Ataxin-3 Deubiquitination Is Coupled to Parkin Ubiquitination via E2 Ubiquitin-conjugating Enzyme

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Background: The deubiquitinating enzyme (DUB) ataxin-3 opposes the E3 ubiquitin-ligase activity of parkin, however, the mechanism involved is unclear.

Results: Ataxin-3 opposes parkin ubiquitination by regulating the E2 ubiquitin-conjugating enzyme.

Conclusion: The components within a E2:E3:DUB complex are functionally coupled to fine-tune the overall extent of ubiquitination.

Significance: The work broadens our understanding of the intricate interplay between E3s, DUBs, and the ubiquitination machinery.

We reported previously that parkin, a Parkinson disease-associated E3 ubiquitin-ligase interacts with ataxin-3, a deubiquitinating enzyme associated with Machado-Joseph disease. Ataxin-3 was found to counteract parkin self-ubiquitination both in vitro and in cells. Moreover, ataxin-3-dependent deubiquitination of parkin required the catalytic cysteine 14 in ataxin-3, although the precise mechanism remained unclear. We report here that ataxin-3 interferes with the attachment of ubiquitin (Ub) onto parkin in real-time during conjugation but is unable to hydrolyze previously assembled parkin-Ub conjugates. The mechanism involves an ataxin-3-dependent stabilization of the complex between parkin and the E2 Ub-conjugating enzyme, which impedes the efficient charging of the E2 with Ub. Moreover, within this complex, the transfer of Ub from the E2 is diverted away from parkin and onto ataxin-3, further explaining how ataxin-3 deubiquitination is coupled to parkin ubiquitination. Taken together, our findings reveal an unexpected convergence upon the E2 Ub-conjugating enzyme in the regulation of an E3/deubiquitinating enzyme pair, with important implications for the function of parkin and ataxin-3, two proteins responsible for closely related neurodegenerative diseases.

Post-translational modification of proteins is central to regulating their stability and activity. One such modification, ubiquitination, involves the attachment of a 76-amino acid ubiquitin (Ub) moiety to a protein via an isopeptide linkage that is formed between the C-terminal glycine of Ub and the ε-amino acid side chain of a lysine (Lys) residue within the target protein (1, 2). Conjugation of Ub to a protein is a multistep process, requiring the concerted activities of three distinct families of enzymes. The first step involves the activation of Ub by the E1 Ub-activating enzyme in an ATP-dependent manner, with the formation of a Ub thioester complex between the active site cysteine in the E1 and the C-terminal glycine of Ub. For Ub, two E1 Ub-activating enzymes, Ube1 and UBA6 (3, 4), can interact with E2 Ub-conjugating enzymes, of which there are ~38 in humans (5), resulting in the formation of an E2:Ub thioester complex. Finally, the E2 thioester interacts with its respective E3 Ub-ligase, which in turn directs Ub onto the target protein. There are two main classes of E3 Ub-ligases: HECT and RING-domain containing E3s. The human genome encodes upwards of 600 predicted RING Ub-ligases, ~30 HECT Ub-ligases, and a smaller subset of non-HECT, non-RING E3s (i.e. U-box or zinc finger E3s). The RING domain provides a scaffold through which the E3 interacts with the E2-Ub, thereby facilitating the transfer of Ub from the E2 onto the substrate protein. In contrast, HECT E3s contain an active site cysteine that receives the Ub directly from the E2, which can then be transferred onto the substrate protein (6). In addition to being conjugated to a lysine residue within a target protein, each of the 7 lysine residues within Ub can serve as an acceptor for the next Ub, leading to the formation of isopeptide-linked Ub chains. Lys48-linked chains are the best studied of the isopeptide-linked chains and direct proteins to the proteasome where they are subsequently degraded. However, Ub chains can be linked through one of the other six lysine residues within Ub, with such chains playing important roles in many cellular processes, including DNA repair, receptor signaling, and endocytosis (7, 8).

E3 Ub-ligases are central in determining the manner in which Ub chains are assembled on substrate proteins and, typ-
Ataxin-3 Regulates Parkin Activity via E2 Enzyme

Physically, E3 ligases can regulate their own activity and stability via self-ubiquitination. MDM2 is a classical example of a RING E3 ligase that can mediate the conjugation of Lys^{48}-linked Ub chains on itself. As a result, MDM2 promotes its own targeting to the proteasome for degradation (9). However, for certain E3s, such as BRCA1 and RING1b, attachment of Ub conjugates does not appear to affect stability. Rather, these RING E3s conjugate non-Lys^{48} linked chains on themselves. BRCA1 mediates the formation of Lys^{6}-linked Ub chains (10, 11), whereas RING1b adds a mix of Lys^{6}, Lys^{27}, and Lys^{48} linked chains (12), resulting in enhanced activity for both E3s to ubiquitinate histone proteins.

Although self-ubiquitination can affect both the activity and stability of an E3, it is also reversible. Indeed, another distinct group of enzymes, collectively called deubiquitinating enzymes (DUBs) can counteract the activity of E3 Ub-ligases. Although there is one family of metalloprotease-type DUBs, the majority of DUBs are cysteine proteases that can be subdivided into four subclasses based on their Ub-protease domain: Ub C-terminal hydrolases, otubain proteases, Ub-specific proteases, and Machado-Joseph disease (MJD) proteases. Three broad functions exist for the DUB enzymes: 1) processing mature Ub precursor proteins to generate free Ub; 2) catalyzing the removal of a Ub from a ubiquitinated substrate; and 3) facilitating the removal of Ub and the subsequent transfer and degradation of a protein through the proteasome (13). Moreover, many DUBs function in partnership with specific E3 ligases. One such E3:DUB pair is MDM2:USP7, with MDM2 being stabilized by USP7-mediated deubiquitination (14). In contrast, Usps can act in partnership with the E6-AP RING-ligase to catalyze the removal of Ub conjugates from RING1b, with E6-AP mediating the attachment of Lys^{48}-linked Ub conjugates that promote the proteasomal degradation of RING1b (15). Although most characterized DUBs have been reported to function via their Ub-protease activity, in some cases DUBs can inhibit ubiquitination and degradation of substrates independent of their catalytic activity (16–18).

We recently characterized an interaction between ataxin-3, an MJD class of DUB, and parkin, a RING-type E3 Ub-ligase (19). Mutations in SCA3, the gene encoding ataxin-3, cause MJD, also known as spinocerebellar ataxia-3 (SCA3) (20), the most common autosomal dominant inherited ataxia worldwide. Mutations in the Parkin gene are responsible for ~50% of early onset Parkinson disease cases (21). Moreover, patients with SCA3 have been reported to present with parkinsonian symptoms further supporting the disease relevance of the interaction between parkin and ataxin-3 (22). We found that ataxin-3 can deubiquitinate parkin directly in vitro and can reduce the extent of parkin ubiquitination in cells. Furthermore, the MJD-associated, polyglutamine (poly(Q))-expanded mutant form of ataxin-3 promotes parkin degradation by the autophagy pathway. However, the precise mechanism involved in ataxin-3-mediated deubiquitination of parkin remains largely unknown.

In the current study, we find that ataxin-3 is unable to hydrolyze preassembled Ub conjugates on parkin. Rather, ataxin-3 appears to edit Ub conjugates as they are being attached to parkin. The mechanism involves stabilizing the parkin:E2 complex and redirecting the transfer of Ub from the E2 onto ataxin-3 rather than on parkin. Both in vitro and in cells, the process requires the active site cysteine of parkin-bound ataxin-3. Taken together, the work reveals a novel mode of coupling between the respective activities within a E2:E3:DUB complex to fine-tune the overall extent of ubiquitination.

EXPERIMENTAL PROCEDURES

Plasmids and Reagents—Antibodies were used to detect ataxin-3 (mouse monoclonal, Chemicon; rabbit polyclonal, gift of H. L. Paulson, University of Michigan), parkin (Santa Cruz Biotechnology), Ub (Covance), ube2G2 (Abnova), His (Novagen), FLAG, HA, and c-Myc (Sigma). Goat anti-mouse light chain-specific antibodies (Jackson ImmunoResearch Laboratories) were used to detect ataxin-3 or parkin from immunoprecipitated SH-SY5 lysates or mouse brain fractions. All constructs used were described previously (19), with the exception of pCMV-Myc-Ubc7 (Addgene). Where indicated Ub-aldehyde (UBAL) (Boston Biochem) was added to a final concentration of 100 mM. DTSSP (Pierce) was added to a final concentration of 0.05 μM in cross-linking assays as described previously (23). 0.5 Units of apyrase (Sigma) was added to stop further monoubiquitination of ataxin-3. All DUBs and reagents used in ubiquitination/deubiquitination/charging assays were purchased from Boston Biochem, with the exception of His-Usp15 and His-Usp25 (EMD Biosciences).

Protein Expression and Binding Assays—GST, MBP, and His fusion proteins were expressed in Escherichia coli strain BL21(DE3) at 23 °C for 3 h and affinity purified using glutathione-Sepharose 4B beads (Amersham Biosciences), amylose resin (New England Biolabs), and nickel-nitrilotriacetic acid agarose (Qiagen), respectively. MBP-parkin was eluted from amylose resin with maltose elution buffer (10% D-maltose, 20 mM Tris, pH 7.4, 100 mM NaCl). Bead-immobilized GST proteins were incubated with 2 μg of purified His proteins at 4 °C for 16 h in 1 ml of 20 mM HEPES-KOH, pH 7.4, 100 mM NaCl, 10% glycerol, 0.1% Triton X-100, 0.5 mM dithiothreitol (DTT).

In Vitro Ubiquitination, Deubiquitination, and Charging Assays—In vitro ubiquitination and charging assays were carried out as described previously (19) with the reducing agent dithiothreitol (DTT) present at a final concentration of 1 mM. Charging reactions with Ubch7 (Fig. 5, B and C) were performed in the absence of DTT using previously described conditions (24). In the absence of DTT, tris(2-carboxyethyl)phosphine was present at a final concentration of 0.1 mM. For sequential reactions, GST-parkin beads were spun down after the indicated time followed by three washes with ubiquitination buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM MgCl₂, 4 mM ATP, 2 mM DTT). Reactions were assembled containing ubiquitination buffer in the presence/absence of ~0.25 μM His-ataxin-3 and left for the indicated time intervals. In sequential reactions with HA- and Myc-Ub, samples were imaged using the two-color LI-COR Odyssey infrared imaging system. FLAG-resin (Sigma) was used to immunoprecipitate ubiquitinated MBP-parkin. For charging assays, reactions were stopped by adding 3× SDS sample buffers (±DTT). Reactions stopped with DTT-containing sample buffer were boiled at 90 °C. For
assays containing Ub-AMC (1 μM), 1 μM of the indicated DUB was added to each reaction in the presence of ubiquitination buffer (−ATP) and incubated at 37 °C for 30 min (Ub-AMC). Reactions were set up in 96-well plates and AMC fluorescence was quantitated on a Victor 3 fluorometer (380 nm excitation and 460 nm).

Cell Culture, Transfections, and Immunoprecipitations—HEK293T and mouse embryonic fibroblasts (ataxin-3 wild-type and knock-out, gift of H. L. Paulson) were maintained under the same conditions in DMEM/F12 (50:50) supplemented to 10% total volume with fetal bovine serum. HEK293T cells were transfected with the indicated plasmids using Lipofectamine 2000. Cells were lysed 48 h after transfection in RIPA lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, and protease inhibitors). HEK293T lysates (1 mg/ml) were incubated with 20 μl of anti-FLAG resin (Sigma) overnight at 4 °C. FLAG-parkin was eluted with 40 μg of FLAG peptide (Sigma) in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl at 4 °C for 20 min. SH-SY5 lysates were incubated overnight at 4 °C with the rabbit anti-ataxin-3 polyclonal or rabbit serum in a 1:250 dilution. Protein G-Sepharose beads were added the following day and after 3 h at 4 °C, antibody bound beads were washed 3 times in lysis buffer and boiled in 3× SDS + DTT sample buffer.

Immunoprecipitations from Mouse Brain Synaptosomal Fractions—Whole mouse brains were fractionated by differential centrifugation as previously described (25, 26). Briefly, brains were homogenized in 0.32 M sucrose, 10 mM HEPES, pH 7.4, supplemented with protease inhibitors. The homogenate was centrifuged for 10 min at 1,000 g, and the supernatant (S1) was collected and centrifuged again for 15 min at 12,000 g to produce a synaptic pellet (P2). 1 mg of the resuspended P2 pellet was spun down as above and resuspended in 1 ml of RIPA lysis buffer. Immunoprecipitations were carried out as described above for SH-SY5 cells.

RESULTS
Ataxin-3 Reduces but Does Not Abolish Parkin Self-ubiquitination—In our previous study, we identified ataxin-3 as a novel DUB partner for the E3 Ub-ligase, parkin, with ataxin-3 directly opposing the ability of parkin to ubiquitinate itself (19, 27). The effect was specific for parkin, as ataxin-3 was much less effective at debiquitinating other RING E3s (19) or the RING-in-between-RING (RIR) domain of HHARI (24), a closely related E3 (Fig. 1A). Although ataxin-3 regulates the ability of parkin to ubiquitinate itself, in vitro time course assays clearly demonstrate that parkin can still ubiquitinate itself in the presence of ataxin-3, albeit at a dramatically reduced rate (Fig. 1B). In these in vitro experiments, both ataxin-3 and parkin were incubated together at the start of the reaction, raising the possibility that ataxin-3 might inhibit parkin self-ubiquitination nonspecifically, rather than via its catalytic deubiquitination activity. Several lines of evidence argue against this notion. First, incubation with the DUB inhibitor UBAL prevents ataxin-3 from debiquitinating parkin, arguing that the effect involves deubiquitination (Fig. 1C). Second, if ataxin-3 was simply sterically hindering ubiquitination by binding parkin, the binding to another UIM containing protein, Eps15 should also suppress parkin self-ubiquitination, because Eps15 binds parkin via domain-domain interactions very similar to those employed by ataxin-3. Yet, Eps15 enhances rather than suppresses parkin self-ubiquitination, arguing that binding per se is not sufficient to inhibit ubiquitination (28, 29). Perhaps, most compellingly, if the effect were purely steric, a C14S catalytically inactive ataxin-3 mutant should also prevent parkin self-ubiquitination because it binds parkin comparably to wild-type ataxin-3 (Fig. 1D). Yet, the C14S ataxin-3 mutant has no effect on parkin self-ubiquitination (Fig. 1B) (19). Hence, ataxin-3 reduces, but does not block parkin self-ubiquitination, via a mechanism that requires the presence of the catalytic cysteine at residue 14 in its active site.

Ataxin-3 Is Unable to Remove Preassembled Ub Chains on Parkin—Given that the effect of ataxin-3 on parkin is catalytic, we next tested whether it could also hydrolyze preformed parkin-Ub conjugates. Remarkably, when ataxin-3 was added to the reaction following washout after 1 h of parkin self-ubiquitination, only minimal deubiquitination was observed (Fig. 2A), even when incubated for up to 8 h (supplemental Fig. S1A). Neither an expansion of the poly(Q) tract (supplemental Fig. S1A), nor the choice of E2 used for Ub conjugation (supplemental Fig. S1B) could confer ataxin-3 with the ability to hydrolyze preassembled Ub conjugates on parkin.

The lack of ataxin-3 activity toward preassembled Ub conjugates suggested that long poly-Ub chains on parkin might preclude the optimal positioning of ataxin-3 required for parkin deubiquitination. To test this possibility, we used a UbK0 mutant, which is unable to support the formation of poly-Ub chains, but that can be efficiently conjugated to parkin (although less so than wild-type Ub) as multiple mono-Ubs. We found that ataxin-3 was unable to remove the preassembled multiple mono-Ubs on parkin after washout (Fig. 2B). However, similar to wild-type Ub, ataxin-3 was able to remove UbK0 from parkin when added during the parkin ubiquitination reaction. These findings were not because of a lack of in vitro hydrolyase activity by ataxin-3 as it was able to cleave Ub-AMC, similarly to several other DUBs (supplemental Fig. S2). Thus, ataxin-3 possesses intrinsic Ub hydrolase activity but is unable to remove preassembled Ub conjugates from parkin, regardless of Ub chain length.

Ataxin-3 Deubiquitination Is Coupled to Parkin Self-ubiquitination—The inability of ataxin-3 to hydrolyze preassembled parkin-Ub conjugates raises the intriguing possibility that ataxin-3 deubiquitination needs to be coupled to active parkin ubiquitination. To characterize the process further, a time course was conducted whereby parkin self-ubiquitination was allowed to proceed for 1–4 h either alone, or in the presence of ataxin-3 added after the first hour (Fig. 3A). When ataxin-3 was present in the reactions for the final 3 h, it did not reduce the level of parkin ubiquitination below the level observed in 1-h reactions with parkin alone (Fig. 3A). Thus, it appears that ataxin-3 can efficiently hydrolyze Ub conjugates, whereas they are actively being assembled (hours 2–4) but is unable to hydrolyze pre-assembled Ub conjugates (those assembled during the initial hour of parkin ubiquitination).
To test more directly whether ataxin-3 and parkin activities are coupled, we carried out sequential ubiquitination reactions consisting of a first reaction using HA-Ub followed by a wash-out and a second reaction using Myc-Ub. As shown in Fig. 3B, when ataxin-3 is added during the second reaction, it efficiently removes Myc-Ub from parkin but is unable to cleave HA-Ub-parkin conjugates pre-assembled during the first reaction. Congruent results were obtained when the order of the HA- and Myc-Ub reactions was reversed (not shown). Thus, ataxin-3 deubiquitination appears to be functionally coupled to parkin self-ubiquitination. In these sequential reactions, ataxin-3 could be targeting a pool of residual non-ubiquitinated GST-parkin rather than newly added Ub conjugates on parkin ubiquitinated during the first hour. To exclude this possibility, MBP-parkin was ubiquitinated for 1 h with FLAG-Ub and ubiquitinated parkin was immunoprecipitated with FLAG-resin. A second reaction was then carried out containing only ubiquitinated MBP-parkin and HA-Ub. As shown in Fig. 3C, wild-type, but not catalytically inactive mutant ataxin-3C14S efficiently removes HA-Ub from parkin but is unable to cleave FLAG-Ub-parkin conjugates pre-assembled during the first reaction. As a whole, these findings suggest that the ability of ataxin-3 to regulate the formation of Ub conjugates on parkin is functionally coupled in real-time to the ability of parkin to ubiquitinate itself.

Ataxin-3 Interacts Directly with the E2-conjugating Enzyme—From our findings, it is apparent that ataxin-3 can deubiquitinate parkin when it is actively ubiquitinating, yet is unable to hydrolyze preformed parkin-Ub isopeptide linkages. This raises the intriguing possibility that ataxin-3 may not deubiquitinate parkin per se, but rather may be interfering with the normal function of another component in the parkin ubiquitination cascade. One candidate is the E2-conjugating enzyme, responsible for mediating the transfer of Ub onto parkin. In vitro ubiquitination reactions were set up containing GST-parkin in the presence or absence of His-ataxin-3 (14Q) and in the presence or absence of the DUB inhibitor UBAL (100 nM). Reactions were incubated for 1 h at 37 °C and immunoblotted for Ub. D, wild-type and catalytically inactive mutant ataxin-3C14S bind comparably to the parkin Ubl and RING domains. GST, GST-Ublparkin, and GST-RIRparkin were added into GST-binding assays with His-ataxin-3 (14Q) or His-ataxin-3C14S and immunoblotted for His. WB, Western blot.

FIGURE 1. Ataxin-3 reduces the rate of parkin self-ubiquitination. A, ataxin-3 is unable to deubiquitinate HHARI, another RIR-containing E3-ligase. GST-parkin or GST-RIRHHARI were incubated either alone or in the presence of His-ataxin-3 (14Q). Reactions were incubated for 2 h at 37 °C, run on SDS-PAGE gels, and transferred to nitrocellulose membranes before staining for Ponceau S to visualize transferred proteins or immunoblotting for Ub and ataxin-3. B, ataxin-3-mediated deubiquitination of parkin over time. GST-parkin was incubated either alone or in the presence of His-ataxin-3 (14Q) or His-ataxin-3C14S. Reactions were incubated for the indicated times at 37 °C and immunoblotted for Ub or analyzed by Coomassie staining. C, UBAL inhibits ataxin-3-mediated deubiquitination of parkin. In vitro ubiquitination reactions were set up containing GST-parkin in the presence or absence of His-ataxin-3 (14Q) or His-ataxin-3C14S, and reactions were quenched for 1 h at 37 °C and immunoblotted for Ub. D, wild-type and catalytically inactive mutant ataxin-3C14S bind comparably to the parkin Ubl and RING domains. WT, Western blot.
Ataxin-3 is unable to hydrolyze preassembled parkin-Ub isopeptide linkages. Ataxin-3 is unable to act on preassembled parkin-Ub conjugates. In vitro ubiquitination reactions were carried out with GST-parkin bound to glutathione beads for 1 h at 37 °C as in Fig. 1B (together). Alternatively, after 1 h, the beads were washed to remove reaction components (sequential). Ubiquitinated GST-parkin was then incubated in the presence or absence of His-ataxin-3 (14Q)/His-ataxin-3C14S for 1 h at 37 °C and reactions were immunoblotted for Ub. Ataxin-3 is unable to hydrolyze preassembled mono-Ub linkages. In vitro ubiquitination assays containing GST-parkin bound to glutathione beads and Ub50 were incubated for the indicated time intervals, followed by washout of reaction components. Monoubiquitinated parkin was incubated in the presence or absence of His-ataxin-3 (14Q) for 1 h at 37 °C and reactions were immunoblotted for Ub. For comparison, His-ataxin-3 was added together in reactions with GST-parkin and Ub50 for 2 h at 37 °C. WB, Western blot.

Remarkably, the inactive C14S ataxin-3 mutant was unable to interact with Ubc7, demonstrating that ataxin-3 requires its active site cysteine to interact with Ubc7. As multiple E2s can support parkin self-ubiquitination, we next tested whether the ataxin-3-E2 complex was specific for Ubc7. Similar to Ubc7, UbcH7 forms a complex with wild-type but not catalytically inactive ataxin-3 (Fig. 4B). In contrast, a C865S mutation of the active site cysteine of UbcH7 had no effect on the interaction between ataxin-3 and UbcH7 (Fig. 4C). Interestingly, DTT-sensitive complexes, likely made up of wild-type ataxin-3 dimers, could also be detected with cross-linking. These dimers were greatly reduced with the inactive ataxin-3 mutant, further supporting a role for C14 in dimerization (Fig. 4A). Taken together, these findings indicate that ataxin-3 interacts directly with the E2 and can homodimerize. Both events are transient as they require cross-linking to be stabilized. They are also dependent on the active site cysteine of ataxin-3 but not on the active site cysteine of the E2. More importantly, they suggest a role for ataxin-3 in regulating the activity of E2s.

Ataxin-3 Reduces the Levels of the E2-Ub Thioester Conjugate in a Parkin-dependent Manner—We next considered the possibility that, by interacting with the E2, ataxin-3 might act at the level of E2-Ub thioester conjugation. This might in turn affect the efficiency of transfer of Ub from the E2 to parkin, thereby explaining the observed decrease in parkin ubiquitination in the presence of ataxin-3. In vitro, the initial cycle of charging of E1 and E2 with Ub can be readily detected within a matter of minutes (supplemental Fig. S3, A and C), resulting in the formation of DTT-sensitive thioester conjugates between the C-terminal glycine of Ub and the active site cysteines in the E1 and E2 (supplemental Fig. S3, A and D). The presence of ataxin-3 in the reaction did not affect the abundance of the E2-Ub thioester complex (Fig. 5A and supplemental Fig. S3B), nor was it able to hydrolyze the E2-Ub thioester once it had formed (supplemental Fig. S3E). Similarly, parkin alone had no affect on the E2-Ub thioester complex (Fig. 5A). However, when parkin was present along with ataxin-3 in the reaction, we observed a clear reduction in the levels of the Ubc7-Ub thioester complex (Fig. 5A). Again, the presence of the active site cysteine within ataxin-3 was essential, as the C14S ataxin-3 mutant was unable to reduce Ubc7-Ub levels (Fig. 5A). This activity was not confined to Ubc7, as ataxin-3 also mediated a reduction in the levels of UbcH7-Ub in a parkin-dependent manner (Fig. 5, B and C). Consistent with our findings for Ubc7, this also required the presence of the active site cysteine (Fig. 5C). Intriguingly, decreased levels of Ubc7-Ub appeared to coincide with the appearance of a monoubiquitinated species of ataxin-3 (Fig. 5A). Thus, in the presence of E2 and parkin, ataxin-3 appears to divert the conjugated Ub away from parkin and onto itself, potentially explaining our findings above (Fig. 2A), whereby ataxin-3 deubiquitination could only be observed in the presence of active parkin self-ubiquitination. However, it is important to note that the effect of parkin and ataxin-3 on the abundance of the E2-thioester was only apparent at the earliest time points (supplemental Fig. S4, A and B). The finding suggests that, in the presence of parkin and ataxin-3, the incoming E2 is charged with Ub at a slower rate but nonetheless, becomes saturated to comparable levels over longer periods. Alternatively, the abundance of E2-Ub thioester may be lower initially, whereas Ub is being diverted onto ataxin-3 but gradually recovers as the transfer of Ub onto ataxin-3 becomes saturated. Both scenarios would be compatible with the observed decreased rate of parkin ubiquitination in the presence of ataxin-3 (Fig. 1B). Taken together, the findings indicate that the typically highly dynamic interaction between E2 and E3 is altered when parkin and its E2 partner are in the presence of ataxin-3, something we wanted to test further in a cellular context.

Ataxin-3 Stabilizes the Interaction between Parkin and ubc7 in HEK293 Cells—In our assays, any functional interaction between parkin and ataxin-3 involved proteins that were either overexpressed in mammalian cells or purified from bacteria. Yet, it remains unclear as to whether endogenous parkin and ataxin-3 interact in cells and tissue. To confirm the existence of a bona fide interaction between endogenous parkin and ataxin-3, an ataxin-3 polyclonal antibody was used to immunoprecipitate endogenous ataxin-3 from cells and brain lysates. This antibody specifically immunoprecipitates ataxin-3, as no band for ataxin-3 was detected with ataxin-3 knock-out mice fibroblasts (supplemental Fig. S5A). Using this antibody, endogenous ataxin-3 was immunoprecipitated from the dop-
aminergic SH-SY5 cell line. Parkin was co-immunoprecipitated with ataxin-3 (Fig. 6A), confirming that endogenous parkin and ataxin-3 interact in cells. Moreover, a small amount of endogenous parkin could also be co-immunoprecipitated with ataxin-3 from synaptosomal fractions prepared from the brains of wild-type but not parkin knock-out mice (Fig. 6B). Thus, endogenous parkin and ataxin-3 interact in cells and brain, further supporting the physiological relevance of our findings.

We showed previously that wild-type but not C14S mutant ataxin-3 reduces the extent of parkin ubiquitination in HEK293 cells (19). As endogenous parkin and ataxin-3 can interact in cells, we next wanted to test whether the ataxin-3 bound to parkin could directly oppose the E2 Ub-conjugating enzyme, thus preventing parkin from ubiquitinating itself.

Interactions between E2s and E3s are typically weak and transient. This property allows the E2 to rapidly cycle between an E3-bound Ub-charged state, in which it can transfer Ub onto the E3 or a substrate, and an uncharged state, in which the E2 dissociates from the E3 and gets reloaded with Ub by the E1 (30). Given these considerations, it was not surprising that we...
were unable to co-immunoprecipitate Myc-Ubc7 with parkin from HEK293T cells, even when both proteins were overexpressed (Fig. 6C). However, when ataxin-3 was co-transfected along with parkin and Ubc7, we could readily co-immunoprecipitate Ubc7 with parkin, even in the absence of cross-linking. Thus, ataxin-3 dramatically stabilizes the interaction between parkin and Ubc7, which in turn would impair the normal cycle of E2-E3 association/dissociation, required for efficient E2 charging and ubiquitination. Such findings are consistent with the reduced levels of E2-Ub thioester observed in vitro (Fig. 5, A and D). Moreover, stabilization of the parkin/Ubc7 interaction and the reduced E2 charging that would ensue could account for the observed decrease in parkin ubiquitination in the presence of ataxin-3 both in vitro (Fig. 1B) and in cells (19).

Remarkably, parkin-bound ataxin-3 was modified only when Ubc7 was overexpressed and stably associated with parkin (Fig. 6C and supplemental Fig. S5B). We speculate that modification of ataxin-3 may represent monoubiquitination, with the parkin-bound Ubc7 transferring its Ub onto ataxin-3 (Fig. 6C). In contrast, overexpression of the C14S catalytically inactive
ataxin-3 mutant, which bound parkin as efficiently as wild-type ataxin-3, was not modified nor did it stabilize the parkin/H18528Ubc7 complex (Fig. 6C). These findings are consistent with our in vitro data indicating that catalytically inactive ataxin-3 could neither bind the E2 (Fig. 4), affect Ubc7-Ub thioester charging (Fig. 5A), nor promote ataxin-3 monoubiquitination (Fig. 5A). Overall, our work suggests a model whereby ataxin-3 binds E2 directly and promotes the stabilization of the E2/parkin complex, which in turn reduces the rate of E2 charging with Ub and diverts Ub away from parkin and onto ataxin-3.

**DISCUSSION**

In the course of characterizing the interaction between parkin and ataxin-3, we identified a novel mechanism explaining how ataxin-3 regulates parkin ubiquitination. We find that ataxin-3 was unable to hydrolyze previously assembled Ub conjugates on parkin (Fig. 2), yet could regulate the assembly of newly forming Ub conjugates on parkin (Fig. 3). We considered the notion that ataxin-3 may inhibit the attachment of these new Ub conjugates on parkin by antagonizing the E2 Ub-conjugating enzyme, an essential component of the ubiquitination cascade. Indeed, ataxin-3 not only bound the E2-conjugating enzyme directly (Fig. 4), but mediated a reduction in the levels of charged E2-Ub thioester, in a strictly parkin-dependent manner (Fig. 5). Interestingly, the reduction in the levels of the E2-Ub thioester conjugate appeared to coincide with the appearance of a monoubiquitinated species of ataxin-3. This monoubiquitinated species of ataxin-3 was also observed in cells. Moreover, in cells, parkin-bound ataxin-3 stabilized the interaction between the E2 and parkin, likely resulting in the formation of an E2-parkin-ataxin-3 complex. Mutation of the active site cysteine in ataxin-3 not only abolished the interaction between ataxin-3 and E2, it also prevented both the monoubiquitination of ataxin-3 and the formation of a stable complex between parkin and E2. From these findings, we now propose a model whereby ataxin-3 stabilizes the association between E2 and parkin, thereby impeding the release of the uncharged E2, whereas in parallel diverting the Ub from the E2-Ub thioester conjugate onto itself, and away from parkin (Fig. 7).
Ataxin-3 Deubiquitinates Parkin in an Unconventional Manner—Even though our model outlines an unconventional mechanism for a DUB to deubiquitinate a substrate protein, it is not unprecedented. The classical mechanism used by the majority of DUBs to deubiquitinate a cognate substrate requires their intrinsic hydrolase activity. However, certain DUBs can mediate the deubiquitination of their substrate independently of their DUB activity. One example is Usp13, a DUB that possesses a Ub-binding domain allowing it to interact directly with the RING E3 Ub-ligase SIAH1, blocking the ability of SIAH1 to ubiquitinate itself (18). In a separate study, the DUB OTUB1 interacted directly with the E2 Ubc13, suppressing the activity of the Ubc13/uev1 to form Ub chains and preventing RNF168 from ubiquitinating its cognate substrate in the DNA-damage response pathway (16).

However, one stark contrast between ataxin-3 and these two DUBs is that ataxin-3 requires the presence of its active site cysteine to impede parkin self-ubiquitination (Fig. 1A). Mutation of the active site cysteine to a serine in OTUB1 and Usp13 had no effect on the ability of both DUBs to inhibit the ligase activity of RNF168 and SIAH1, respectively. Inactivation of ataxin-3 by mutating its active site cysteine to a serine abolishes the activity of ataxin-3 toward parkin (Fig. 1B). Furthermore, inactive ataxin-3 was not monoubiquitinated in our assays (Figs. 5A and 6A), nor did it stabilize the binding between parkin and the E2 (Fig. 6A). Thus, when cysteine 14 is mutated to a serine, ataxin-3 can no longer redirect the Ub from the E2-Ub thioester away from parkin (Fig. 5A) onto itself, whereas at the same time being unable to interact with and stabilize the binding of the E2 to parkin (Figs. 4A and 6A).

Model of Ataxin-3-mediated Deubiquitination of Actively Ubiquitinatating Parkin—From our findings, we propose a model whereby ataxin-3 reduces the ability of both DUBs to inhibit the ligase activity of RNF168 and SIAH1, respectively. Inactivation of ataxin-3 by mutating its active site cysteine to a serine abolishes the activity of ataxin-3 toward parkin (Fig. 1B). Furthermore, inactive ataxin-3 was not monoubiquitinated in our assays (Figs. 5A and 6A), nor did it stabilize the binding between parkin and the E2 (Fig. 6A). Thus, when cysteine 14 is mutated to a serine, ataxin-3 can no longer redirect the Ub from the E2-Ub thioester away from parkin (Fig. 5A) onto itself, whereas at the same time being unable to interact with and stabilize the binding of the E2 to parkin (Figs. 4A and 6A).
by 2) diverting the Ub from the E2 within the parkin/E2 complex away from parkin and onto ataxin-3. Together, these effects reduce the efficiency of parkin ubiquitination (Fig. 7). From our in vitro data, it is clear that, in the presence of parkin, ataxin-3 redirects the Ub in the E2-Ub complex away from parkin and onto itself, in a manner dependent on its active site cysteine (Figs. 5A and 6). How could ataxin-3 divert the Ub onto itself in the presence of parkin? We have considered two distinct scenarios that may explain our findings. In the first scenario, C14 within ataxin-3 interacts directly with the Ub-thioester when the E2 is bound to parkin, and directs Ub onto a lysine within ataxin-3 and away from parkin. Alternatively, instead of acquiring the Ub directly from the E2, parkin might mediate the transfer of Ub onto ataxin-3. A recent study suggests that RBR-type E3-ligases such as parkin function as hybrids of RING- and HECT-type ligases, with the E2 transferring the Ub onto a cysteine within one of their RING domains to form an E3-Ub thioester conjugate, before the Ub is transferred to a lysine within the E3 (24). In this scenario an intermediate parkin-Ub thioester conjugate might be involved in transferring Ub onto a lysine within ataxin-3.

In addition to directing the Ub from the E2 onto itself, ataxin-3 can also promote the formation of a stable complex between the E2 and parkin (Fig. 6A). By keeping the E2 bound to parkin, ataxin-3 can impede the recharging of the E2 to form a new E2-Ub thioester conjugate, whereas at the same time preventing the association of parkin with newly charged E2-Ub from the free pool. Studies with other E3-ligases demonstrate clearly that dissociation of the uncharged E2 from the E3 is an essential step for the E2 to become charged again (30). If the E2 remains bound to the E3, it is unable to interact with the E1 Ub-activating enzyme and become recharged. The cycling between binding and dissociating from parkin not only maintains the available pool of free E2 that can be charged with a Ub, but helps provide parkin with a constant pool of charged Ub that can be directed toward the assembly of Ub chains.

Consistent with the notion that ataxin-3 may affect the charging of the E2, a reduction in the levels of E2-Ub was noted in the presence of parkin (Fig. 5, A and C). However, as the reaction proceeded over longer times, levels of the E2-Ub returned to normal (supplemental Fig. S4). We speculate that the initial reduction in levels of the E2-Ub thioester conjugate (Fig. 5, A and C) is a direct result of the Ub being diverted onto ataxin-3, with ataxin-3 retaining the E2 on parkin (Fig. 6A), thus preventing its release and subsequent recharging. Moreover, the retention of the E2 on parkin may also be acting as a barrier preventing newly charged free E2-Ub from associating with and conjugating Ub onto parkin. Taken together, we present evidence for a novel mechanism through which ataxin-3 diverts the Ub from the E2-Ub thioester complex away from parkin and onto itself, thereby counteracting the ability of parkin to ubiquitinate itself.

**Implications for Parkin Activity and Other DUBs**—Prior to this study, ataxin-3 was considered a classical DUB. It was demonstrated to hydrolyze both free Ub chains and Ub conjugates from the U-box E3 CHIP, another recently identified substrate for ataxin-3 (31, 32). Indeed, by deubiquitinating CHIP, ataxin-3 regulates the ligase activity of CHIP, whereas at the same time trimming newly forming Ub chains on CHIP substrates. Through these actions, ataxin-3 promotes the termination of the CHIP-mediated ubiquitination reaction, whereas preventing excessive ubiquitination by CHIP of its cognate sub-
strate (32). In contrast, ataxin-3 appears to use a distinct mechanism, not previously observed for other DUBs, to regulate the activity of parkin. What advantages might such a mechanism offer over a more conventional mode of deubiquitination as described for CHIP (32)? In our previous study, we demonstrated that wild-type ataxin-3 had no effect on parkin stability (19), raising the possibility that ataxin-3 regulates the activity of parkin within the cell. Recently, parkin has been demonstrated to interact with and promote the clearance of mitochondria (19), raising the possibility that ataxin-3 regulates the activity of mitochondria-associated ubiquitin ligase activity could represent an exciting therapeutic target in Parkinson's disease. If indeed the inappropriate regulation of parkin promotes neuronal cell loss in MJD, then targeting parkin E3 Ub-ligase activity could represent an exciting therapeutic target in the future.

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