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Rhes, a Physiologic Regulator of Sumoylation, Enhances Cross-sumoylation between the Basic Sumoylation Enzymes E1 and Ubc9*[S]

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We recently reported that the small G-protein Rhes has the properties of a SUMO-E3 ligase and mediates mutant huntingtin (mHtt) cytotoxicity. We now demonstrate that Rhes is a physiologic regulator of sumoylation, which is markedly reduced in the corpus striatum of Rhes-deleted mice. Sumoylation involves activation and transfer of small ubiquitin-like modifier (SUMO) from the thioester of E1 to the thioester of Ubc9 (E2) and final transfer to lysines on target proteins, which is enhanced by E3s. We show that E1 transfers SUMO from its thioester directly to lysine residues on Ubc9, forming isopeptide linkages. Conversely, sumoylation on E1 requires transfer of SUMO from the thioester of Ubc9. Thus, the process regarded as "autosumoylation" reflects intermolecular transfer between E1 and Ubc9, which we designate "cross-sumoylation." Rhes binds directly to both E1 and Ubc9, enhancing cross-sumoylation as well as thioester transfer from E1 to Ubc9.

Huntington disease (HD)[3] is an autosomal dominant disorder caused by an expansion of glutamine residues in the gene encoding the protein huntingtin (Htt) (1). Htt and mutant Htt (mHtt) are ubiquitously expressed throughout the brain and peripheral tissues, yet HD is associated with highly selective degradation of the striatum with no notable alterations in peripheral tissues. Rhes, a member of the Ras family of small G-proteins, was identified on the basis of its selective localization to the corpus striatum of the brain, hence its designation as Ras homolog enriched in striatum (2). We recently reported that Rhes binds mHtt selectively and with high avidity to enhance mHtt sumoylation, with Rhes acting as an apparent E3 ligase for mHtt as well as other substrates such as RanGAP and SP100 (3). Sumoylation of mHtt leads to its disaggregation and augmented neurotoxicity (3, 4). Thus, selective striatal neuronal loss in HD may reflect the tissue-specific influence of Rhes on mHtt sumoylation and cytotoxicity.

Sumoylation involves a multistep enzymatic cascade analogous to ubiquitination, beginning with the activation and attachment of SUMO as a thioester bond to E1, the Aos1/Uba2 heterodimer. The SUMO group is then transferred from E1 to Ubc9, the E2-conjugating enzyme, as a thioester bond. In the final step, SUMO is transferred to lysine residues of target proteins forming an isopeptide bond, a process enhanced by E3 ligases (5).

Sumoylation can regulate protein function through changes in localization, stability, and activity. Because the sumoylation cascade employs only a single E1 (Aos1/Uba2) and a single E2 (Ubc9), regulation of these enzymes could have substantial effects upon sumoylation. SUMO E1 and Ubc9 are themselves sumoylated in yeast and mammalian cells (6, 7). Recently, it was reported that attachment of SUMO at lysine 14 on Ubc9 acts as a molecular switch to modulate sumoylation of target proteins (8). Molecular mechanisms underlying "autosumoylation" of SUMO-E1 and Ubc9 have not hitherto been elucidated.

We now report that mice with deletion of Rhes manifest a pronounced reduction in the overall sumoylation of proteins selectively in the corpus striatum. We identify novel features of the sumoylation cascade, including isopeptide sumoylation of Ubc9 by direct transfer of SUMO from the thioester of E1, and characterize the reciprocal reaction whereby the thioester SUMO of Ubc9 is transferred to E1, forming an isopeptide bond. These findings suggest that what has been regarded as autosumoylation reflects intermolecular transfer of SUMO between E1 and Ubc9, which we designate as "cross-sumoylation." Rhes enhances cross-sumoylation as well as the canonical transfer of SUMO from the E1 thioester to the thioester of Ubc9.

EXPERIMENTAL PROCEDURES

Reagents, Plasmids, and Antibodies—Unless otherwise noted, reagents were obtained from Sigma. Antibodies for Ubc9 (ab21193) and E1 (ab16849) were from Abcam. SP100 antibody (PW0325-0100) was from Biomol. GST (G7781) and His (H1029) antibodies were from Sigma. Plasmid vectors pGEX-SUMO-1, pGEX-SUMO-2, His-E1 (Aos1/Uba2), pGEX-Ubc9, and pGEX-RanGAP1 (for bacterial purification) were a generous gift from Michael J. Matunis (Johns Hopkins University, Baltimore, MD). GST-SP100 vector was a gift from Andreas Pichler (Medical University of Vienna, Vienna). Rhes full length (1–266) and fragments (1–171 and 171–266), Ubc9 lysine

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[3] The abbreviations used are: HD, Huntington disease; Htt, huntingtin; mHtt, mutant huntingtin; SUMO, small ubiquitin-like modifier; Ubc, ubiquitin-conjugating enzyme; GST, glutathione *S*-transferase; DTT, dithiothreitol; WT, wild-type; Bis-Tris, 2-(bis(2-hydroxyethyl)amino)-2-(hydroxymethyl)propane-1,3-diol; LDS, lithium dodecyl sulfate.

mutants (K14R, K49R, K153R, and K14R,K49R,K153R), and Ubc9-C93A were generated by PCR and cloned into pGEX-vectors as before (3).

Recombinant Protein Production, In Vitro Binding, and Immunoblotting—These procedures were followed as described before (3) (supplemental text).

Sumoylation Assay—In vitro sumoylation assays were performed as before (3). Briefly, sumoylation was carried out in 20 μl of volume reaction mix in 1× reaction buffer (20 mM HEPES, pH 7.4, 2 mM magnesium acetate, 110 mM KCl), 1 μg of E1(Aos1/Uba2), 500 ng of Ubc9, 2 μg of SUMO-1/2, 5 mM ATP, 0.2 mM DTT, and 200 ng of Rhes at 32 °C for the indicated time, unless otherwise noted. For detecting thioester bonds, reactions were stopped by adding LDS loading buffer (Invitrogen) with no reducing agents. For the detection of isopeptide bonds, 100 mM DTT was added for 10 min at the end of the reaction followed by LDS loading buffer containing 20 mM β-mercaptoethanol. Samples were heated at 98 °C for 2 min, separated on a 4–12% Bis-Tris gel (Invitrogen), and transferred to 0.45 μm of polyvinylidene difluoride. Reactions detecting only isopeptide bonds contained 2 mM DTT during reaction.

Single turnover sumoylation assay was performed as described previously with the following modifications (8). Briefly, 15 μl of volume reaction mix containing 1 μg of E1, 500 ng of Ubc9 (tag-less), 2 μg of SUMO-1/2, 5 mM ATP, and 0.2 mM DTT in 1× reaction buffer was incubated for 30 min at 32 °C in the presence and absence of 200 ng of Rhes. Where indicated, E1 was then inactivated with 10 mM EDTA for 10 min followed by incubation with either 250 ng of GST-tagged-Ubc9 C93A or 250 ng of SP100 for 1 h or followed by incubation with 250 ng of GST-RanGAP for 4 min. Reaction was stopped in reducing LDS loading buffer.

Liquid Chromatography-Tandem Mass Spectrometry Analysis—LTQ-Orbitrap mass spectrometry was performed at the Taplin Mass Spectrometry Facility, Harvard Medical School (supplemental text).

Statistical Analysis—Statistical analysis was performed as indicated in the supplemental text.

RESULTS

Rhes Regulates Sumoylation, Enhancing Thioester and Isopeptide Sumoylation on Ubc9—We wondered whether Rhes physiologically regulates sumoylation in brains of intact animals. We examined sumoylation of proteins in the striatum and cerebellum of 1-year-old wild-type and Rhes-deleted mice (9). Virtually all sumoylated protein bands above 30 kDa are markedly decreased in intensity in the striatum of Rhes knock-out mice, with no alterations in the cerebellum (Fig. 1*A*). Thus, Rhes is a critical determinant of protein sumoylation with its effects selectively evident in the striatum and not the cerebellum, which lacks Rhes protein. In 6-month-old mice, striatal sumoylation is not altered in Rhes mutants, suggesting that the influence of Rhes on sumoylation is age-related (data not shown).

Evidence that Rhes is a determinant of striatal sumoylation prompted us to further investigate the underlying molecular mechanism. Rhes differs from most members of the Ras family by the presence of a C-terminal extension of 95 amino acids

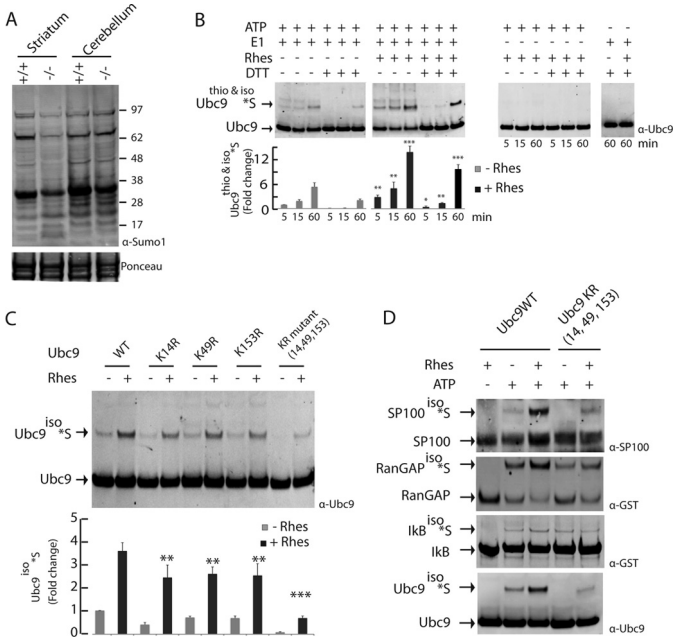

FIGURE 1. **Rhes regulates sumoylation, enhancing thioester and isopeptide sumoylation on Ubc9.** *A*, reduced sumoylation in Rhes knock-out mice striatum. One-year-old Rhes knock-out mice striatum and cerebellum were dissected and lysed in radioimmune precipitation buffer with 20 mM *N*-ethylmaleimide. Cell lysates were immunoblotted with SUMO-1 antibody. *B*, Rhes enhances thioester and isopeptide sumoylation on Ubc9. An *in vitro* sumoylation reaction was performed for 5, 15, or 60 min in the presence and absence of Rhes (200 ng), E1 (1 μg), and ATP (5 mM) as indicated under "Experimental Procedures." Samples were immunoblotted and probed with Ubc9 antibody. To distinguish isopeptide from thioester linked SUMO, 100 mM DTT was added at the end of the reaction. Rhes could not modify Ubc9 in the absence of E1 or ATP. *, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$ *versus* without Rhes. *C*, effect of Ubc9 lysine mutants on isopeptide sumoylation. Ubc9 WT and Ubc9 (single K14R, K49R, or K153R or the triple K14R,K49R,K153R) mutants were subjected to *in vitro* sumoylation as in *B* in the presence and absence of Rhes (200 ng) and then reduced with 100 mM DTT. **, $p < 0.01$ and ***, $p < 0.001$ *versus* Rhes + Ubc9 WT. *D*, effect of Ubc9 K14R,K49R,K153R (*Ubc9 KR (14,49,153)*) mutation on substrate sumoylation. *In vitro* sumoylation in the presence of Ubc9 WT and Ubc9 triple KR mutants was carried as in *C* with 500 ng of substrates (SP100, GST-RanGAP, or IκB) and then probed with SP100, GST or Ubc9 antibodies.

(supplemental Fig. S1*A*). In our earlier study, we showed that Rhes binds Ubc9 independent of its GTPase activity (3). Both N-terminal and C-terminal portions of Rhes directly bind Ubc9 comparable with full-length Rhes (supplemental Fig. S1*B*). We examined the effect of Rhes on Ubc9 sumoylation *in vitro*. Rhes enhances sumoylation on Ubc9 both at thioester linkages (DTT-sensitive) and at isopeptide linkages (DTT-insensitive) (Fig. 1*B*). In reactions with longer incubation times, Rhes enhancement of both thioester and isopeptide linkages is more pronounced (supplemental Fig. S1*C*). No modification of Ubc9 is seen in the absence of E1 or ATP, confirming the requirement of E1 for Ubc9 modification. Stimulation by Rhes of thioester and isopeptide linkages is time- and concentration-dependent (Fig. 1*B* and supplemental Fig. S1*D*).

Like Rhes, RSUME stimulates thioester bond formation on Ubc9 (10). However, no proteins are known to enhance isopeptide bond formation on Ubc9. We tested PIAS1 and RanBP2, known E3 ligases, neither of which affects Ubc9 isopeptide linkage (supplemental Fig. S1*F*). In control reactions, PIAS1 and RanBP2 enhance sumoylation of p53 and SP100, respectively (data not shown). Although both N-terminal and C-terminal

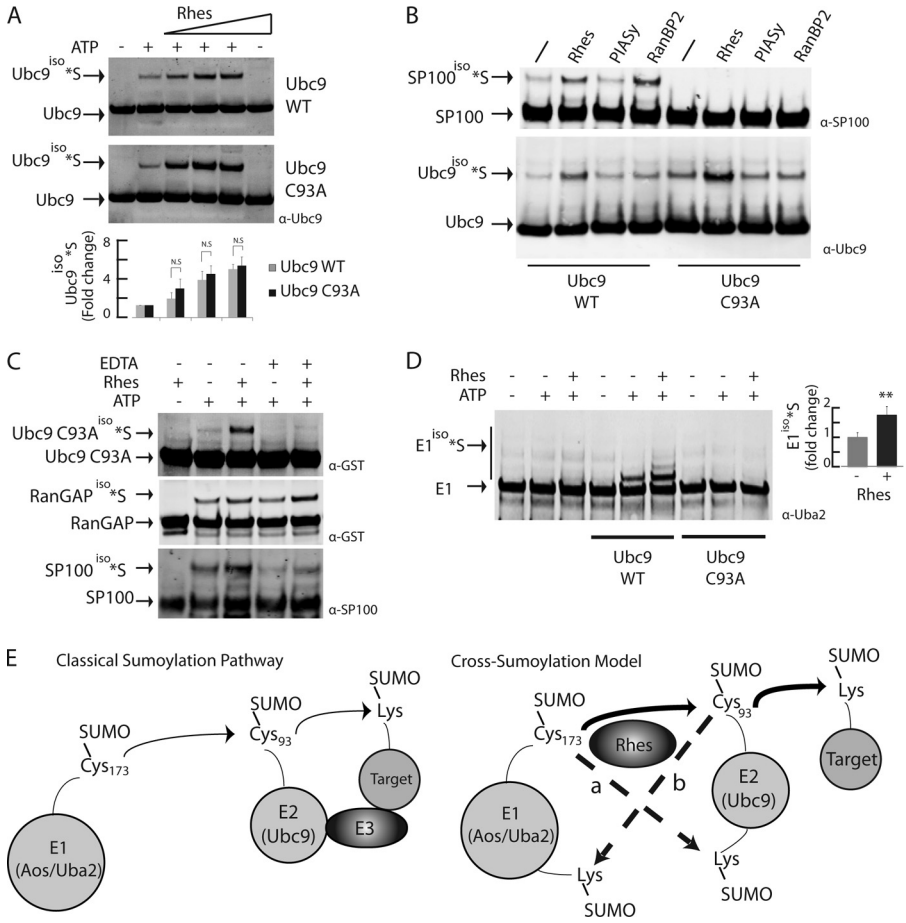

FIGURE 2. **Cross-sumoylation of E1 and Ubc9 is enhanced by Rhes.** *A*, effect of Rhes on Ubc9 C93A isopeptide sumoylation. An *in vitro* sumoylation reaction was carried out in 1× reaction buffer containing 1 μg of E1, 500 ng of Ubc9 (WT or C93A), 0.2 mM DTT, 5 mM ATP, 2 μg of SUMO-1/2, and Rhes (50, 100, 200 ng) for 1 h at 32 °C and then reduced with 100 mM DTT. Blots were probed with Ubc9 antibody. *N.S.*, not significantly different. *B*, effect of E3 ligases on Ubc9 C93A and SP100 isopeptide sumoylation. *In vitro* sumoylation was carried as in *A* in the presence of 250 ng of Rhes, PIASy, or RanBP2 and 500 ng of SP100. *C*, Ubc9 cannot perform intermolecular transfer of SUMO to Ubc9. Single-turnover *in vitro* sumoylation was carried out as indicated under "Experimental Procedures." 10 mM EDTA was then added to stop E1 activity followed by incubation with 500 ng of either GST-Ubc9 C93A (for 1 h), GST-RanGAP (for 5 min), or SP100 (1 h). 100 mM DTT was added at the end of the reaction followed by Western blotting and probing with GST or SP100 antibody. *D*, Rhes enhances Ubc9 WT-dependent E1 isopeptide sumoylation. *In vitro* sumoylation was carried out as in *A* in the presence and absence of Ubc9 or Ubc9 C93A and 200 ng of Rhes. Samples were reduced with 100 mM DTT followed by Western blotting and probing with Uba2 antibody. **, $p < 0.01$ without Rhes. *E*, cross-sumoylation model. Analogous to ubiquitination, in the classical sumoylation pathway, SUMO is transferred from the thioester of E1 (Cys-173) to a thioester on Ubc9 (Cys-93) followed by transfer of thioester-linked SUMO from Ubc9 to isopeptide linkage on target lysines; the last step is enhanced by E3s. In the cross-sumoylation model, besides transferring SUMO to Ubc9 in a thioester linkage, E1 can directly attach SUMO to lysines on Ubc9 forming an isopeptide linkage (*a*). Besides attaching SUMO to its targets, Ubc9 can attach SUMO to lysines on E1 as an isopeptide linkage (*b*). Rhes enhances all the principle steps of the pathways, including cross-sumoylation and thioester transfer from E1 to Ubc9.

fragments of Rhes bind Ubc9, the C-terminal fragment augments Ubc9 isopeptide formation to a similar extent as full-length Rhes, whereas the N-terminal fragment is substantially less active (supplemental Fig. S1*F*). We also evaluated whether other small G-proteins affect Ubc9 sumoylation. None of those tested had a significant effect on Ubc9 isopeptide linkage, including DexRas1, the closest relative of Rhes, which shares a 50% homologous C-terminal extension (supplemental Fig. S1*G*). We examined whether Ubc9 sumoylation occurs differentially with SUMO-1 or SUMO-2. Rhes enhancement of Ubc9 isopeptide or thioester formation is the same with both paralogs of SUMO (supplemental Fig. S1*G*).

Ubc9 Isopeptide Sumoylation on Lys-14, Lys-49, and Lys-153 Is Increased by Rhes—We sought to identify the site of Rhes-stimulated isopeptide bond formation on Ubc9. Knipscheer *et al.* (8) reported lysine 14 in mammalian Ubc9 and lysine 153 in yeast Ubc9 as the primary sites of sumoylation. Another study identified Lys-146 as a site of human Ubc9 isopeptide modification (6). Our mass spectrometric analysis of human Ubc9 identified Lys-14, Lys-49, and Lys-153 as sites modified by SUMO-2. Both in the presence and in the absence of Rhes, all three lysines were modified by SUMO-2 (supplemental Fig. S2, *A* and *B*). To ascertain the relative contributions of these three lysine residues, we prepared single Lys-Arg mutations of the three sites as well as a construct in which all three are mutated. Individual mutations of Lys-14, Lys-49, and Lys-153 modestly reduce sumoylation to an equal extent, whereas mutation of all three virtually abolishes sumoylation (Fig. 1*C*). The pattern of reduced sumoylation is the same in the presence of Rhes, confirming that Rhes predominately targets Lys-14, Lys-49, and Lys-153 of Ubc9. SUMO-1 also produced the same pattern of reduced sumoylation in Ubc9 lysine mutants (supplemental Fig. S2*C*). Knipscheer *et al.* (8) reported that isopeptide sumoylation of Ubc9 influences its ability to sumoylate different targets. Mutating lysines 14, 49, and 153 to block sumoylation of Ubc9 greatly reduces sumoylation of SP100, with negligible changes for RanGAP and IκB (Fig. 1*D*). The altered sumoylation activity of the triple Lys-Arg mutant Ubc9 cannot be ascribed to effects on binding to E1, Rhes, or SUMO as mutant and wild-type Ubc9 bind similarly to these proteins (supplemental Fig. S2*D*). Thus, we confirm that sumoylation of Ubc9 differentially influences its target preferences.

Ubc9 Catalytic Cysteine 93 Is Not Required for Ubc9 Isopeptide Sumoylation—Because Ubc9 sumoylation is critical for its function, we explored the source of the isopeptide linkage in Ubc9. Basal isopeptide linkage and its marked enhancement by Rhes are the same for wild-type Ubc9 and the catalytically inactive Ubc9-C93A (Fig. 2*A*). Thus, Ubc9 sumoylation does not require thioester modification at Cys-93.

We explored the effect of Rhes and known E3s on SP100 and Ubc9 sumoylation, with or without the active site Cys-93 of Ubc9 (Fig. 2*B*). With wtUbc9 (containing Cys-93), Rhes stimulates isopeptide sumoylation on both SP100 and Ubc9; PIASy has no effect on either SP100 or Ubc9; and RanBP2, a known E3 ligase for SP100 (11), augments SP100 sumoylation but does not affect Ubc9 isopeptide formation. With Ubc9-C93A, isopeptide linkage on SP100 does not occur, and only Rhes enhances Ubc9-C93A sumoylation. This confirms that the mechanism of action of Rhes is different from other E3s and that Cys-93 on Ubc9 is required for target sumoylation. We wondered whether another cysteine on Ubc9 is responsible for Ubc9 isopeptide modification. Accordingly, we examined thioester bond formation on Ubc9-C93A (supplemental Fig. S3*A*). In reactions with wtUbc9, a substantial portion of SUMO attachment to Ubc9 is lost with DTT treatment, indicating thioester formation. However, in reactions with the C93A mutant of Ubc9, SUMO attachment is the same in the presence or absence of DTT, supporting Cys-93 as the probable site of thioester bond formation on Ubc9. These findings establish that isopeptide sumoylation on Ubc9 does not derive from an intramolecular transfer.

To determine whether Cys-93 influences the site of isopeptide formation on Ubc9, we examined isopeptide formation on Ubc9-C93A with individual and triple Lys-Arg mutations at Lys-14, Lys-49, and Lys-153. The pattern of isopeptide formation in both WT and C93A is nearly identical, with individual mutations modestly reducing sumoylation and triple mutation abolishing sumoylation on Ubc9 (supplemental Fig. S3*B*). We also examined thioester bond formation on Ubc9 and Ubc9-C93A containing triple mutations of lysines 14, 49, and 153 (supplemental Fig. S3*A*). Ubc9 with mutations at Lys-14, -49, and -153 (Ubc9 KR mutant) maintains significant DTT-sensitive SUMO bonds indicative of thioester formation but still displays marked reduction in DTT-insensitive isopeptide bonds when compared with wtUbc9. In Ubc9-C93A with mutations at Lys-14, -49, and -153 (Ubc9-C93A KR mutant), DTT-sensitive bonds are abolished, and DTT-insensitive bonds are markedly diminished when compared with wtUbc9 (supplemental Fig. S3*A*). This further supports Cys-93 as the site of thioester formation and lysines 14, 49, and 153 as the primary sites of isopeptide formation on Ubc9.

Ubc9 isopeptide sumoylation is augmented with increasing concentrations of E1 (supplemental Fig. S3*C*). Although suggestive that the E1 thioester is the sole contributor of SUMO for Ubc9 isopeptide modification, these findings do not rule out the possibility that Ubc9 can transfer SUMO to another Ubc9 through an intermolecular reaction involving two molecules of Ubc9. To test this possibility, we performed single turnover experiments similar to those reported by Knipscheer *et al.* (8). In this experiment, E1, Ubc9, SUMO-1/2, and ATP were incubated together to allow formation of Ubc9-SUMO thioesters. EDTA was added to block further thioester formation on E1 and Ubc9. This Ubc9-SUMO thioester was incubated with SP100, GST-RanGAP, or GST-Ubc9 C93A to monitor the transfer of the single thioester-linked SUMO to target proteins. Transfer readily occurs to both RanGAP and SP100 but not to Ubc9-C93A, ruling out intermolecular transfer of SUMO between molecules of Ubc9 (Fig. 2*C*). Taken together, the above experiments establish that isopeptide sumoylation on Ubc9 occurs via direct transfer of SUMO from the thioester of E1 to lysine residues on Ubc9, not through intra- or intermolecular transfer from the thioester of Ubc9.

Rhes Augments E1 Isopeptide Sumoylation—As Rhes markedly increases the transfer of SUMO from E1 to Ubc9, we considered a possible influence of Rhes on E1. Rhes directly binds E1 and the binding is not inhibited by increasing concentrations of Ubc9, suggesting separate binding sites for E1 and Ubc9 on Rhes (supplemental Fig. S3*D*). Sumoylation of E1 at multiple lysines has previously been reported (6). To determine whether E1 is modified through intramolecular autosumoylation, similar to ubiquitin E1 and E2 (12), or whether it is dependent on transfer of SUMO from Ubc9, we performed *in vitro* sumoylation assays in the presence and absence of Ubc9 and with the catalytically inactive Ubc9-C93A (Fig. 2*D*). Sumoylation of E1 occurs only with wild-type Ubc9, and is nearly doubled in the presence of Rhes. Rhes enhancement of E1 sumoylation occurs in a time- and concentration-dependent manner (supplemental Fig. S3, *E* and *F*). Therefore, E1 sumoylation does not occur through intramolecular autosumoylation and is dependent on the transfer of SUMO from the thioester of Ubc9.

DISCUSSION

In the 10 years since the discovery of Rhes as a striatal-enriched transcript, studies of its functions have focused on signal transduction cascades at the plasma membrane (2, 13). Rhes regulates the sensitivity of G-protein-coupled receptors including dopamine D1 and D2 receptors (14, 15), and its expression is coupled to the level of dopamine innervation (16). Rhes influences $G\alpha_i$ signaling to modulate N-type calcium channels (17). Our previous study demonstrated that Rhes behaves like a SUMO E3 ligase, interacting with Ubc9 and enhancing the transfer of SUMO to substrates including mHtt, Sp100, and RanGAP (3). We now show that Rhes is a physiologic regulator of sumoylation. Rhes-deleted mice manifest markedly diminished sumoylation selectively in the striatum. Moreover, Rhes robustly enhances cross-sumoylation between E1 and Ubc9 as well as all principal steps of the sumoylation pathway (Fig. 2*E*).

Multiple classes of SUMO-E3 ligases have been identified, which lack structural homology and do not resemble Rhes. E3s bind both Ubc9 and target proteins, catalyzing the transfer of SUMO by acting as scaffolds to stabilize the protein interaction or positioning the proteins in optimal orientation for transfer (5). We demonstrate the unique ability of Rhes to enhance by up to 400% both thioester and isopeptide sumoylation in Ubc9. Thus, Rhes does not fit the classification of E3s as simple scaffolding proteins, bridging Ubc9 and targets. Rhes appears to act on both E1 and Ubc9 to increase cross-sumoylation, where E1 directly transfers its thioester-linked SUMO to lysine residues on Ubc9, and Ubc9 transfers its thioester-linked SUMO to lysine residues on E1. This mechanism suggests that for both E1 and Ubc9, autosumoylation does not arise by intramolecular transfer. As isopeptide formation in Ubc9 involves intermolecular transfer of SUMO directly from E1, we speculate that E1 might also act

as a SUMO-conjugating enzyme for other unidentified substrates.

Cdc34 and Ubc9 are both members of the "ubiquitin-conjugating" (Ubc) family of enzymes (18). In contrast to our findings in the SUMO system, "automodification" is known to occur in the ubiquitin system (19, 20). Cdc34, an ubiquitin E2, undergoes autoubiquitination via two distinct pathways with different functional consequences depending on the presence of the ubiquitin E3 ligase, SCF (Skp1, Cdc53/Cul1, F-box protein). Without SCF, Cdc34 is autoubiquitinated via intramolecular modification with no effect on enzyme function. However, when SCF is present, Cdc34 transfers ubiquitin to separate Cdc34 molecules via an intermolecular reaction, which inhibits their activity (12). We find that Ubc9 is incapable of either reaction and depends on E1 for sumoylation. Why is Cdc34, but not Ubc9, capable of automodification? Differences in physical interactions might play a role. Cdc34 can self-associate due to the presence of an extended C-terminal region (21). Ubc9 cannot self-associate (22) and lacks the extended C-terminal region of Cdc34 (18). Furthermore, SUMO E1 and Ubc9 interact more strongly than ubiquitin E1 and E2 (23). High affinity binding between SUMO E1 and Ubc9 may facilitate functional interactions of the two proteins.

E1 and Ubc9 are essential enzymes whose deficiency leads to embryonic lethality and cell cycle abnormalities (24–29). There are hundreds of SUMO substrates but few E3s. Modification of E1 and Ubc9 may be important points of regulation to affect target discrimination. We speculate that cross-sumoylation is a "symbiotic" mechanism in which E1 and Ubc9 regulate each other's function and stability.

RSUME is a general enhancer of sumoylation, increasing thioester transfer from E1 to Ubc9 (10). Rhes and RSUME may regulate sumoylation by a similar mechanism, modulating both cross-sumoylation and thioester transfer. Within the brain, RSUME levels are highest in the cerebellum and might serve a role analogous to that of Rhes in the striatum. Perhaps other proteins serve similar tissue-specific functions in other brain areas, contributing to the selective neuronal loss seen in various neurodegenerative diseases.

Functions of Rhes may not be restricted to the corpus striatum. Rhes was recently reported as a vulnerability gene for certain neuropsychological features of schizophrenia (30). Rhes was also identified in a screen for proteins uniquely enriched in the endothelium of tumors (31). As abundant evidence links sumoylation to cancer (32), it is tempting to speculate a role for Rhes in tumor progression via its regulation of sumoylation.

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