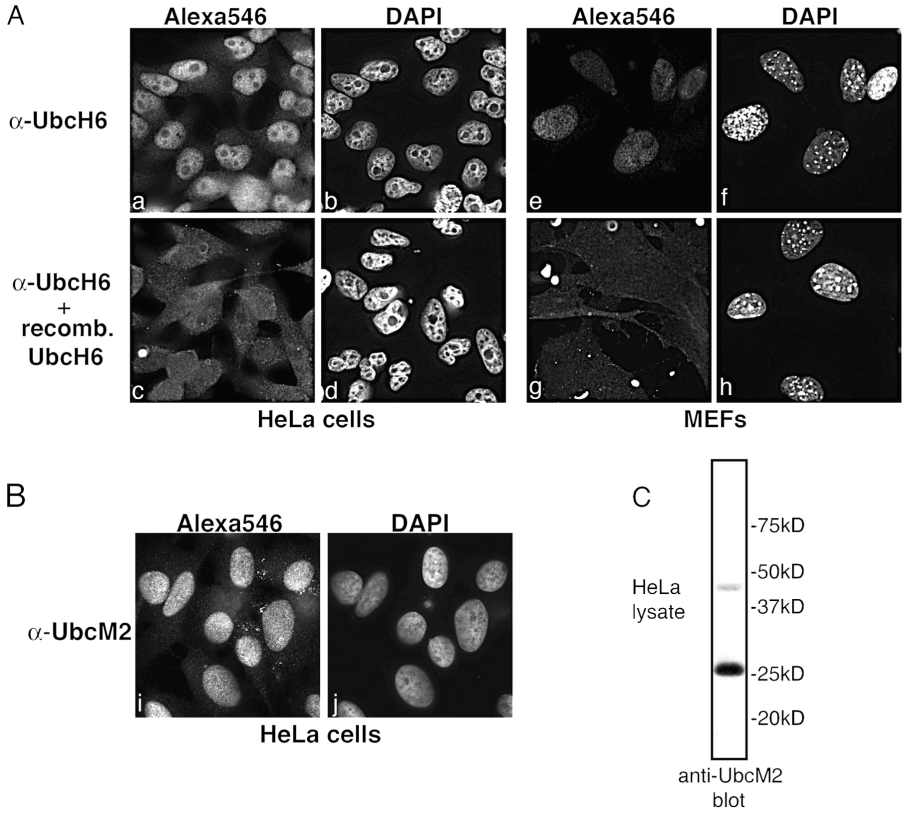

Figure 6. **Endogenous UbcH6 and UbcM2 are resident nuclear enzymes.** (A) Mouse embryonic fibroblasts, from a 12.5-d mouse embryo, and HeLa cells were fixed, permeabilized, and immunostained for endogenous UbcH6 using an anti-UbcH6 antibody and a goat anti–rabbit-Alexa$_{546nm}$ secondary antibody (a 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 None of the class III enzymes were coprecipitated by a different HA^3-tagged transport receptor (importin-β; unpublished data).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