Inj marker; 1mg/ml), GFP-UbcH6-H$_6$ (A), or GFP-UBE2E2-H$_6$ (B), and a 20-molar excess of GST-UbcM2 (C145A) (a, b, e, and f) or GST-UbcM2 (wt) (c, d, g, and h). After injection, cells were incubated for 15 min at 37°C and analyzed live by fluorescence microscopy. 50–75 cells were injected for each condition. Bar, 10 μm. (C) Cells injected with GST-UbcM2 (C145A) (i–k) or GST-UbcM2 (wt) (l–n) were incubated for 30 min at 37°C and fixed. The localization of the GST fusions was determined by indirect immunofluorescence using an anti-GST antibody and a FITC-conjugated secondary antibody. DNA was stained with DAPI to mark the nuclear compartment for each cell.

inhibited by addition of (Q69L) Ran, a mutant of the Ran GTPase that is constitutively loaded with GTP (Bischoff et al., 1994; Klebe et al., 1995) and inhibits karyopherin–cargo complex formation (Jakel and Gorlich, 1998; S.M. Plafker and Macara, 2000) (Fig. 4 B, lane 5); and (3) (C145S) UbcM2 that was preincubated in a loading reaction lacking Ub and then mixed with lysate was not detectably charged with Ub or bound by HA[3]-importin-11 (Fig. 4 B, lanes 1 and 4). These data show that importin-11 selectively interacts with UbcM2 that is charged with Ub at the active site. However, this interaction may not be direct, as it could not be reconstituted with recombinant proteins (unpublished data).
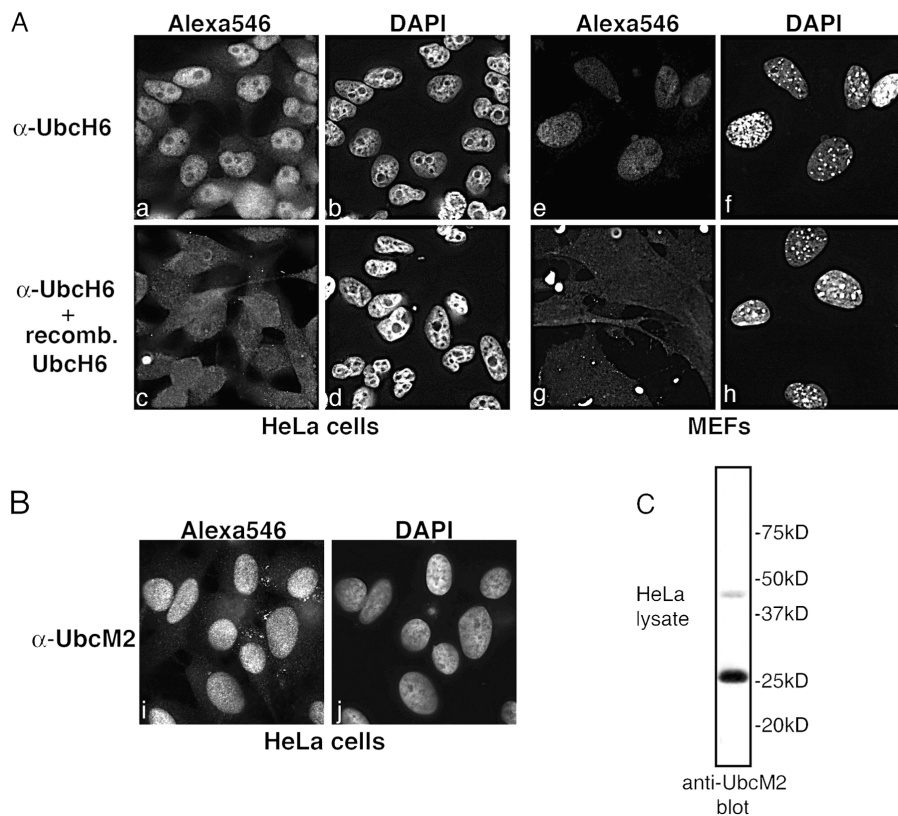
A

| Alexa546 | DAPI | | Alexa546 | DAPI |

α-UbcH6 — a, b, e, f

α-UbcH6 + recomb. UbcH6 — c, d, g, h

HeLa cells — **MEFs**

B

| Alexa546 | DAPI |

α-UbcM2 — i, j

HeLa cells

C

HeLa lysate

-75kD
-50kD
-37kD
-25kD
-20kD

anti-UbcM2 blot

Figure 6. **Endogenous UbcH6 and UbcM2 are resident nuclear enzymes.** (A) Mouse embryonic fibroblasts, from a 12.5-d mouse embryo, and HeLa cells were fixed, permeabilized, and immunostained for endogenous UbcH6 using an anti-UbcH6 antibody and a goat anti–rabbit-Alexa$_{546nm}$ secondary antibody (a and e). The specificity of the immunostaining was verified by blocking the anti-UbcH6 antibody with recombinant UbcH6 (c and g). (B) HeLa cells stained with an anti-UbcM2 antibody (i). Nuclei were counterstained with DAPI (b, d, f, h, and j). (C) HeLa whole-cell extracts probed with an anti-UbcM2–specific antibody followed by a goat anti–rabbit-HRP conjugate and ECL. The antibody detects a primary band at the estimated size for UbcM2 and a faint, slower migrating band.

To examine whether importin-11 interacts with other E2 enzymes in an activation-dependent fashion, we performed similar coimmunoprecipitation experiments from transfected cell lysates expressing HA[3]-tagged importin-11 and myc-tagged forms of UbcH6, UBE2E2, UbcM2, UbcH7, UbcH5B, or hCDC34. Because UbcM2 is identical to human UBE2E3 (Ito et al., 1999), UbcH6, UBE2E2, and UbcM2/UBE2E3 represent three human class III E2s, whereas UbcH7 and UbcH5B are class I E2s and hCDC34 is a class II E2. Importin-11 specifically coprecipitated Ub-charged UbcH6, UBE2E2, and UbcM2, but not UbcH7, UbcH5B, or hCDC34 (Fig. 4 C; Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200406001/DC1). We reproducibly found that relatively less Ub-charged UbcH6 was coprecipitated with importin-11, as compared with the other class III E2s. None of the class III enzymes were coprecipitated by a different HA[3]-tagged transport receptor (importin-β; unpublished data). These data demonstrate that importin-11 specifically binds the Ub-charged forms of these human class III E2s.

The coimmunoprecipitation data predict that all three human class III E2s can localize to the nucleus by accessing the importin-11 pathway. We reasoned that the nuclear import of any of these E2s should be specifically prevented by saturating the importin-11 pathway with an excess of a second class III enzyme. To test this prediction, BHK cells were injected in the cytoplasm with GFP-UbcH6-H$_6$ or GFP-UBE2E2-H$_6$ and a 20-molar excess of either GST-UbcM2(C145A) or GST-UbcM2(wt). After a 15-min incubation at 37°C, the cells were analyzed live by fluorescence microscopy. The GFP-E2 fusions localized efficiently to the nucleus in the presence of the

GST-UbcM2(C145A) competitor (Fig. 5 Ab; Fig. 5 Bf), but their import was effectively competed by GST-UbcM2(wt) (Fig. 5 Ad; Fig. 5 Bh). Similar results were found using His-S-tagged UbcM2 (wt or C145A) as competitors (Fig. S1 B). The import of both GFP-E2 fusions was also inhibited by coinjecting Ran (Q69L) (unpublished data). When this experiment was done using an excess of GST-UbcH7 as a competitor, both GFP fusions localized efficiently to the nucleus (Fig. S1 C). Therefore, the differential effects of the wt and inactive UbcM2 competitors was not simply a consequence of the wt UbcM2 competitor overwhelming the Ub-charging capacity of endogenous E1 and preventing activation of the GFP-E2s. The localizations of the competitor GST fusions were validated in a separate micro-injection experiment. As expected, GST-UbcM2(C145A) was distributed throughout the cytoplasm (Fig. 5 C, i–k), and GST-UbcM2(wt) accumulated in the nucleus (Fig. 5 C, l–n). Together, these injection data demonstrate that these three human class III E2s can access the nucleus by the importin-11 pathway.

To determine if endogenous class III E2s are resident nuclear proteins, we examined the subcellular distribution of UbcH6 and UbcM2. HeLa and 12-d mouse embryonic fibroblasts were fixed, permeabilized, and exposed to anti-UbcH6 antibodies and an Alexa$_{546nm}$-conjugated anti–rabbit secondary antibody. The distribution of UbcH6 was then assessed by fluorescence microscopy. In both cell types, UbcH6 immunostaining revealed a nuclear distribution (Fig. 6 A, a and e), and this nuclear signal was ablated by coincubation of the antibody with recombinant UbcH6 (Fig. 6 A, c and g). Similarly, immunostaining of HeLa cells with an antibody against the unique

NH$_2$-terminal extension of UbcM2 revealed that this enzyme also resides in the nucleus (Fig. 6 B, i and j).

Discussion

The covalent attachment of Ub and Ub-like modifiers to protein targets occurs through coordinated enzyme cascades, the salient features of which are conserved in all eukaryotes. These cascades are comprised of three main classes of enzymes; E1, E2, and E3. Numerous studies have established the hierarchical nature of this enzymatic cascade, but many important questions remain regarding the regulation of these enzymes. Are they constitutively active or do other cellular factors trigger or abrogate their catalytic activity? Are E2–E3 pairs present in the cell in an "inactive" state (Deffenbaugh et al., 2003), or does loading of an E2 promote E2–E3 complex assembly? How are these enzymes delivered to their sites of action?

Answers to these questions have begun to emerge, particularly with respect to the influence of intracellular localization on E3 function. For example, the E3 ligase Nedd4 has been shown to harbor a Crm-dependent nuclear export signal that maintains the steady-state cytoplasmic localization of this E3 (Hamilton et al., 2001). Interestingly, Nedd4 has both cytoplasmic (Dinudom et al., 1998; Murillas et al., 2002; Debonneville and Staub, 2004) and nuclear (Hamilton et al., 2001; Murillas et al., 2002) substrates, implying that its Crm-mediated export may need to be blocked or delayed under certain conditions. The von Hippel-Lindau (VHL) tumor suppressor protein is an E3 ligase that is remarkable for its Ran-, ATP-hydrolysis–, RNA pol II activity-dependent, Crm-independent export. Although the export receptor for VHL is unknown, its importance is highlighted by the finding that the export-targeting region of VHL is frequently mutated in renal cell carcinoma (Groulx et al., 2000). In addition, the MDM2 E3 ligase, which ubiquitinates p53, is regulated by changes in its intracellular distribution mediated by nuclear import, export, and nucleolar localization signals present in MDM2 and its binding partners (Chen et al., 1995; Tao and Levine, 1999; Weber et al., 1999; Zhang and Xiong, 1999; Lohrum et al., 2000).

Recent insights into how E2s are targeted to their putative sites of action have been revealed through the identification of novel nuclear transport pathways. We have found that UbcM2 nuclear import is mediated by the importin-11 transport receptor (S.M. Plafker and Macara, 2000), and Mingot et al. (2001) have shown that the SUMO-conjugating enzyme Ubc9 is translocated by importin-13. Interestingly, both UbcM2 and Ubc9 are small enough (~18–25 kD) to diffuse freely through nuclear pore complexes, the diffusion cutoff of which is 40–60 kD (Feldherr, 1971; Paine and Feldherr, 1972). Why then has the cell assigned particular receptor proteins to deliver these enzymes to their targets? An enticing answer to this question may be that the transport receptors provide a means of coupling enzyme localization to catalytic state. This certainly appears to be the case for the human class III E2s (UbcH6, UBE2E2, and UBE2E3). Importin-11 selectively interacts with the Ub-charged forms of these enzymes (Fig. 4 C), linking the catalytic activation of these E2s to their translocation from the cyto-

plasm to the nucleus. The need for such a mechanism may be to ensure the delivery of activated enzyme to its appropriate nuclear targets and/or to prevent the interaction of the activated E2 with cytoplasmic E3s or substrates.

The ability of importin-11 to interact with these three human class III E2s is presumably a consequence of the fact that the UBC domains (i.e., ~150-aa catalytic domain) of these enzymes are 96% identical. We have previously reported that this domain is necessary and sufficient for the interaction of UbcM2 with importin-11 (S.M. Plafker and Macara, 2000). Therefore, although these E2s each contain a distinct 40–60-aa NH$_2$-terminal extension appended to their UBCs, these extensions do not appear to play a primary role in the importin-11 interaction. The apparent specificity of importin-11 for the UBC domains of the class III enzymes was further corroborated by testing if UbcH5B or hCDC34 could bind to importin-11. The UBC domain of UbcH5B is 66% identical to the class III E2s and hCDC34 is 34% identical, and both have presumed nuclear substrates (Scheffner et al., 1994; Stancovski et al., 1995; Pati et al., 1999; Stroschein et al., 2001). However, neither enzyme was coprecipitated by importin-11 from transfected cell lysates (Fig. S1 A). Thus, unlike the class III E2s, neither of these enzymes in the forms tested appears to interact with importin-11.

The NLS that is recognized by importin-11 has not been defined. Its identification has been confounded by an inability to reconstitute the binding of importin-11 to Ub-charged UbcM2 using recombinant proteins (unpublished data). This may be a result of improper importin-11 folding in bacteria, or it may indicate that in the cell, a cofactor contributes to the preference of the import receptor for loaded enzyme. This cofactor could be a partner E3 ligase, an importin-11–specific adaptor protein, or a post-translational modification of importin-11. If an E3-ligase is involved, then Ub charging of the E2s would be predicted to recruit this E3 ligase, which in turn would promote importin-11 binding. Translocation of the complex and its subsequent release in the nucleus might provide the cell with a mechanism for efficiently targeting particular nuclear substrates for ubiquitylation. Alternatively, this factor might be a protein containing any of the known Ub-binding domains such as a Ub-interacting domain (UIM) (Hofmann and Falquet, 2001), a UBA (Hofmann and Bucher, 1996), or a CUE domain (Kang et al., 2003). Further work is required to determine if a bridging factor is involved; however, our findings demonstrate that Ub charging can, either directly or indirectly, trigger the nuclear import of these class III E2s. A complementary function for Ub in nuclear export has recently been reported in studies analyzing the functional consequence of p53 mono-ubiquitylation by Mdm2 (Li et al., 2003).

Collectively, our data reveal a new regulatory mechanism governing enzymatic components of the Ub system. We have defined a link between the enzymatic activation and nuclear transport of class III E2s that results in delivery of the Ub-loaded E2s to the nucleus. Furthermore, we have uncovered a novel function for Ub charging as a trigger for nuclear import. Finally, our data are consistent with the idea that independent of their role as transporters, karyopherins may function as chaperones to protect a specific state of their cargo in the com-

partment of its origin until delivery and release in the target compartment. Jakel et al. (2002) showed that several importins could behave as chaperones for ribosomal protein subunits to prevent nonspecific aggregation. Our data expand on this theme by suggesting that karyopherins may have a broader role in regulating specific enzyme or other protein activities.

Materials and methods

Cloning and recombinant protein expression
Mutation of the active site cysteine of UbcM2 was done with the QuikChange mutagenesis kit (Stratagene) and a set of complementary 53mer oligonucleotides coding for either Cys to Ser or Cys to Ala at the active site (residue 145). UbcH7, UbcH6, and UBE2E2 were all cloned from a human brain–derived cDNA library (CLONTECH Laboratories, Inc.) using primers specific for each cDNA. UbcH5B and hCDC34 were PCR subcloned from mammalian expression vectors (Lorick et al., 1999). The coding sequences for the E2s were subcloned into pRK7 (Lounsbury and Macara, 1997) for expression in mammalian cells, pQE60 (QIAGEN) and pGEX-2T (Amersham Biosciences) for expression in bacteria, and pVP16 and pGBT10 (S.M. Plafker and Macara, 2000) for expression in yeast. The RING finger domains of RNF5 and Ariadne-2 were each recovered from a two-hybrid screen done with (C145S) UbcM2 as the bait. The cDNAs coding for each RING finger protein were subcloned into the pGBT10 bait vector. Construction of the mammalian expression vector pKH3-importin-11, which codes for a triple HA-tagged importin-11, has been described (S.M. Plafker and Macara, 2000).

All recombinant proteins, except the GFP-E2-H_6 fusions, were expressed in *Escherichia coli* BL21 (DE3) grown in Terrific Broth supplemented with 2.5% ethanol and the appropriate antibiotic. The GFP-E2-H_6 fusions were expressed in *E. coli* XL1Blue cells. Cultures were grown at 37°C to an OD_{600} of ~0.8 and protein expression was induced with 0.5 mM IPTG at 18°C overnight. 6× His-tagged proteins were purified with either Ni^{2+}-agarose beads (QIAGEN) or Talon beads (QIAGEN) and eluted with imidazole. GST fusion proteins were purified over glutathione Sepharose beads (Amersham Biosciences) and eluted with 10 mM reduced glutathione.

Yeast conjugation assays
Mating assays were performed as described previously (Neudauer et al., 1998). Importin-11, RNF5 (1–105), and Ariadne-2 (30–220) were expressed in *Saccharomyces cerevisiae* HF7c (MAT**a**) strain as COOH-terminal fusions to the DBD of the GAL4 protein. UbcM2, UbcM2 (C145S), UbcM2 (C145A), and UbcH7 were expressed in the W303α (MATα) strain as COOH-terminal fusions to the TA domain of VP16.

Transient transfections
BHK cells and HEK293T cells were seeded 18–20 h before transfection and were transfected by standard calcium phosphate precipitation (K. Plafker and Macara, 2000).

Microinjections and immunofluorescence
Microinjections were done as described previously (K. Plafker and Macara, 2000) using TRITC-labeled dextran (1 mg/ml) as an injection site marker. Wt and mutant UbcM2-GGH_6 were injected at 9 µM. After injection, cells were incubated for the indicated times at 37°C and then were imaged live or fixed, permeabilized, and stained with DAPI before analysis by fluorescence microscopy. Fixed samples were treated with 4% PFA in PBS and were permeabilized with −20°C methanol for 2 min at RT. DNA was stained with DAPI (0.01 µg/ml in PBS). For Fig. 2 C, cells were injected and maintained at 37°C on a heated stage and subjected to time-lapse fluorescence microscopy. For the competition injection experiments, the GFP-E2 fusions were each injected at 2.2 µM and the competitors at 44 µM (except where noted in the figure legend). The injections were done over a 10-min period at RT after which the cells were incubated for an additional 15 min at 37°C and then imaged live by fluorescence microscopy. GST fusions were detected with a mouse monoclonal anti-GST antibody (a gift of Dr. T. Parsons, University of Virginia, Charlottesville, VA) and a FITC-conjugated goat anti–mouse secondary antibody.

Immunostaining of endogenous UbcH6 was done in a HeLa-derived cell line (CLONTECH Laboratories, Inc.) and in mouse embryo fibroblasts from 12.5-d embryos of an inbred strain (Xu et al., 2000). Cells were fixed in 3.7% formaldehyde in PBS, permeabilized with −20°C methanol, blocked in 3% BSA in PBS, and immunostained with anti-UbcH6 (Boston

Biochem; diluted 1:1,000 in 3% BSA in PBS) and goat anti–rabbit-$Alexa_{546nm}$ secondary antibody. To block anti-UbcH6 staining, the antibody was preincubated with 50 µg recombinant GFP-UbcH6-his_6 for 30 min at 4°C before being added to the cells. Endogenous UbcM2 staining in HeLa cells was done with a rabbit pAb raised against the unique NH_2-terminal 58 residues of UbcM2. The specificity of the antibody was determined by Western blotting a whole-cell extract from HeLa cells. DNA was counterstained with DAPI (0.01 µg/ml in PBS).

Cells were examined at RT (except for Fig. 2 B, which was done at 37°C) using an inverted microscope (Eclipse TE2000; Nikon) equipped with a 60× water immersion objective lens (NA 1.20). Images were captured with a charge-coupled device camera (Orca, C4742-95-12NRB; Hamamatsu Corporation) and were processed with Openlab software (Improvision) and Adobe Photoshop version 8.0. Cells were imaged live in Ringer's solution.

Binding assays
All RRL-based, importin-11 binding assays were performed at 4°C in a thermomixer (Eppendorf) as described previously (S.M. Plafker and Macara, 2000). ^{35}S-importin-11 was detected by fluorography and bead-bound, recombinant proteins by CBB staining after SDS-PAGE.

For the immunodepletion experiments, ^{35}S-importin-11–containing reticulocyte lysates were incubated with either anti-E1 (Affinity BioReagents, Inc.) or control rabbit IgG antibodies and protein A–Sepharose beads before being mixed with bead-bound myc-UbcM2-H_6 or Ran (Q69L)-His_6. 0.5 µg purified rabbit E1 (Calbiochem) was added back to the indicated samples.

To catalytically inactivate E1, purified E1 was treated for 10 min at 37°C with 10 mM iodoacetamide. The reactions were then quenched with DTT for 15 min at 25°C (Haas et al., 1982; Rose and Warms, 1987). E1 was detected by immunoblotting with the anti-E1 antibody (diluted 1:1,000 in 1% goat serum/PBS), a goat anti–rabbit secondary antibody (1:20,000), and ECL.

To deplete ATP, reticulocyte lysates were supplemented with 50 U/ml hexokinase/12.5 mM glucose and incubated at 25°C for 30 min.

Coimmunoprecipitations were done from transfected HEK293T cells expressing HA_3-importin-11 and myc-tagged E2s. Cells were harvested 3 d after transfection under nonreducing conditions to maintain thiolester linkages between E2s and Ub. Cells were collected, pelleted for 5 min at 300 g, washed once in ice-cold PBS, and resuspended in 100 µl of 2× concentrated transport buffer (20 mM Hepes/KOH, pH 7.4, 110 mM potassium acetate, 2 mM magnesium acetate, and 0.5 mM EGTA) plus 0.5% vol/vol Tween 20 and 2 mM PMSF, and were incubated on ice for 30 min with occasional mixing. The resulting lysate was clarified by centrifugation (14,000 g for 15 min at 4°C). Each clarified lysate was combined with mouse mAb 12CA5 for 90 min at 4°C, and then with protein A–Sepharose beads for an additional 90 min. Unbound proteins were solubilized in nonreducing SDS-PAGE buffer (50 mM Tris-HCl, pH 6.8, 4 M urea, 2% SDS, 10% glycerol, and 0.001% bromophenol blue) (Jahngen-Hodge et al., 1997) and were incubated for 15 min at 30°C. Half of each solubilized sample was then reduced by the addition of β-ME and heated for 3 min in a boiling water bath. Immune complexes were rinsed two times with ice cold transport buffer/0.25% Tween 20 and once with PBS before being solubilized and treated as described above for the unbound proteins. Non-reduced and reduced proteins were resolved by SDS-PAGE at 4°C and were detected by immunoblotting with a 12CA5-HRP conjugate or with an anti-Myc antibody (Santa Cruz Biotechnology, Inc.) and ECL.

For the pull-down experiment in Fig. 4 B, recombinant (C145S) UbcM2 was preloaded with Ub in vitro at 37°C. In vitro loading reactions all contained 5.3 µM His-S-(C145S) UbcM2, 83 nM E1, and 0.02 units of pyrophosphatase in reaction buffer (50 mM Tris-HCl, pH 7.5, 0.2 mM ATP, 0.5 mM $MgCl_2$, 0.1 mM DTT, 1 mM creatine phosphate, 0.002 U/µl creatine phosphokinase, and 0.1% Tween 20). 83 µM Ub was included in the indicated loading reactions, and all reactions were incubated at 37°C for 3 h before being combined with a transfected cell lysate containing HA_3-importin-11 and mixed with 12CA5 and protein A–Sepharose beads at 4°C. One lysate was spiked with (Q69L) Ran, which is constitutively loaded with GTP, to demonstrate that complex formation between importin-11 and loaded UbcM2 is Ran:GTP-sensitive. Unbound and bead-associated proteins were solubilized in 4× concentrated Laemmli buffer, resolved by SDS-PAGE, and detected by Western blotting with 12CA5 (HA_3-importin-11) or anti-UbcM2 (His-S-UbcM2), goat anti–mouse (for 12CA5) or anti–rabbit (for anti-UbcM2) secondary antibodies, and ECL. Reducing SDS-PAGE could be used for these experiments because the oxy-ester bond between the serine at the active site of (C145S) UbcM2 and the COOH-terminal glycine of Ub is largely resistant to reduction with β-ME.

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