Supplemental figures

Supplemental figure 1: AT3-Ub cleaves mixed-linkage Ub4 chains.

Unmodified or ubiquitinated, GST-tagged AT3 was incubated with K63-linked tetra-Ub chains (K63-Ub4; left) or mixed-linkage tetra-Ub chains (K63-48-63-Ub4; right). Similar to unmodified AT3, AT3-Ub does not cleave homotypic Ub4 chains, but does cleave mixed-linkage Ub4 better than its unmodified counterpart.

Supplemental figure 2: Multi-mono-ubiquitinated AT3 shows activation.

GST-AT3 was ubiquitinated *in vitro* with wild type E2 (UbcH5c(WT)) or a mutant variant (UbcH5c(S22R)) that cannot extend Ub chains and therefore produces only mono-ubiquitinated or multi-mono-ubiquitinated AT3. In both cases, AT3-Ub is more active than its unmodified version. Note that in the presence of UbcH5c(S22R), AT3 ubiquitination does not reach completion.

Supplemental figure 3: Mutating two conserved residues on each UIM of AT3 eliminates its ability to bind Ub chains with high affinity.

GST, GST-AT3(WT) or GST-AT3(UIM*) prebound to glutathione sepharose beads was incubated with K48- or K63-linked Ub3-7 chains at 4˚C for 30 minutes. Mutating the conserved alanine and serine residues in each UIM to glycine and aspartic acid, respectively, eliminates high affinity binding of AT3 to either type of Ub chain. P: pellet fractions that had been washed four times and immunoblotted with anti-Ub antibody; S: supernatant.

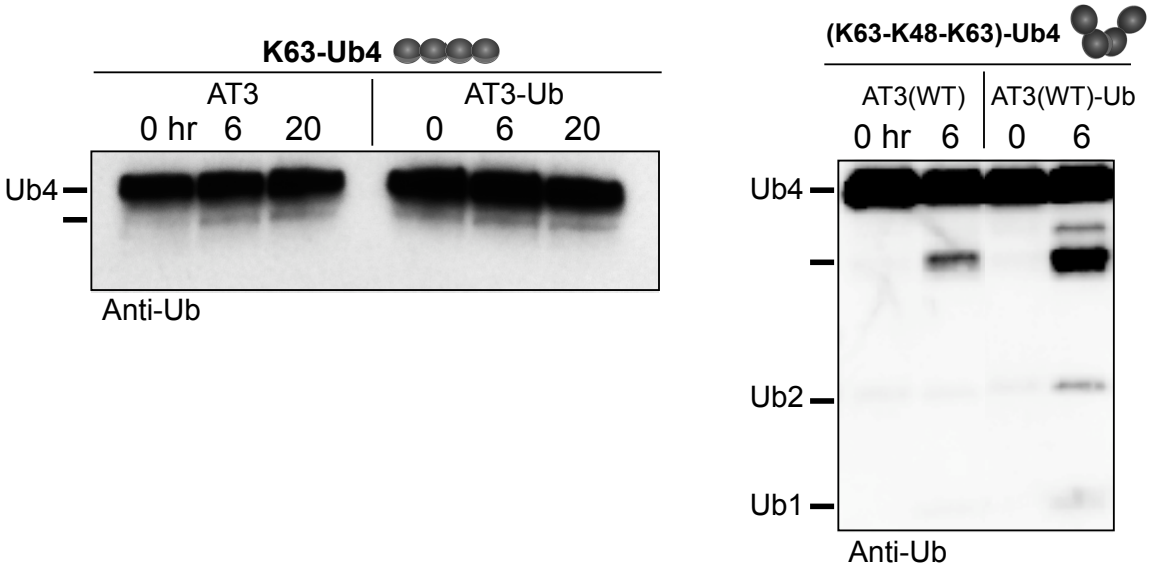
Supplemental figure 4: AT3(WT)-Ub and AT3(UIM*)-Ub show switched preference for K63- and K48-linked Ub chains.

Wild type AT3 and UIM-deficient AT3 were ubiquitinated *in vitro,* then incubated with either K63-Ub6 or K48-Ub5 chains. Only the 6 hr time points are shown for each reaction to highlight differences in activity. All lanes are from the same exposure of the same blot, rearranged to highlight differences. Shown are representative results from three independent experiments. Anti-AT3 blot shows GST-AT3 species used in deubiquitination reactions.

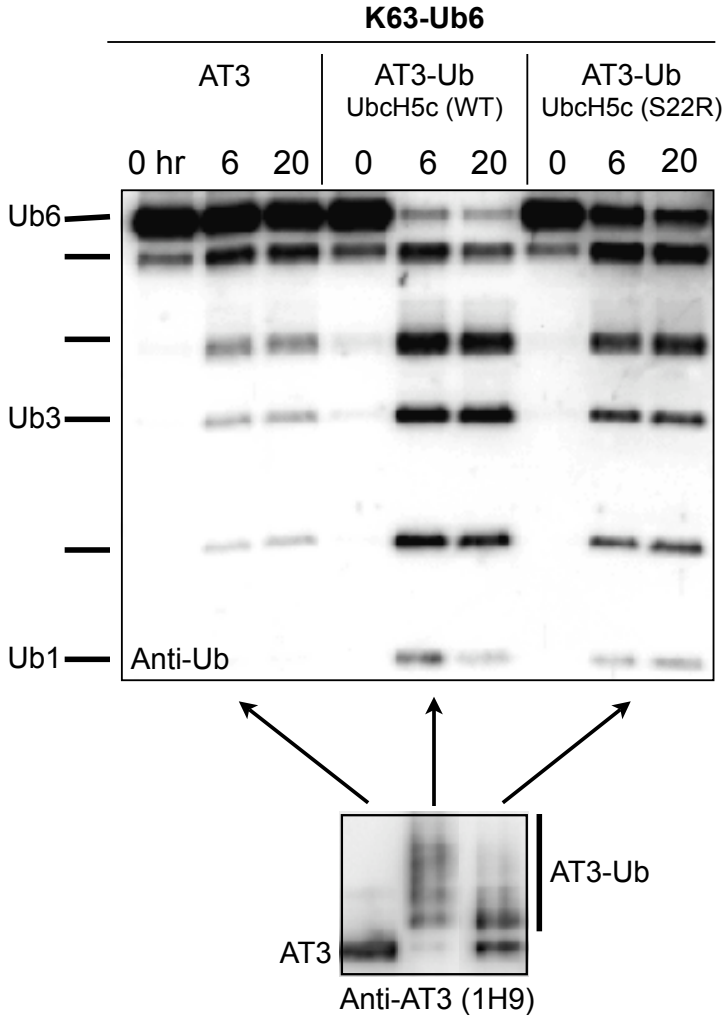
Supplemental figure 5: AT3 ubiquitination does not induce large conformational changes.

Left: AT3 was ubiquitinated *in vitro*. Right: AT3 and AT3-Ub were gel-filtered through a Superdex 200 column. Fractions were collected and resolved through SDS-Page gels and western blotted with anti-AT3 antibody.

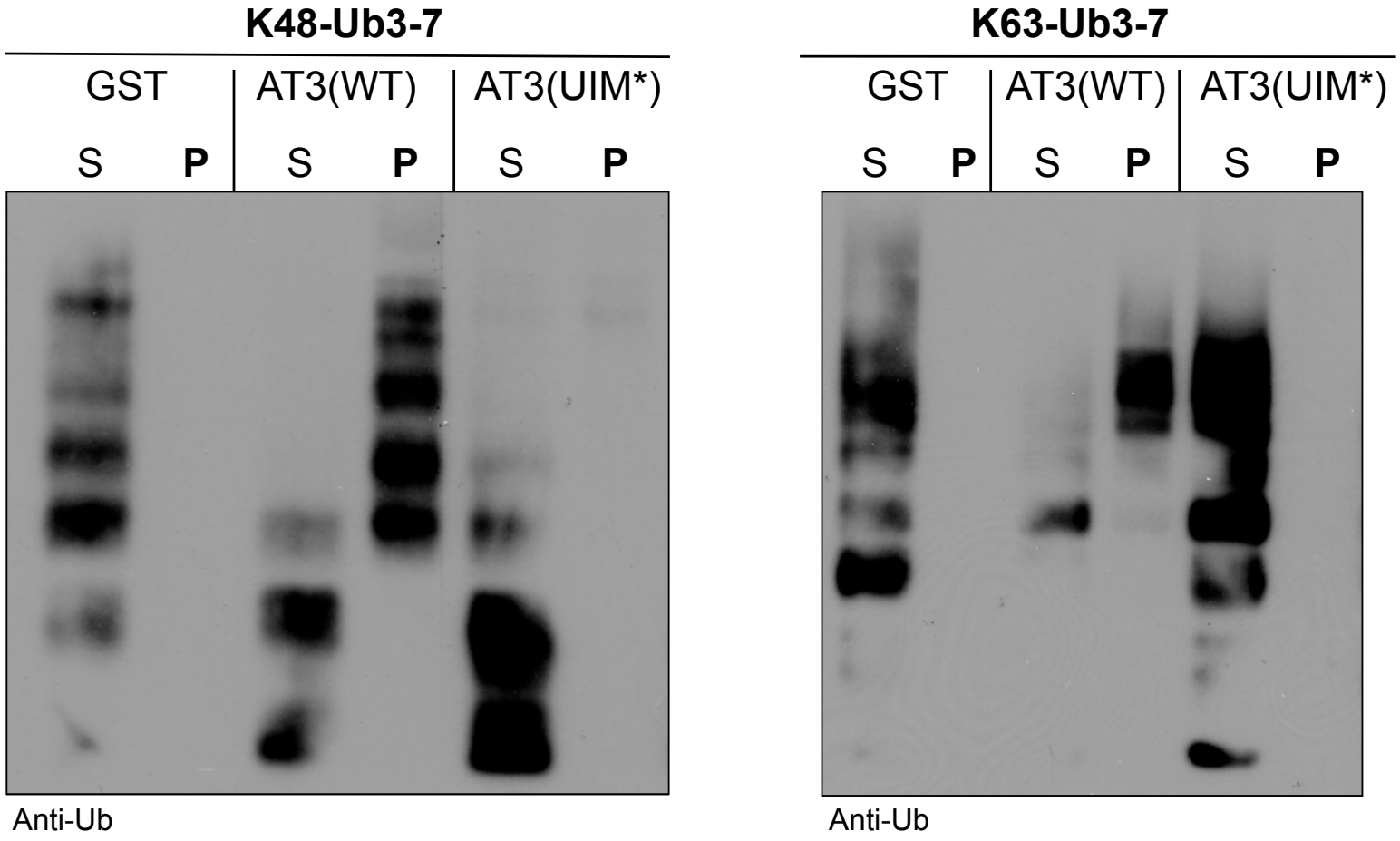
Todi et al., Supplemental Figure 1



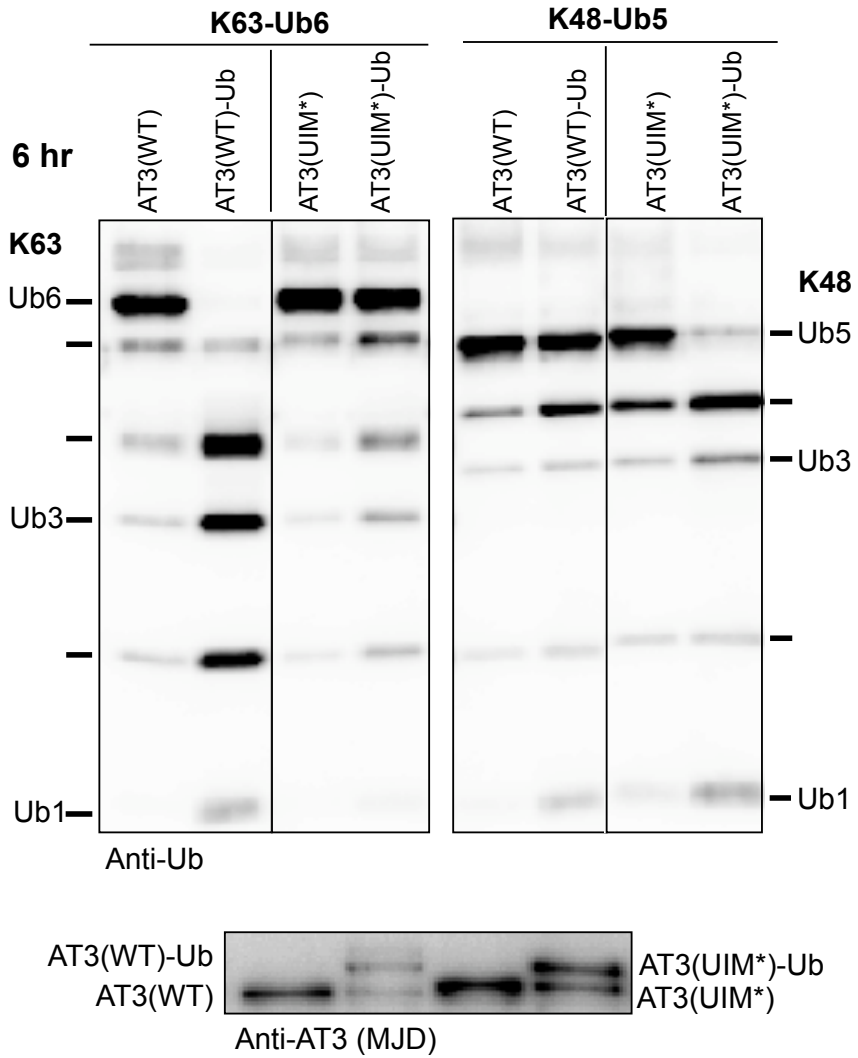
Todi et al., Supplemental Figure 2



Todi et al., Supplemental Figure 3



Todi et al., Supplemental Figure 4



Todi et al., Supplemental Figure 5