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HSJ1 Is a Neuronal Shuttling Factor for the Sorting of Chaperone Clients to the Proteasome

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Summary

Protein degradation in eukaryotic cells usually involves the attachment of a ubiquitin chain to a substrate protein and its subsequent sorting to the proteasome. Molecular mechanisms underlying the sorting process only recently began to emerge and rely on a cooperation of chaperone machineries and ubiquitin-chain recognition factors [1–3]. Here, we identify isoforms of the cochaperone HSJ1 as neuronal shuttling factors for ubiquitylated proteins. HSJ1 combines a J-domain that stimulates substrate loading onto the Hsc70 chaperone with ubiquitin interaction motifs (UIMs) involved in binding ubiquitylated chaperone clients. HSJ1 prevents client aggregation, shields clients against chain trimming by ubiquitin hydrolases, and stimulates their sorting to the proteasome. In this way, HSJ1 isoforms participate in ER-associated degradation (ERAD) and protect neurons against cytotoxic protein aggregation.

Results and Discussion

A hallmark of most neurodegenerative diseases, including those caused by polyglutamine expansion, is the formation of ubiquitin-positive inclusions of aggregated protein [4, 5]. This highlights the importance of chaperoning ubiquitylated proteins in neurons [6, 7]. The data presented here identify isoforms of the Hsc70 cochaperone HSJ1 as key components in neuronal protein quality control. Human HSJ1a and HSJ1b both belong to the family of J-proteins that stimulate ATP hydrolysis by Hsc70. On the basis of this Hsc70-regulating activity and an intrinsic chaperone function (see the Supplemental Results and Discussion and Figure S1 in the Supplemental Data available with this article online), HSJ1 isoforms facilitate substrate loading onto Hsc70 [8–10]. HSJ1a and HSJ1b are preferentially expressed in neurons and display distinct intracellular localizations [11, 12]. HSJ1a is cytoplasmic and nuclear, whereas the larger HSJ1b isoform is targeted to the cytoplasmic face of the ER by C-terminal geranylgeranylation [12].

To elucidate the function of HSJ1 proteins, we performed a yeast two-hybrid screen and identified the ubiquitin carboxyl extension proteins Uba52 and Uba80 as novel binding partners of the cochaperone (Figure 1A). Uba52 and Uba80 are fusions of ubiquitin to the ribosomal proteins S27a and L40, respectively [13]. We detected two ubiquitin interaction motif (UIM) domains within the primary structure of both cochaperone isoforms (Figures 1B and 1C), consistent with an interaction of HSJ1 with ubiquitin fusion proteins. UIMs have an important role in both ubiquitylation and in binding mono and/or polyubiquitylated proteins [14–16]. They are present in a wide variety of proteins, ranging from the neurodegenerative Machado-Joseph disease protein (MJD1) to epsin, a protein involved in endocytosis. To test whether the interaction between ubiquitin fusion proteins and HSJ1b was dependent upon the UIMs, we mutated conserved serine and glutamic acid residues in each UIM to alanine (S219A/E222A-ΔUIM1; S262A/E265A-ΔUIM2; double mutant S219A/E222A/S262A/E265A-ΔUIM) (Figure 1B). Mutation of UIM1 had only a minor effect on growth in the two-hybrid assay, whereas mutation of UIM2 significantly reduced growth (Figure 1D). Interaction with the ubiquitin fusion proteins was abolished when both UIMs were mutated. UIM-dependent recognition of ubiquitin moieties was confirmed in vitro with purified components (Figure 1E). Notably, HSJ1a preferentially bound to ubiquitin chains that contained at least four ubiquitin moieties. High-molecular-mass polyubiquitylated polypeptides were also detected in HSJ1a complexes immunoisolated from HeLa cells (Figure 2A). Moreover, an association of HSJ1a with the proteasome was detected. Both interactions were dependent on functional UIMs, revealing the essential role of the domains for the engagement of HSJ1 in proteasomal sorting. In contrast, binding to Hsc70 was UIM independent. After immunoprecipitation, we also noted a modified, possibly ubiquitylated form of HSJ1a (Figure 2A, arrow; see below for further analyses). In light of this observation, we sought to verify that the high-molecular-mass ubiquitylated polypeptides detected in HSJ1a complexes were bound to HSJ1a and not modified forms of the cochaperone itself. Immunoprecipitation performed under non-native conditions led to an almost complete loss of coprecipitated polyubiquitylated polypeptides, clearly revealing their identity as HSJ1a bound chaperone clients (Figure 2B).

Recently, the cochaperone CHIP was identified as a central component in chaperone/proteasome cooperation [17–22]. CHIP acts as a chaperone-associated ubiquitin ligase and labels chaperone clients, such as oncogenic ErbB2, immature CFTR, and hyperphosphorylated tau, for degradation by the proteasome. Coexpression of CHIP and HSJ1a in HeLa cells led to a significant increase in the amount of HSJ1a bound ubiquitylated polypeptides (Figure 2B), suggesting a close cooperation of the two cochaperones in the pro-

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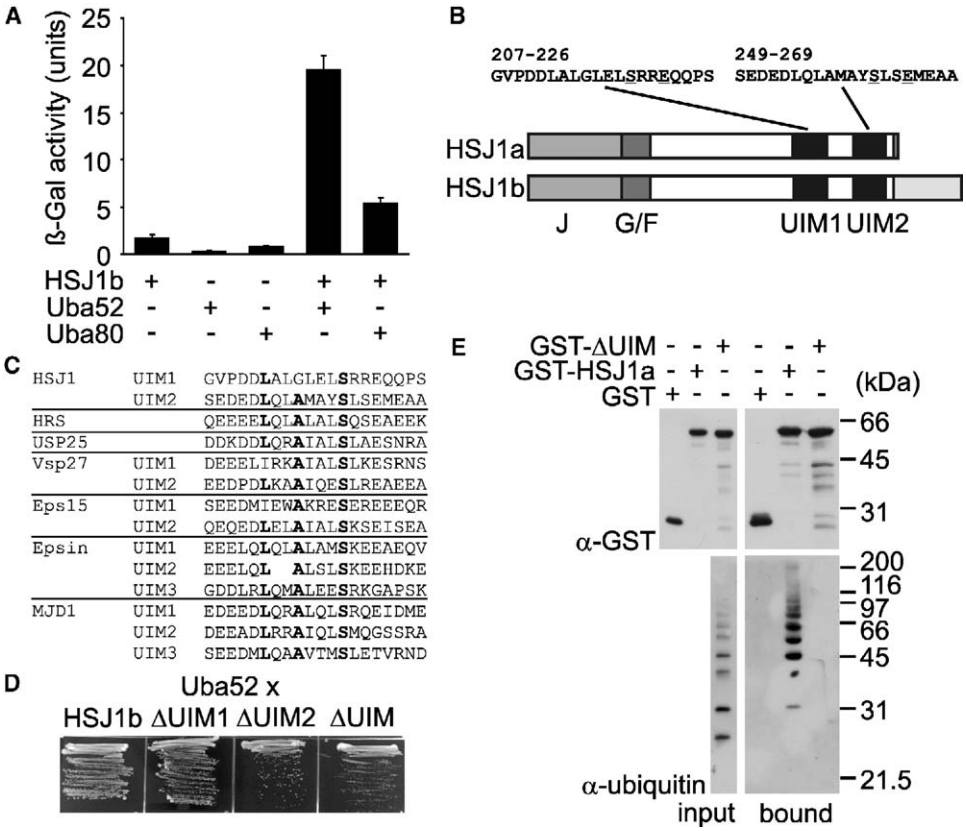

Figure 1. HSJ1 Interacts with Ubiquitin Fusion Proteins and Purified Ubiquitin Chains through Its UIM Domains

(A) Yeast cells expressing HSJ1b(Δ1–103) fused to the Gal4-DNA binding domain (HSJ1b) and the ubiquitin fusion proteins Uba52 and Uba80 fused to the Gal4-activation domain were assayed for β-galactosidase activity. Bars represent the mean of four independent experiments ± the standard deviation.

(B) Domain arrangement of HSJ1 isoforms (J, J-domain; G/F, glycine- and phenylalanine-rich region) and primary structure of the two UIM domains. Underlined residues were mutated to alanine to generate UIM-deficient forms of HSJ1.

(C) Alignment of UIM domains. Conserved residues are shown in bold.

(D) Yeast two-hybrid interactions between UIM mutants of HSJ1 and Uba52 (ΔUIM1-S219A/E222A; ΔUIM2-S262A/E265A; ΔUIM-S219A/E222A/S262A/E265A).

(E) Purified K48-linked ubiquitin chains were incubated with HSJ1a or ΔUIM immobilized on affinity resins via GST tags. Retained proteins were analyzed by SDS-PAGE and immunoblotting. "Input" represents 5% of added protein, and "bound" corresponds to 20% of total fraction.

teasomal sorting of chaperone clients. Upon overexpression of CHIP, the modified form of HSJ1a became more abundant and could be detected with an anti-ubiquitin antibody (Figures 2B and 2C, arrow). This identifies HSJ1a as a cellular target of CHIP. However, CHIP did not induce HSJ1a degradation, and HSJ1a levels were not increased upon proteasome inhibition (Figures 2D and 2E). We hypothesize, therefore, that CHIP-mediated ubiquitylation of HSJ1 does not regulate HSJ1 degradation but promotes client sorting to the proteasome. That HSJ1-associated client proteins are, in fact, on a sorting pathway to the proteasome is evident from the increased association observed upon proteasome inhibition (Figure 2E).

In vitro assays were performed to elucidate a potential modulating activity of HSJ1 on CHIP-mediated ubiquitylation. As previously established, CHIP cooperates with the ubiquitin-conjugating enzyme UbcH5b in mediating ubiquitylation of the protein kinase Raf-1 when presented by Hsc70 [17, 19]. In this assay, HSJ1a and also the UIM-deficient mutant form stimulated CHIP-mediated ubiquitylation (Figure 3A). This was expected because ΔUIM retains the ability to promote substrate loading onto Hsc70 owing to its functional J-domain and intrinsic chaperone activity. However, HSJ1a displayed a more pronounced stimulating activity than ΔUIM. UIM-mediated binding of HSJ1a to CHIP-generated ubiquitin chains seems to increase the processivity of ubiquitylation within the assembled chaperone/cochaperone complex.

We entertained the hypothesis that binding of HSJ1 to polyubiquitin chains may protect those chains against trimming by ubiquitin hydrolases, similar to observations for other ubiquitin binding factors [23]. To verify this hypothesis, we generated ubiquitylated Raf-1 in vitro in the presence of the CHIP ubiquitin conjugation machinery and Hsc70 and then added the ubiquitin hydrolase UBP1 [24]. Ubiquitin chains attached to the chaperone client were shortened by UBP1 (Figure 3B). Intriguingly, HSJ1 efficiently protected chains against

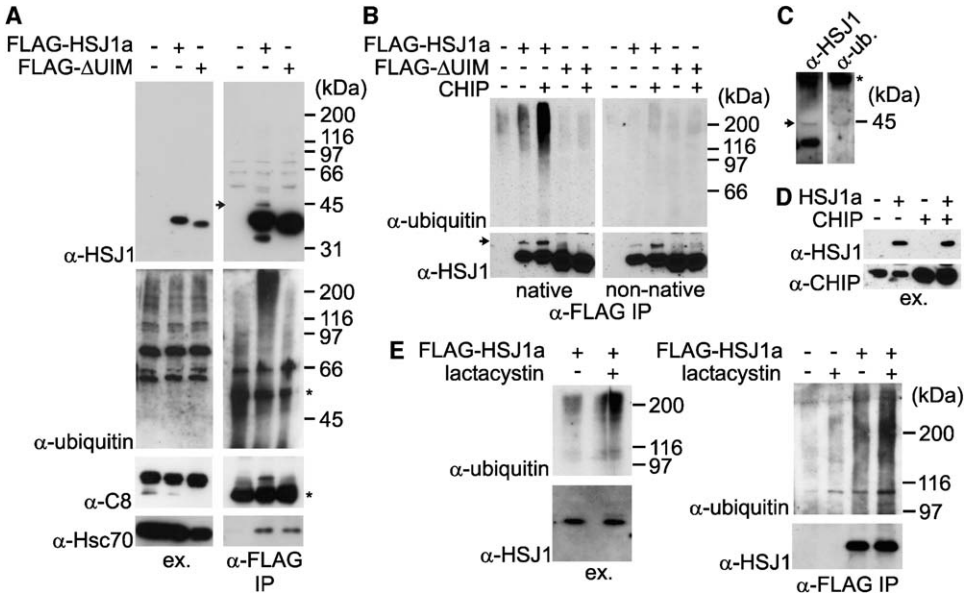

Figure 2. HSJ1 Is Involved in Chaperone-Assisted Degradation

(A) HeLa cells were transfected with FLAG-tagged HSJ1a, and cochaperone complexes were isolated by immunoprecipitation. Complex composition was analyzed by immunoblotting with antibodies against HSJ1, ubiquitin, the proteasomal subunit C-8, and Hsc70 as indicated. Asterisks highlight immunoglobulin chains. The arrow points to a monoubiquitylated form of HSJ1a.

(B) HSJ1a-containing complexes were isolated as in (A) and compared with complexes isolated from cells coexpressing CHIP. Immunoprecipitations were performed under native (left panel) and non-native (right panel) conditions.

(C) HSJ1a complexes isolated from CHIP-coexpressing cells under non-native conditions were probed with an anti-HSJ1 and anti-ubiquitin antibody to verify that modified HSJ1a is ubiquitylated (arrow). The immunoglobulin heavy chain is highlighted.

(D) Coexpression of CHIP does not alter HSJ1a expression levels in transfected HeLa cells.

(E) HeLa cells expressing FLAG-HSJ1a were treated with 20 μM lactacystin for 12 hr, and then immunoprecipitation of HSJ1a complexes was performed. Complexes were analyzed by immunoblotting with the indicated antibodies.

trimming, dependent on functional UIM domains. Taken together, these findings demonstrate a dual activity of HSJ1: promoting chaperone-assisted ubiquitylation and protecting ubiquitylated clients against the activity of ubiquitin hydrolases. This dual activity led to a strong accumulation of high-molecular-mass ubiquitylated polypeptides upon addition of HSJ1a to cell extracts incubated with the CHIP conjugation machinery (Figure 3C). Moreover, ubiquitylated polypeptides that accumulated in this situation could be precipitated with an anti-Hsc70 antibody (Figure 3D). These polypeptides might represent chaperone-associated ubiquitylated clients and/or ubiquitylated Hsc70 itself because the chaperone was also efficiently ubiquitylated in the extract in the presence of HSJ1a (Figure 3E). A comparison of immunoprecipitations performed under native and non-native conditions revealed that the majority of precipitated ubiquitylated polypeptides represent Hsc70-associated client proteins (Figure 3E). Notably, Hsc70 was preferentially modified by attachment of fewer than four ubiquitin moieties, whereas chaperone clients accumulated in high-molecular-mass polyubiquitylated form. Hsc70 is a physiological substrate of CHIP, but the ubiquitin ligase does not trigger the proteasomal degradation of Hsc70, which led to the conclusion that ubiquitylation of Hsc70, instead of serving as a degradation signal, promotes docking of the chaperone complex onto the proteasome during client delivery [25, 26]. On the basis of its UIM domains, HSJ1 apparently stimulates the association of Hsc70 with ubiquitylated clients and directs Hsc70 onto a proteasomal delivery pathway.

Chaperone-assisted ubiquitylation plays an important role in the quality control of ER membrane proteins, such as cystic fibrosis transmembrane conductance regulator (CFTR), that expose large domains into the cytoplasm [20, 27]. We therefore analyzed whether HSJ1 could affect CFTR turnover by coexpressing the ER-associated isoform HSJ1b (Figure 4A). Coexpression led to a strong decline of cellular CFTR levels. Such a decline was not observed upon proteasome inhibition, and ubiquitylated forms of CFTR became detectable (Figure 4A). Apparently, HSJ1b stimulates the ubiquitylation of the chaperone client at the ER membrane and in this way triggers its proteasomal degradation.

Considering the mainly neuronal expression of HSJ1a and HSJ1b, we investigated whether the cochaperones could modulate aggregation and inclusion formation in a cellular model of a polyglutamine disease, i.e. Huntington's disease. SK-N-SH neuroblastoma cells were transiently transfected with exon 1 of a pathogenic form of huntingtin, containing 103 glutamines, fused to enhanced green fluorescent protein (termed Q103 hereafter) [28]. Confocal microscopy showed that in the presence of polyglutamine inclusions, HSJ1a was recruited from its diffuse cellular distribution to a “ring” of immunoreactivity that surrounded the inclusion (Figure 4B). A similar

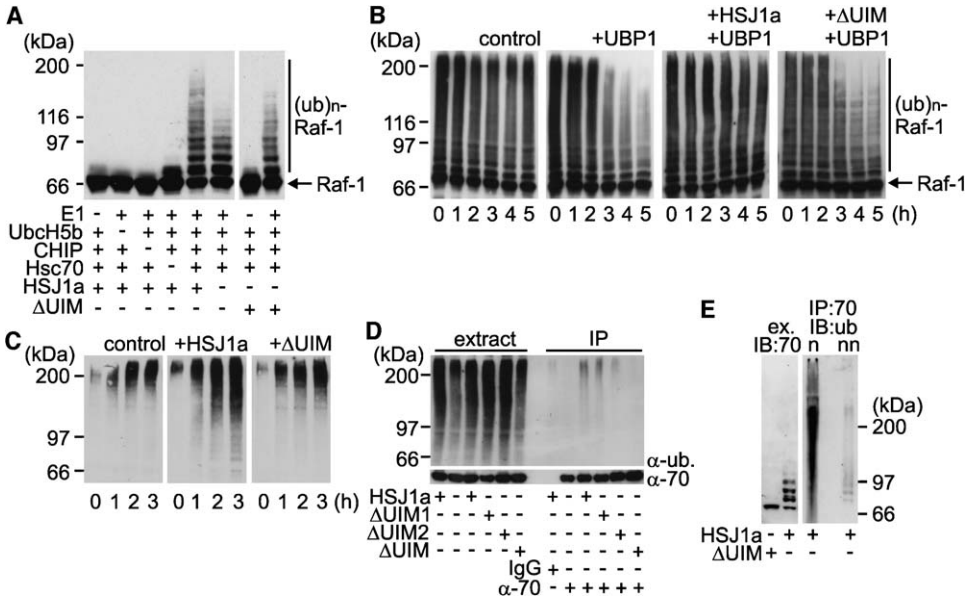

Figure 3. HSJ1 Stimulates CHIP-Mediated Ubiquitylation

(A) Ubiquitylation assays were performed in the presence of the indicated purified proteins. Raf-1 and ubiquitylated forms of the kinase ([ub]n-Raf-1) were detected after SDS-PAGE and immunoblotting.

(B) Ubiquitylated Raf-1 was incubated with the ubiquitin hydrolase UBP1, HSJ1a, and ΔUIM as indicated.

(C) The CHIP conjugation machinery was added to HeLa cell extracts in the presence of HSJ1a and ΔUIM as labeled, and the accumulation of ubiquitylated polypeptides was analyzed over time by SDS-PAGE and immunoblotting with an anti-ubiquitin antibody.

(D) After ubiquitylation in HeLa cell extracts in the presence of the indicated proteins, Hsc70 complexes were isolated by immunoprecipitation and analyzed for the presence of ubiquitylated polypeptides.

(E) Ubiquitylation in HeLa cell extracts was performed by addition of the CHIP conjugation machinery and HSJ1a or ΔUIM as indicated. After incubation, Hsc70 and ubiquitylated forms of the chaperone were detected in the extract (ex.) by SDS-PAGE and immunoblotting with an anti-Hsc70 antibody (IB:70). Extracts were subsequently subjected to immunoprecipitation with an anti-Hsc70 antibody. Immunoprecipitations were performed under native (n) and non-native (nn) conditions from 6 mg protein extract. Ubiquitylated polypeptides were detected in precipitated material (IP:70) by immunoblotting with an anti-ubiquitin antibody (IB:ub). Extract corresponds to 40 μg of cell extract. Immunoprecipitated samples represent 16% of total fraction.

ring-like staining was observed upon coexpression of ΔUIM, suggesting that the association of HSJ1 with polyQ inclusions mainly relies on the UIM-independent intrinsic chaperone activity of the cochaperone (see Supplemental Results and Discussion; Figure S1). Quantitative assessment revealed that coexpression of HSJ1a led to a significant reduction in the incidence of inclusions ($p < 0.0001$) (Figure 4C). ER-associated HSJ1b had no effect, unless it was released from the cytoplasmic face of the ER through a mutation that interferes with its C-terminal geranylgeranylation (C321S). The activity of HSJ1a in this assay was dependent on its ability to cooperate with Hsc70 and to bind ubiquitylated chaperone clients, as is evident from the use of the J-domain mutant H31Q and ΔUIM (Figure 4C). Its combined function as a regulator of Hsc70 and ubiquitin-chain recognition factor apparently enables HSJ1 to counteract aggregation processes that underlie neurodegeneration. In contrast to HSJ1a, expression of CHIP did not reduce inclusion formation (Figure 4D). However, CHIP enhanced the activity of HSJ1a in a manner dependent on the interaction of CHIP with Hsc70 (abolished in the TPR-domain-deficient mutant of CHIP-ΔTPR) and on its ubiquitin ligase activity (abolished in the U-box-deficient mutant-ΔU) (Figure 4D). Again, this confirmed the ability of the two cochaperones to cooperate in the processing of chaperone clients. The inclusion data were complemented with filter-trap assays of aggregated protein [29]. The amount of trapped Q103 was reduced by over 50% when HSJ1a was expressed in neuronal cells, and a further reduction was observed upon coexpression of CHIP. In contrast, CHIP alone or CHIP in combination with the ΔUIM mutant did not result in a significant decrease of filter-trapped Q103 (Figure 4E). J-proteins were previously shown to fulfill protective functions in experimental models of protein aggregation diseases [6, 7, 28, 30]. For example, the J-protein Hsp40 cooperates with Hsc70 in preventing mutant huntingtin from adopting toxic conformational states during aggregation [31–33]. Intriguingly, HSJ1a but not Hsp40 was found associated with soluble Q103 (Figure 4F). This underlines the unique function of HSJ1, which not only regulates the association of Hsc70 with mutant huntingtin but accompanies the aggregation-prone protein during further processing, that is, sorting to the proteasome. Indeed, proteasome inhibition revealed that HSJ1a bound Q103 was destined for degradation (Figure 4F).

HSJ1 represents a novel escort pathway to the proteasome in neuronal cells (Figure 5). This pathway is entered through a direct interaction of HSJ1 with the chaperone client, which relies on the intrinsic chaper-

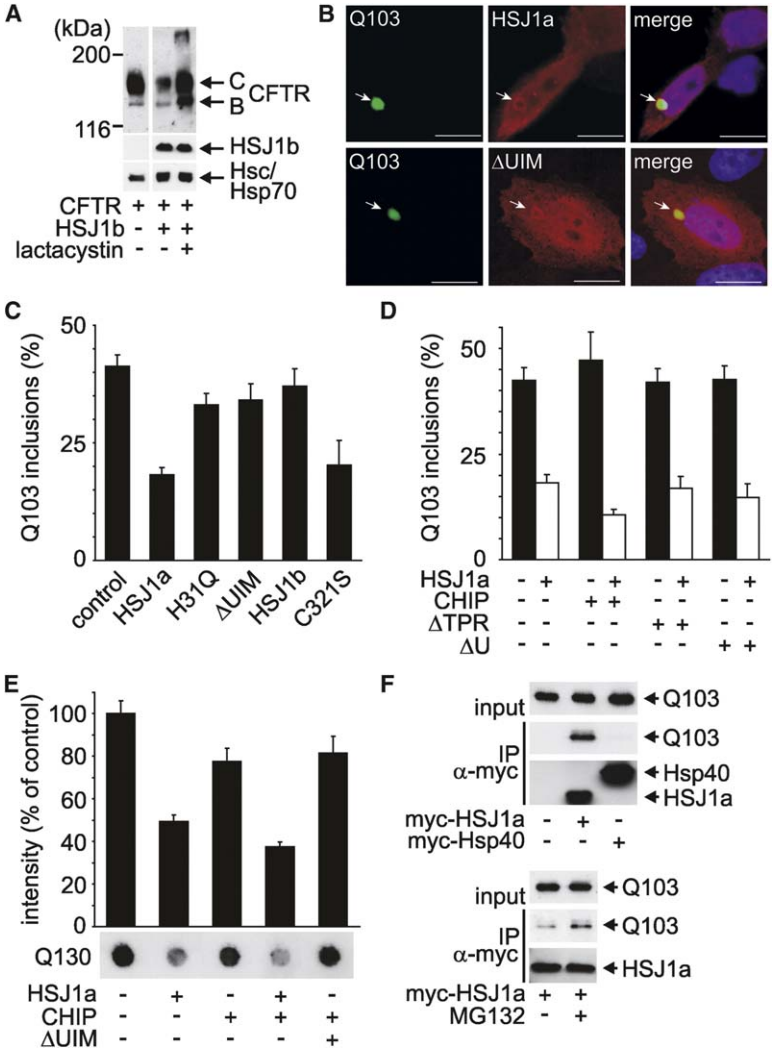

Figure 4. HSJ1 Induces the Degradation of Chaperone Clients and Reduces Q103 Inclusion Formation

(A) HEK293 cells were transfected with CFTR- and HSJ1b-expressing plasmids, and steady-state protein levels were analyzed by SDS-PAGE and immunoblotting. Each lane corresponds to 60 μg protein.

(B) Confocal immunofluorescence microscopy of SK-N-SH cells showing recruitment of HSJ1a or ΔUIM (red) to a ring of immunoreactivity surrounding the Q103 inclusion (green). The scale bar represents 10 μm.

(C) Quantification of Q103 inclusions in SK-N-SH cells 24 hr after transfection with HSJ1 proteins or HSJ1 mutants, as indicated. Bars are the mean percentage of inclusions in four independent counts by an observer blind to experimental status ± the standard deviation.

(D) Quantification of Q103 inclusions in SK-N-SH cells 24 hr after transfection with HSJ1a and/or CHIP and CHIP mutants, as indicated. Bars are the mean percentage of inclusions in four independent counts ± the standard deviation.

(E) Filter-trap experiment showing reduction of Q103 aggregation in SK-N-SH cells 24 hr after transfection with HSJ1a, ΔUIM, and/or CHIP, as labeled. The graph shows the mean intensity of GFP immunoreactivity of three independent experiments; the intensity was quantified by ImageJ and is shown ± the standard deviation. A representative filter trap is shown below.

(F) Coimmunoprecipitation of myc-tagged HSJ1 bound Q103 from the soluble fraction of SK-N-SH cells 24 hr after transfection as indicated. "Input" corresponds to 1% of added protein, and the precipitated fraction was 20% of the total fraction. Proteasome inhibition with MG132 was for 3 hr prior to cell lysis.

one activity of the escort protein, and subsequent J-domain-facilitated transfer of the client onto Hsc70. Once bound to Hsc70, the CHIP/Ubc5 ubiquitin conjugation machinery associates with the chaperone complex to mediate ubiquitin-chain attachment to the chaperone client (Figure 5). The generated chains are recognized by HSJ1 and protected against deubiquitylation. In the conjugation complex, HSJ1 also stimulates the attachment of a few ubiquitin moieties or short chains onto Hsc70. These modifications are not efficiently recognized by the HSJ1 UIM domains but may facilitate association with the proteasome. The observed CHIP-mediated ubiquitylation of HSJ1 may be of similar functional consequence. The chaperone complex thus exposes multiple sorting signals for recognition by the diverse ubiquitin-chain and ubiquitin-domain receptors present in the regulatory particle of the proteasome [34, 35]. Upon nucleotide exchange, the chaperone complex dissociates. If this occurs prior to docking onto the proteasome, HSJ1 stabilizes the released ubiquitylated client against aggregation based on interactions mediated via the UIMs and on its intrinsic chaperone activity. At the same time, the cochaperone stimulates reloading onto Hsc70 through its J-domain. In this way, HSJ1 ensures an engagement of the chaperone system until docking is achieved. The close cooperation of Hsc70 and HSJ1 in the sorting of ubiquitylated clients might be compared with the recently unravelled interplay between CDC48/p97 and ubiquitin binding factors [1–3]. Both cellular machineries achieve efficient proteasomal sorting by coordinating ubiquitylation, chaperone activity, and processing of the degradation signal.

Supplemental Data

Supplemental Results and Discussion, detailed Experimental Procedures, and a supplemental figure are available at http://www.current-biology.com/cgi/content/full/15/11/1058/DC1/.

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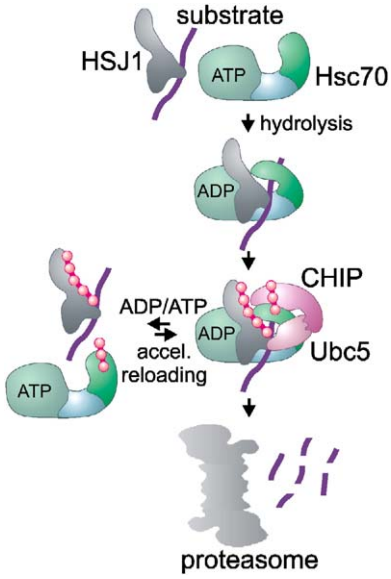

Figure 5. Model for the Involvement of HSJ1 in Chaperone-Assisted Degradation

See text for details.

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