Supplementary Figures

Figure S1. (A) Comparison of Ras and Rhes domains. Rhes contains an extended C-terminal domain in addition to consensus N-terminal GTP-binding domain. **(B)** Both N- and C-terminal Rhes fragments bind Ubc9. Equimolar concentrations of purified Rhes full length, N-terminal domain (1-171) or C-terminal domain (171-266) were incubated with GST or GST-Ubc9 in tris-IP-buffer, pulled down with Glutathione (GSH) beads and immunoblotted with GST or Rhes antibody. **(C)** Rhes enhances thioester and isopeptide bond formation on Ubc9. In vitro sumoylation reaction was performed for 90 to 150 minutes in 1x reaction buffer containing 1μg E1, 500ng of Ubc9, 0.2mM DTT, 5mM ATP, 2μg of SUMO-2 and 200ng of Rhes. To distinguish isopeptide from thioester-linked SUMO, 100mM DTT was added at the end of the reaction. Samples were immunoblotted and probed with Ubc9 antibody. **(D)** Concentration dependent effect of Rhes. In vitro sumoylation reaction was carried out as in (C) for 60 minutes with 25, 100, 200 and 500ng of Rhes. $p^{*}<0.05$, $p^{**}<0.01$ and $p^{***}<0.001$ vs. without Rhes. **(E)** Effect of small G-proteins on Ubc9 isopeptide sumoylation. In vitro sumoylation was carried as in (D) for 90 minutes in presence of GST tagged Rac1, RhoA, Ras, Grab, Cdc42 or Rheb (500ng each upper 2 panels) or with Rhes full length or C-terminal fragment, 171-266 (200ng in upper 2 panels) or with GST-Rhes and GST-DexRas1 (200ng and 1μg in lower panel). 100mM DTT was added at the end of the reaction. **(F)** SUMO paralog effect on Ubc9 thioester/isopeptide sumoylation. In vitro sumoylation was carried out as in (C) for 90 minutes with either 2μg of SUMO-1 or SUMO-2.

Figure S2. (A) Samples for mass spectrometric analysis. In vitro sumoylation was carried out with 10μg E1, 5μg of GST-Ubc9, 2mM DTT, 5mM ATP, 20μg of SUMO-2 and 2μg of Rhes. Gel fragments 1 & 2 were excised and subjected to orbitrap tandem mass spectrometric analysis. **(B)** Identification of isopeptide linked-SUMO to lysines 14, 49 and 153 in Ubc9. Tryptic digestion of sumoylated Ubc-9 was subjected to LTQ-Orbitrap mass spectrometer and peptide sequences were determined by the software program Sequest. One set of searches was performed using the modification of 599.2663 mass units to lysine (*QQQTGG*-K) to determine sumoylation sites on Ubc9. Another set of searches was performed using predicted tryptic fragments of Ubc9 as modifications on to the C-terminus of SUMO-2. Only data from Ubc9-SUMO in the presence of Rhes is shown, as identical sites were identified in the absence of Rhes. **(C)** Rhes attaches SUMO-1 to lysine 14, 49 and 153 in Ubc9. In vitro sumoylation was carried out in 1x reaction buffer containing 1μg E1, 0.2mM DTT, 5mM ATP, and 2μg of his-SUMO-1 in the presence and absence of Rhes (200ng) for 60 min with 500ng of either wtUbc9, Ubc9-K14R, Ubc9-K49R, Ubc9 K153R or Ubc9-K14, 49, 153R (KR mutant). 100mM DTT was added at the end of the reaction. Samples were immunoblotted and probed with Ubc9 antibody. **(D)** Binding of Ubc9 mutants to E1, Rhes and SUMO-2. Equimolar concentrations of GST, GST-Ubc9 WT or GST-Ubc9 mutant KR (14, 49, 153) incubated with E1, Rhes, and SUMO-2 in tris-IP-buffer and glutathione pulled down, followed by western blotting and probing for Uba2, Rhes, SUMO-2 or GST.

Figure S3.
(A) Cys-93 is not required for Ubc9 isopeptide sumoylation. In vitro sumoylation was carried out for 90 minutes in 1x reaction buffer containing 1μg E1, 500ng of either wtUbc9, Ubc9-C93A, Ubc9-K14, 49, 153R (KR mutant), or Ubc9-C93A KR mutant, 0.2mM DTT, 5mM ATP, 2μg of SUMO1/2 and 200ng of Rhes. To distinguish isopeptide sumoylation from thioester, 100mM DTT was added at the end of the reaction. Samples were immunoblotted and probed with Ubc9 antibody. **(B)** Effect of lysine mutations on Ubc9-C93A isopeptide sumoylation. 500ng of either single (K14R, K49R, K153R) or triple (K14, 49, 153R) mutants of Ubc9 WT or C93A were subjected to in vitro sumoylation as in (A) in the presence and absence of 200ng Rhes. 100mM DTT was added at the end

of the reaction. p**<0.01 and p***<0.001 vs. Rhes + Ubc9 WT/C93A. **(C)** E1 concentration dependent effect on Ubc9 isopeptide sumoylation. In vitro sumoylation was carried as in (B) with different amount of E1 (0.1, 0.5, 1.0, 1.5µg). **(D)** Rhes directly binds E1. Equimolar concentration of GST, GST-Rhes, E1, and up to 10x increasing molar concentrations of Ubc9 was incubated in tris-IP buffer and glutathione pulled down, followed by western blotting and probing with Uba2 or Rhes antibody. **(E)** Rhes induces E1 isopeptide sumoylation in a time dependent -manner. In vitro sumoylation reaction was carried out as in (B) for 5, 15 and 60 min and 200ng of Rhes followed by western blotting and probing with Uba2 antibody. **(F)** Rhes induces E1 isopeptide sumoylation in a concentration dependent manner. In vitro sumoylation reaction was carried out as in (E) with increasing Rhes (50, 100, 200, 300 or 500ng) concentrations for 60 min, followed by western blotting and probing for E1 with His antibody.

Supplementary Methods

Recombinant Protein Production.

In brief, recombinant proteins were produced in bacteria (DH5a or BL21) by transforming appropriate vector and inducing with 0.1mM isopropyl-β-D-1-thiogalactopyranoside at 37°C for 3h. Rhes expression improved with IPTG induction at room temperature. Recombinant proteins were purified by affinity chromatography on glutathione-sepharose beads (Amersham Biosciences) for GST-tagged proteins or TALON beads (clontech) for His-tagged proteins. GST-Rhes and GST-SUMO-1, SUMO-2 were cleaved by precision protease (Amersham Biosciences). Ubc9 wild type and mutants were cleaved by Factor Xa (Calbiochem). Purified GST-SP100, GST-IkB and His-SUMO-1 were from Biomol international (PA, USA).

In Vitro Binding.

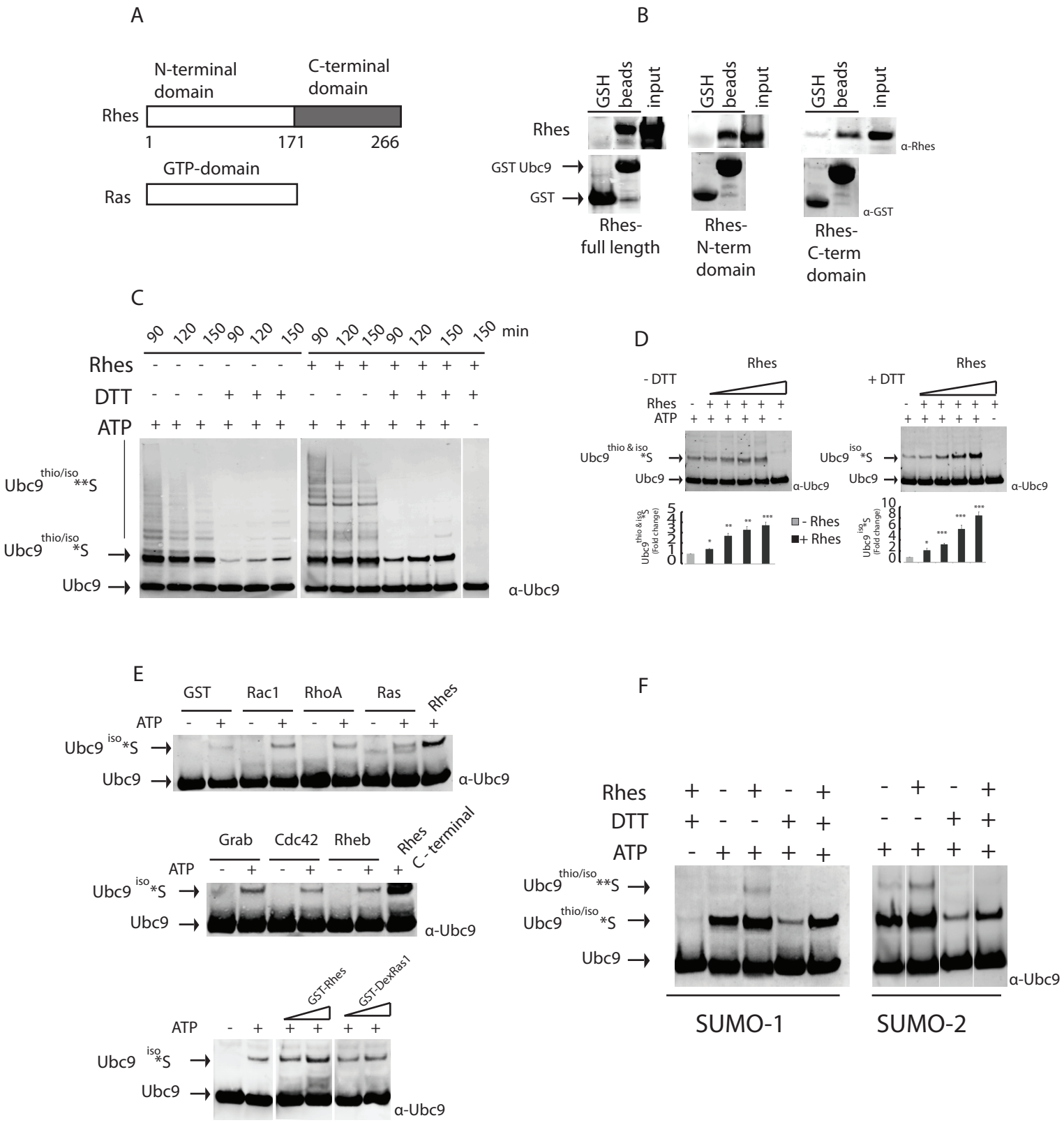
Binding of Rhes (fragments)/Ubc9 was carried out by incubating equimolar concentration of purified GST or GST-Ubc9 and Rhes full length or fragments (1-171 / 171-266). Binding of Rhes/E1/Ubc9 was examined by incubating equimolar concentration of purified GST or GST-Rhes, with purified E1 heterodimer (Aos1/Uba2) and increasing Ubc9 concentration, up to 10 fold excess. Binding was done in Tris buffer (50 mM Tris, pH 7.6, 150 mM Nacl, 1% Triton X-100 and 10% glycerol) for 3 h at 4°C. GST tagged proteins were precipitated with GSH beads, and immunoblotted with anti-Rhes, anti-Uba2 or anti-GST antibody after western blotting.

LC-MS/MS Analysis.

Briefly, Excised Gel pieces were subjected to a modified in-gel trypsin digestion procedure (1). Samples were reconstituted in 5 µl of HPLC solvent A (2.5% acetonitrile, 0.1% formic acid) and loaded onto a nano-scale reverse-phase HPLC capillary column with a flame-drawn tip. As each peptide was eluted they were subjected to electrospray ionization and then entered into an LTQ-Orbitrap mass spectrometer (ThermoFinnigan, San Jose, CA). Eluting peptides were detected, isolated, and fragmented to produce a tandem mass spectrum of specific fragment ions for each peptide. Peptide sequences were determined by a matching protein database with the acquired fragmentation pattern by the software program, Sequest (ThermoFinnigan, San Jose, CA). One set of searches was performed using the modification of 599.2663 mass units to lysine (QQQTGG-K) to determine sumoylation sites on UBC9. Another set of searches was performed using predicted tryptic fragments of UBC9 as modifications on to the C-terminal of Sumo2. For example the masses 541.3120 and 526.32270 were used for the tryptic fragments QQQTGG-KAWR (aa 14-17) and QQQTGG-KKAQA (aa 154-150) of UBC9, respectively. Each modified peptide that was determined by the Sequest program was also manually inspected to ensure confidence.

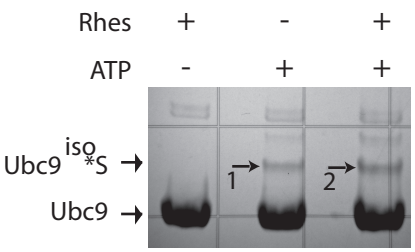
Supplementary Reference

1. Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996) *Anal Chem* **68**, 850-858



Supplementary Figure 1

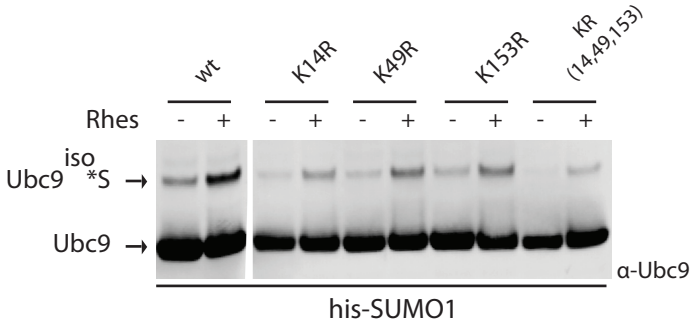
A



B

Peptide	Expected m/z	Observed m/z	Mass accuracy (ppm)	Notes	Lysine modified on Ubc9
K#GTPW	594.2938	594.2954	2.61	# represents QQQTGG of Sumo2	49
QQQTGG@	572.8071	572.8084	2.22	@ represents KKAQA of UBC9	153
QQQTGG#	580.3019	580.3037	2.97	# represents KAWR of UBC9	14

C



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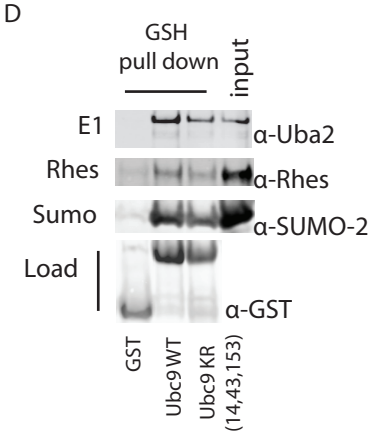
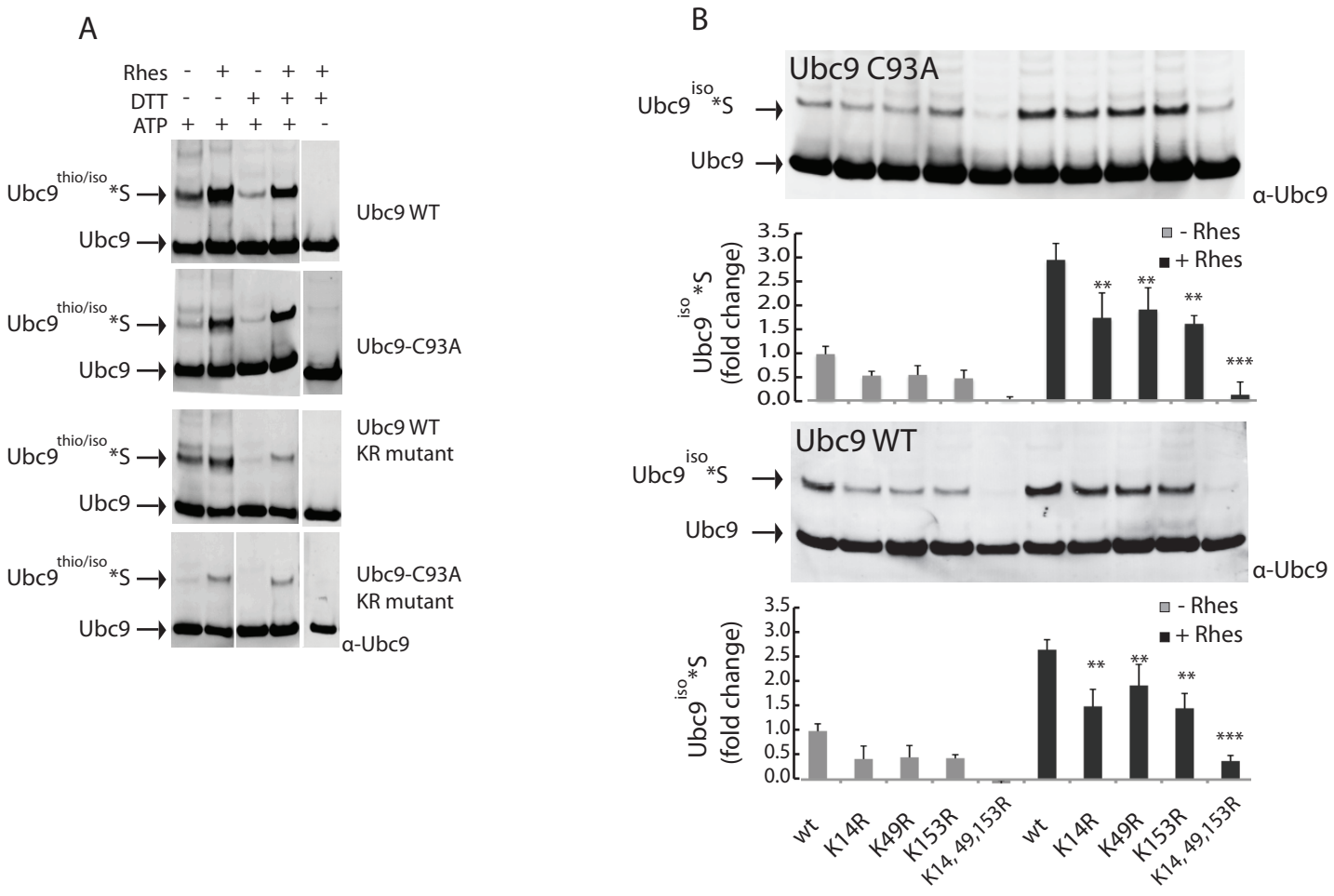
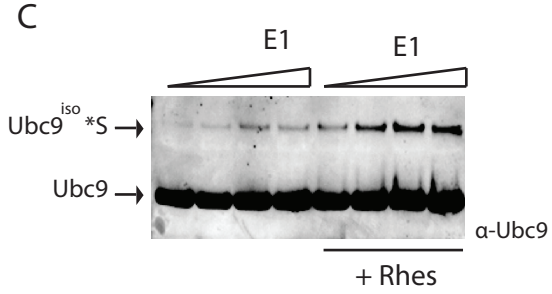
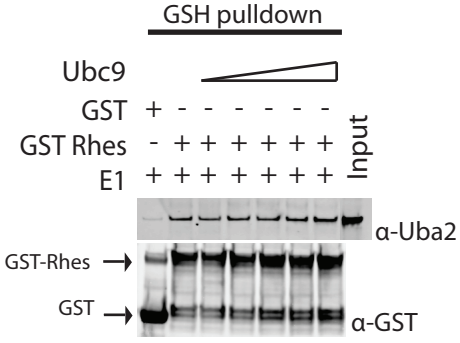
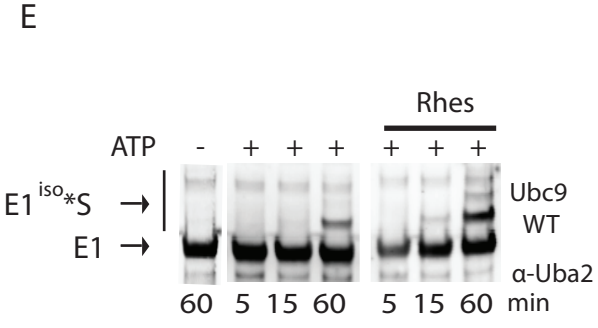


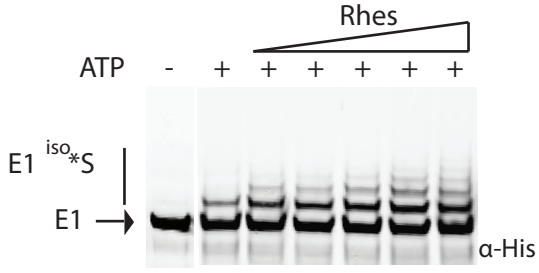
Supplementary Figure 2











Supplementary Figure 3