Development of a Highly Sensitive *In Vitro* Assay to Characterize the Interplay of Multiple Components of the CHIP Ubiquitin Ligase Complex

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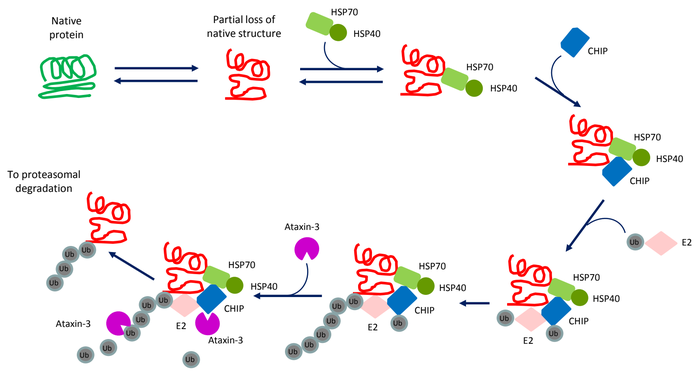
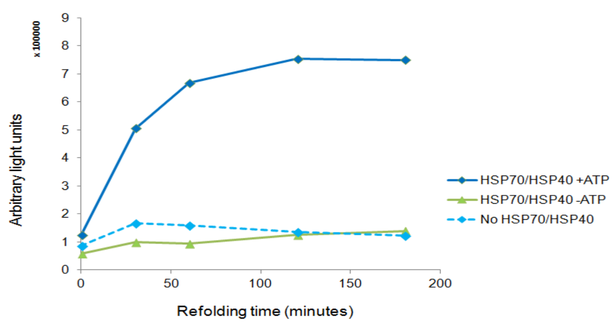
Introduction



CHIP plays a central role in linking protein folding and degradation pathways
Nascent polypeptides are folded into native proteins by the concerted action of several chaperones and co-chaperones. A variety of factors associated with cellular stress or disease states can cause native proteins to become partially unfolded. The chaperone system will attempt to refold these polypeptides back to their native states, but when this is not successful, some chaperones associate with the E3 ubiquitin ligase CHIP to polyubiquitinate these proteins and direct them to proteasomes for degradation. The deubiquitinating enzyme Ataxin-3 also interacts with CHIP and may regulate the length of polyubiquitin chains on unfolded substrate proteins and/or CHIP itself.
We recreated the CHIP ubiquitination pathway from active purified components to study their interactions *in vitro*. We show that a chaperone complex consisting of HSP70 and HSP40 can refold heat-denatured Glow-Fold (a luciferase derivative), to an enzymatically active state. Inclusion of CHIP in the refolding assay results in the polyubiquitination of multiple proteins, including the Glow-Fold substrate. Finally, recombinant Ataxin-3 was found to reduce the chain length of both polyubiquitinated Glow-Fold and CHIP.

Denatured Glow-Fold is Reactivated *In Vitro* by HSP70/HSP40 Complex

1 A



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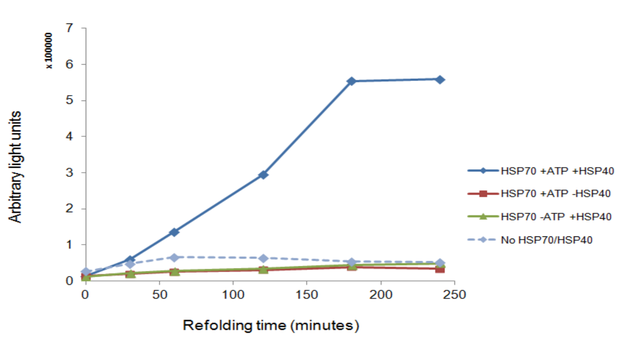


Figure 1: Refolding of heat-denatured Glow-fold by HSP70/HSP40
1.2 µM Glow-Fold was heat-denatured at 45°C for 7 minutes in the presence of 3 µM HSP70/HSP40 and 5 mM ATP in 50 mM HEPES pH 8.0, 50 mM NaCl, 2 mM $MgCl_2$ and 1 mM TCEP in 20 µl total volume. After cooling on ice, refolding was initiated by incubation at 30°C. At indicated time points, luciferase activity was determined on 2µl aliquots.
A: Refolding reaction is ATP-dependent.
B: Refolding reaction is HSP40-dependent

Ubiquitination of Denatured Glow-Fold is HSP70- and ATP-Dependent

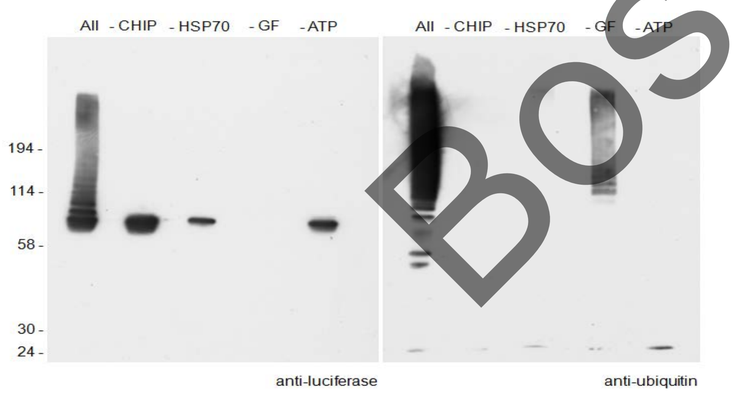


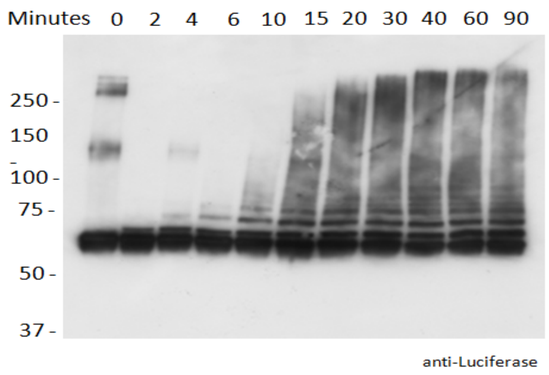
Figure 2: Polyubiquitination of Glow-Fold by CHIP
Glow-Fold, HSP70/HSP40 and ATP (as indicated) were heat-denatured as in **Figure 1**. After cooling on ice, 50 nM UBE1, 1 µM UBE2D3, 1 µM CHIP and 100 µM ubiquitin were added. Ubiquitination reaction was performed by incubation at 30ºC for 60 minutes. Individual reaction tubes contained all the components, or leaving out CHIP, HSP70, Glow-Fold (GF) or ATP as indicated.
Western blot analysis was performed on aliquots of the reactions above run on a 7.5 % gel. The membrane was probed with anti-luciferase (left panel) or anti-ubiquitin (right panel) antibodies.

Conclusions:

- HSP70 and HSP40 are functional and required for the refolding of heat denatured Glow Fold *in vitro*.
- Heat-denatured Glow-Fold is polyubiquitinated by HSP70/HSP40 in an ATP dependent reaction.
- HSP70 is extensively polyubiquitinated by CHIP.
- Ubiquitination of Glow-Fold and HSP70 is evident at 2 minutes and progresses steadily up to 90 minutes with accumulation of highly polyubiquitinated species and concomitant depletion of unmodified substrates.

CHIP Ubiquitinates Glow-Fold and HSP70

3 A



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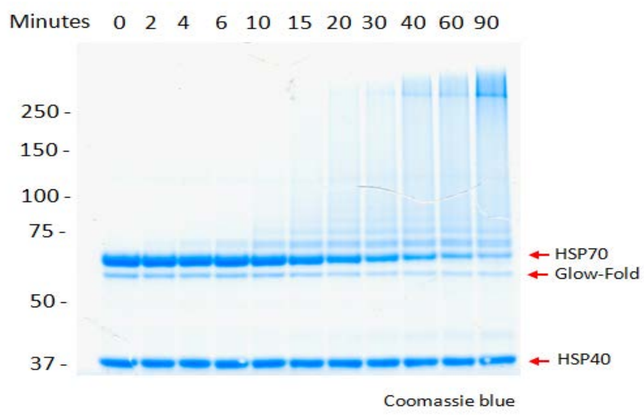
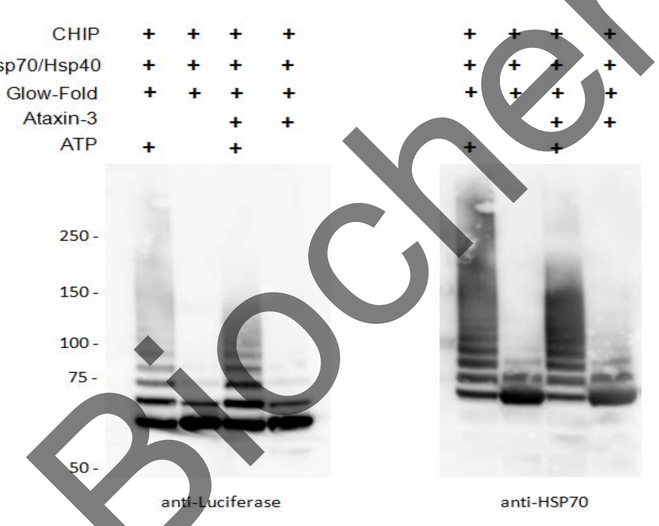


Figure 3: Time course of *in vitro* ubiquitination of Glow-fold by CHIP
Reaction was conducted as described in **Figure 2**. At the indicated time points, aliquots were taken and the reaction stopped with SDS-PAGE sample buffer.
A: Western blot analysis was performed on aliquots of the reactions above run on a 7.5 % SDS-PAGE. The membrane probed with anti-luciferase antibody.
B: Aliquots of the reactions above were run on a 7.5 % gel and stained with Coomassie Blue

Ataxin-3 Alters CHIP-Dependent Ubiquitination of Glow-Fold and HSP70

4 A



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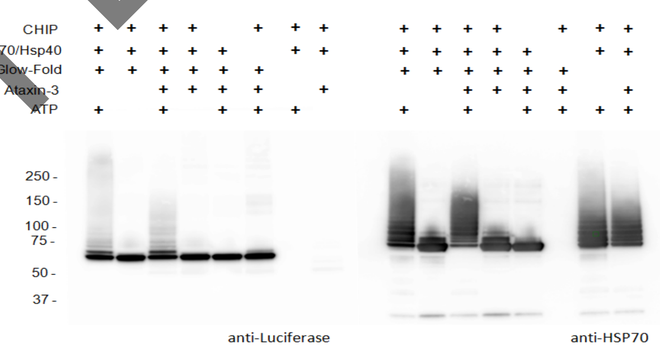

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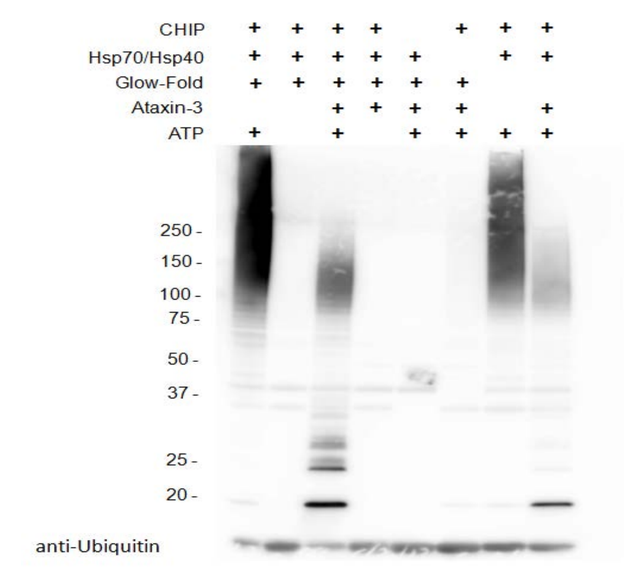
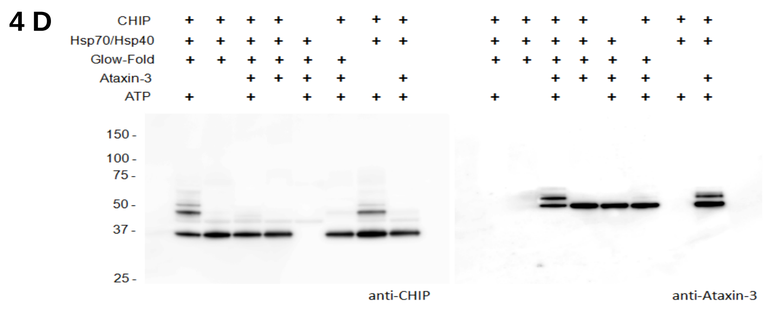


Figure 4: *In vitro* effect of Ataxin-3 on ubiquitination of Glow-Fold by CHIP.
Heat shock reactions were conducted as described in **Figure 2,** with the addition of 1 µM Ataxin-3 after the heat shock where indicated. Reactions were then continued for an additional 70 minutes at 30°C. Aliquots were run on a 4-20 % SDS-PAGE. Western blot analysis was performed on aliquots of the reactions and the membrane probed with the indicated antibodies
A and B: anti-luciferase or anti-HSP70
C: anti-ubiquitin

4 D



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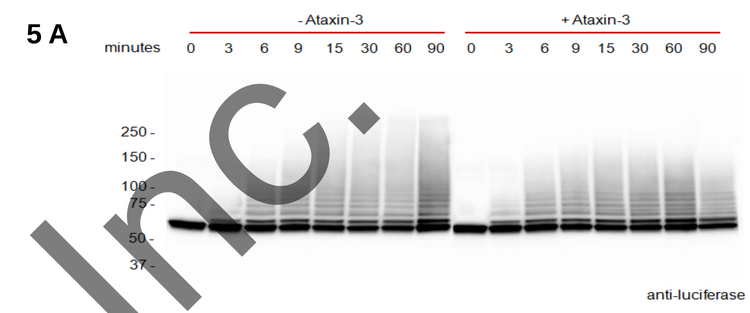
D: anti-CHIP or anti-Ataxin-3

Time Course of Glow-Fold Ubiquitination in Reactions with CHIP and Ataxin-3

5 A



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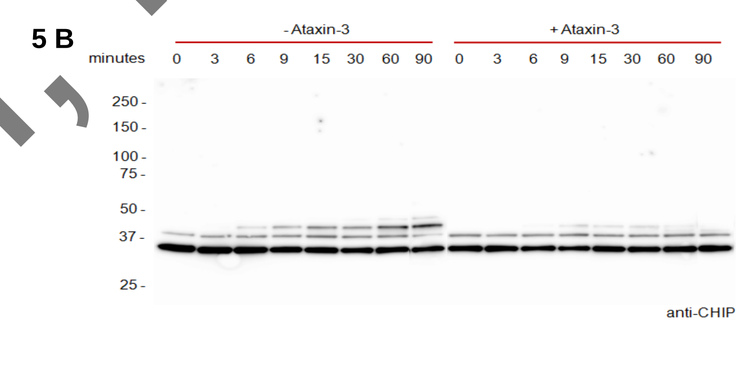

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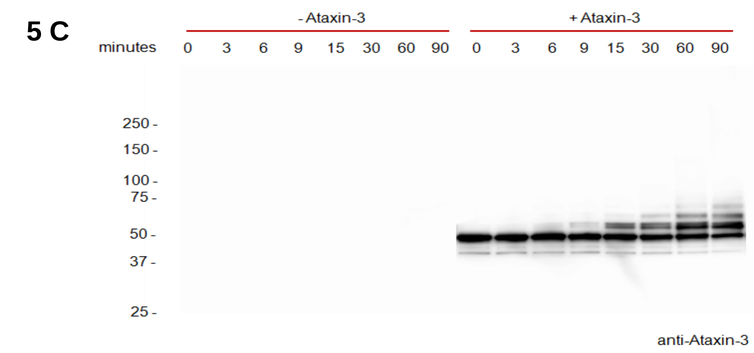

Figure 5: *In vitro* effect of Ataxin-3 on ubiquitination of Glow-Fold by CHIP: time curve
Reaction was conducted as described in **Figure 4** with the addition of 1 µM Ataxin-3 after the heat shock . Incubation was performed at 30°C. At the indicated time points aliquots were taken and stopped with SDS-PAGE sample buffer and run on a 4-20 % gel. Western blot analysis was performed on aliquots of the reactions and the membrane probed with the indicated antibodies
A : anti-luciferase
B: anti-CHIP
C: anti-Ataxin-3.

Conclusions:

- Ataxin-3 reduces the size of polyubiquitinated chains both on Glow Fold and HSP70.
- Ataxin-3 inhibits to a great extent the autoubiquitination of CHIP. In turn, Ataxin-3 is progressively ubiquitinated by CHIP.
- The accumulation of short oligoubiquitin chains over time in the presence of Ataxin-3 suggests that the reduction of longer polyubiquitin chains on Glow-Fold and HSP70 is due to deubiquitination by Ataxin-3 rather than inhibition of CHIP

Summary

We generated an *in vitro,* multi-component system that recreates many of the protein triage properties described for its constituents *in vivo.*

CHIP polyubiquitinates the heat-denatured artificial substrate Glow-Fold in an HSP70/HSP40- and ATP-dependent manner *in vitro*. The chaperone HSP70 is highly polyubiquitinated as well.

In this system, addition of Ataxin-3 reduces the length of polyubiquitin chains on both Glow-Fold and HSP70. The accumulation of short ubiquitin oligomers suggests that this action is due to trimming of longer chains produced by CHIP in the absence of Ataxin-3.

In the absence of Ataxin-3, CHIP progressively autoubiquitinates during *in vitro* reactions. Ataxin-3 greatly reduces this and in the process becomes increasingly ubiquitinated itself.

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