Supplemental Material

A molecular basis for phosphorylation-dependent SUMO conjugation by the E2 Ubc9

Firaz Mohideen[1], Allan D. Capili[1], Parizad M. Bilimoria[2], Tomoko Yamada[2], Azad Bonni[2], and Christopher D. Lima[1]*

[1]Program in Structural Biology, Sloan-Kettering Institute, Box 414, 1275 York Avenue, New York, NY 10065, USA

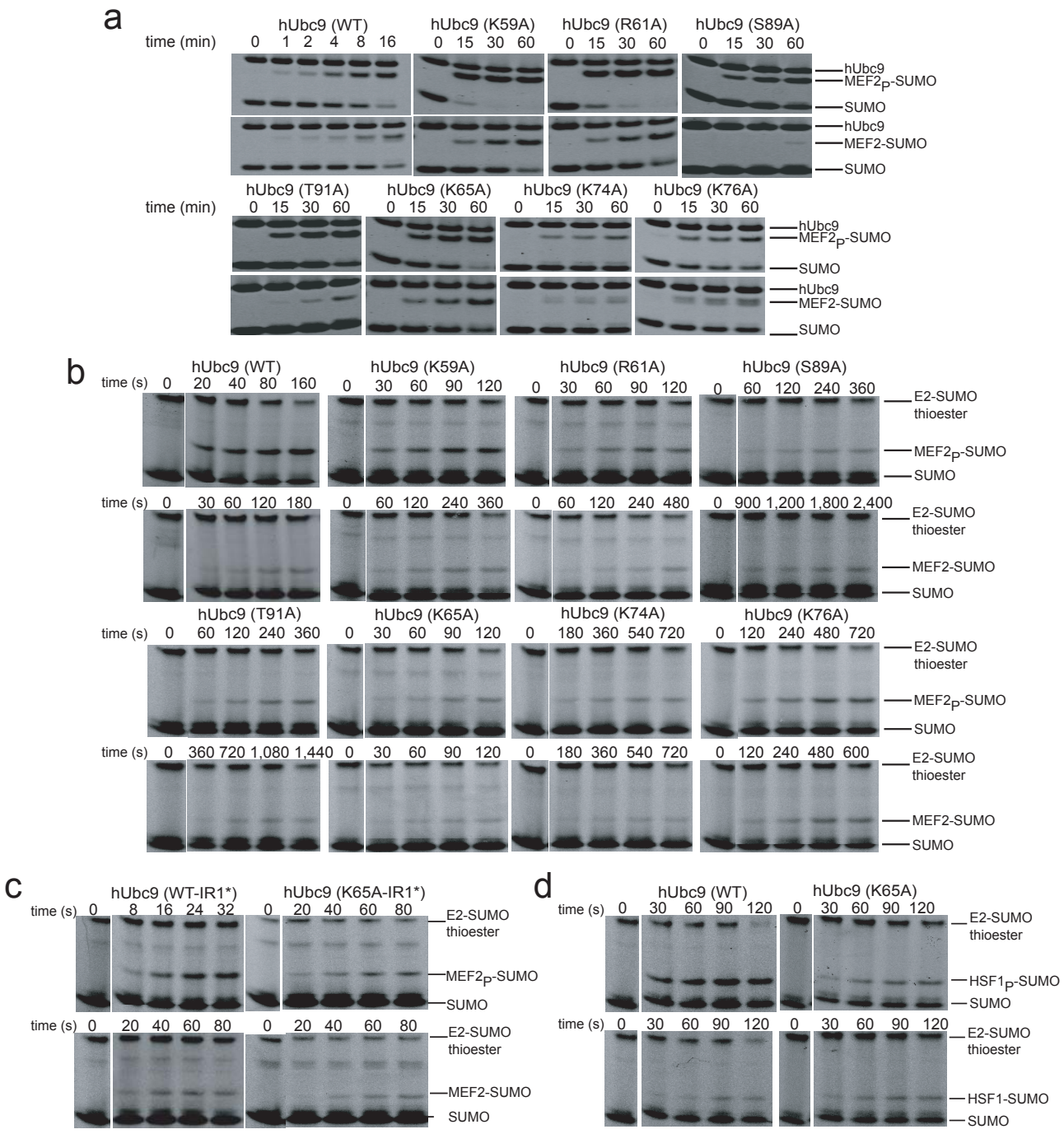
[2]Department of Pathology, Harvard Medical School, New Research Building, 8th Floor, 77 Ave. Louis Pasteur, Boston, MA 02115,USA

*To whom correspondence should be addressed.

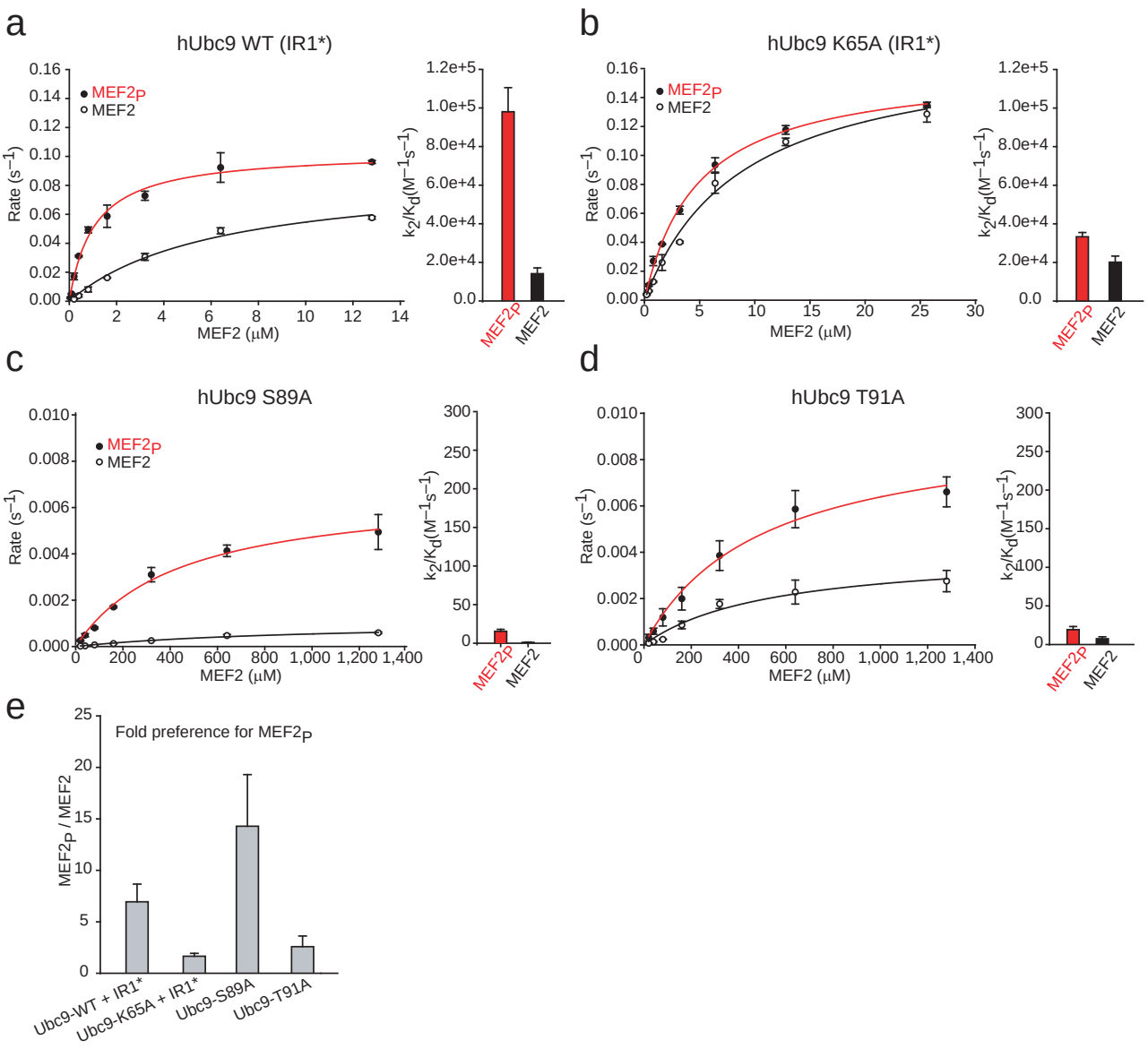
Email: limac@mskcc.org

Ph: (212)639-8205

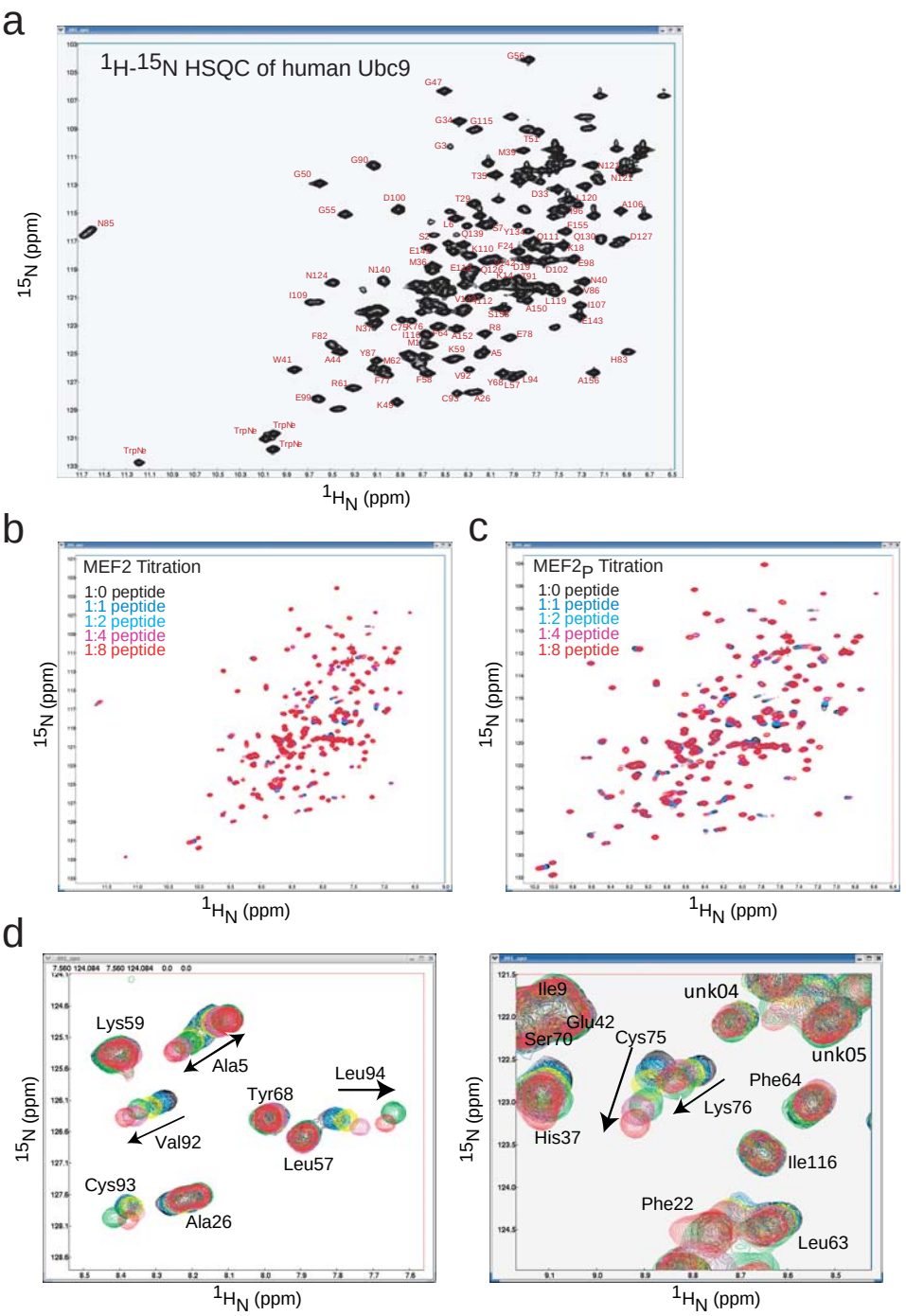
FAX: (212)717-3047



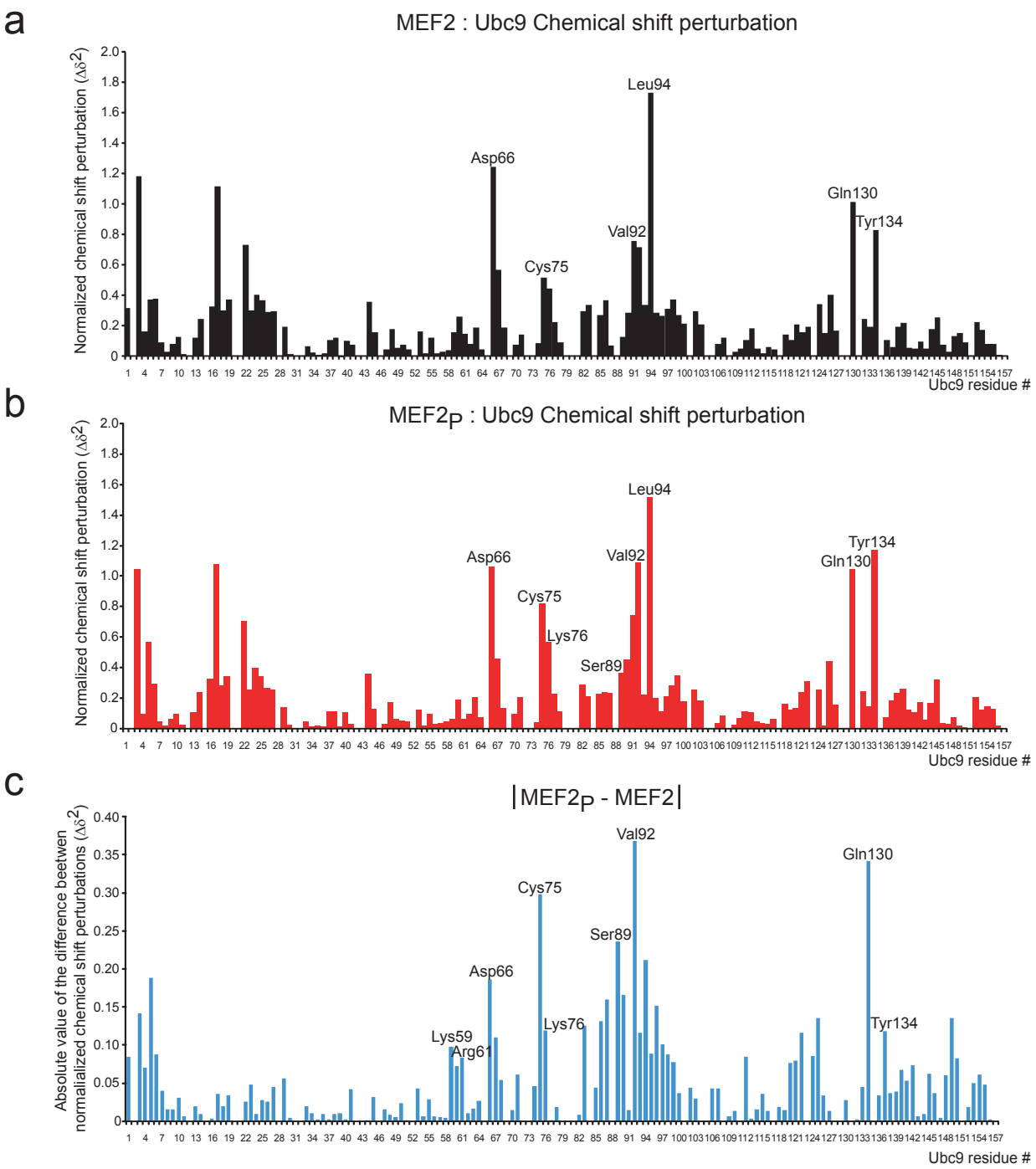
Supplementary Figure 1. SDS-PAGE analysis and detection of products in SUMO conjugation reactions under conditions of multiple and single turnover for MEF2. (a) Insets representing SDS-PAGE analysis for a subset of reactions obtained under multiple turnover conditions for SUMO conjugation assays using $MEF2_P$ or MEF2 substrates at concentrations of 80μM for wild-type or mutant Ubc9. The positions indicated at the right are for Ubc9, the MEF2-SUMO product and SUMO. MEF2 is not visible in the gel. (b) Insets representing SDS-PAGE analysis for reactions obtained under single turnover conditions for SUMO conjugation using $MEF2_P$ or MEF2 substrates with wild-type Ubc9 or mutant Ubc9. a and b represent data obtained at substrate concentrations of 80 μM. (c) Insets representing SDS-PAGE analysis for reactions obtained under single turnover conditions for SUMO conjugation to $MEF2_P$ or MEF2 substrates in the presence of Nup358/RanBP2 IR1* with wild-type Ubc9 or Ubc9-K65A at substrate concentrations of 0.4 μM. (d) Insets representing SDS-PAGE analysis for reactions obtained under single turnover conditions for SUMO conjugation to $HSF1_P$ or HSF1 substrates with wild-type Ubc9 or Ubc9-K65A at substrate concentrations of 80 μM. Relative positions for the E2-SUMO thioester, the MEF2-SUMO product, and SUMO are indicated at the right.



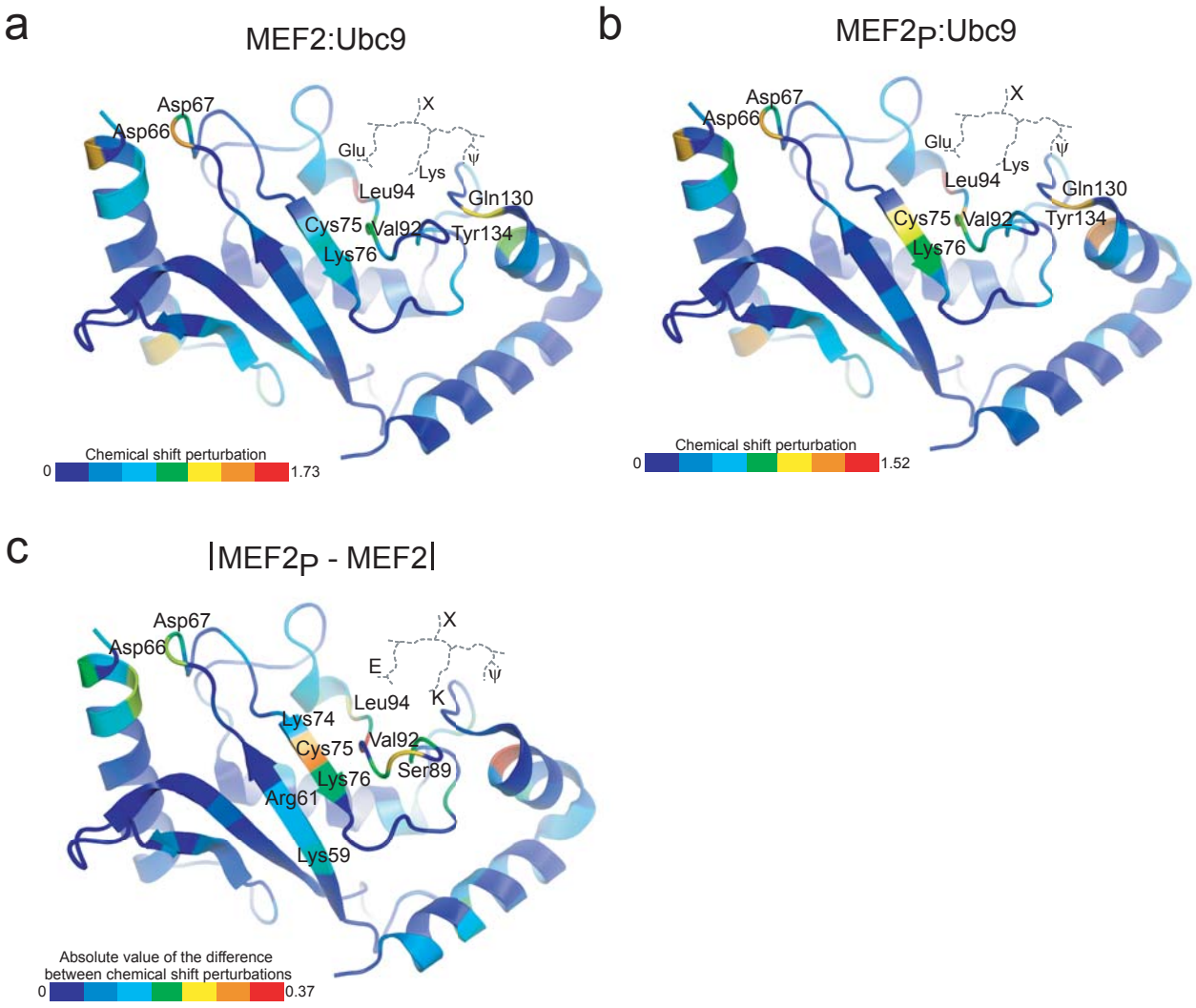
Supplementary Figure 2. Kinetic analysis of SUMO conjugation to MEF2. (a) The plot on the left indicates the initial rates of reaction versus substrate concentration in the presence of the Nup358/RanBP2 SUMO E3 ligase IR1* with the bar chart on the right indicating the specificity constants (k_2/K_d) for wild-type Ubc9 mediated SUMO conjugation to $MEF2_P$ and MEF2 . (b) Plot and bar chart as in a, but for Ubc9-K65A. (c) The plot on the left indicates the initial rates versus substrate concentration with the bar chart on the right indicating the specificity constants (k_2/K_d) for $MEF2_P$ and MEF2 for the reaction mediated by Ubc9-S89A. (d) Plot and bar chart as in a, but for Ubc9-T91A. Error bars (±1 s.d) were obtained by performing at least three independent reactions. (e) Ratio of specificity constants as determined for data presented in a-d.



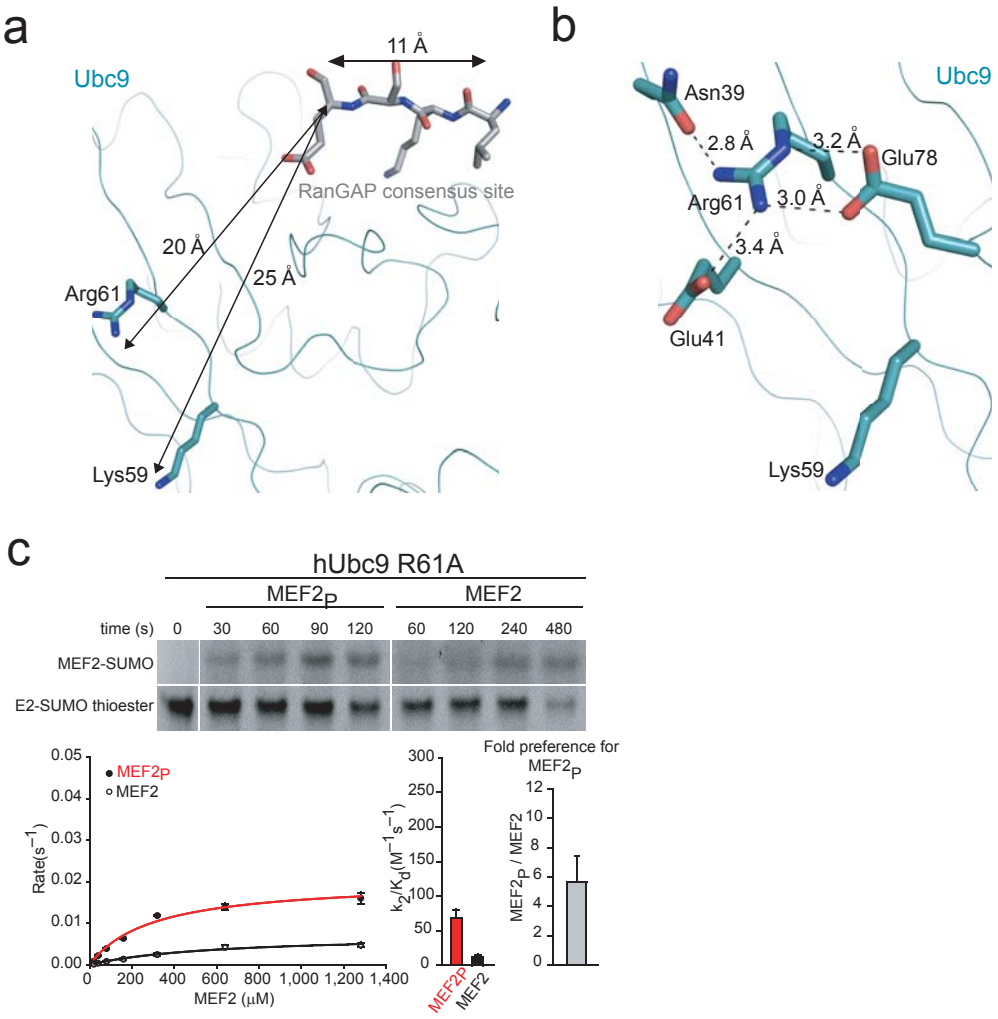
Supplementary Figure 3. NMR analysis and substrate titrations. (a) ^{1}H-^{15}N HSQC spectra obtained for human Ubc9 with assigned peaks denoted by the individual amino acid code and its corresponding number within the linear Ubc9 sequence. (b) Overlay of ^{1}H-^{15}N HSQC spectra for Ubc9 with increasing concentrations of the MEF2 substrate. Spectra are color coded based on the molar ratio between Ubc9 and substrate. (c) Similar spectra as in b but using increasing MEF2$_P$ concentrations. (d) Close-up views for two regions in b and c to highlight chemical shifts for some residues discussed in the text. Axes are labeled and spectra color-coded to correspond to unique molar ratios between Ubc9:MEF2 or Ubc9:MEF2$_P$. Amino acids are labeled by three letter code and number within the Ubc9 sequence.



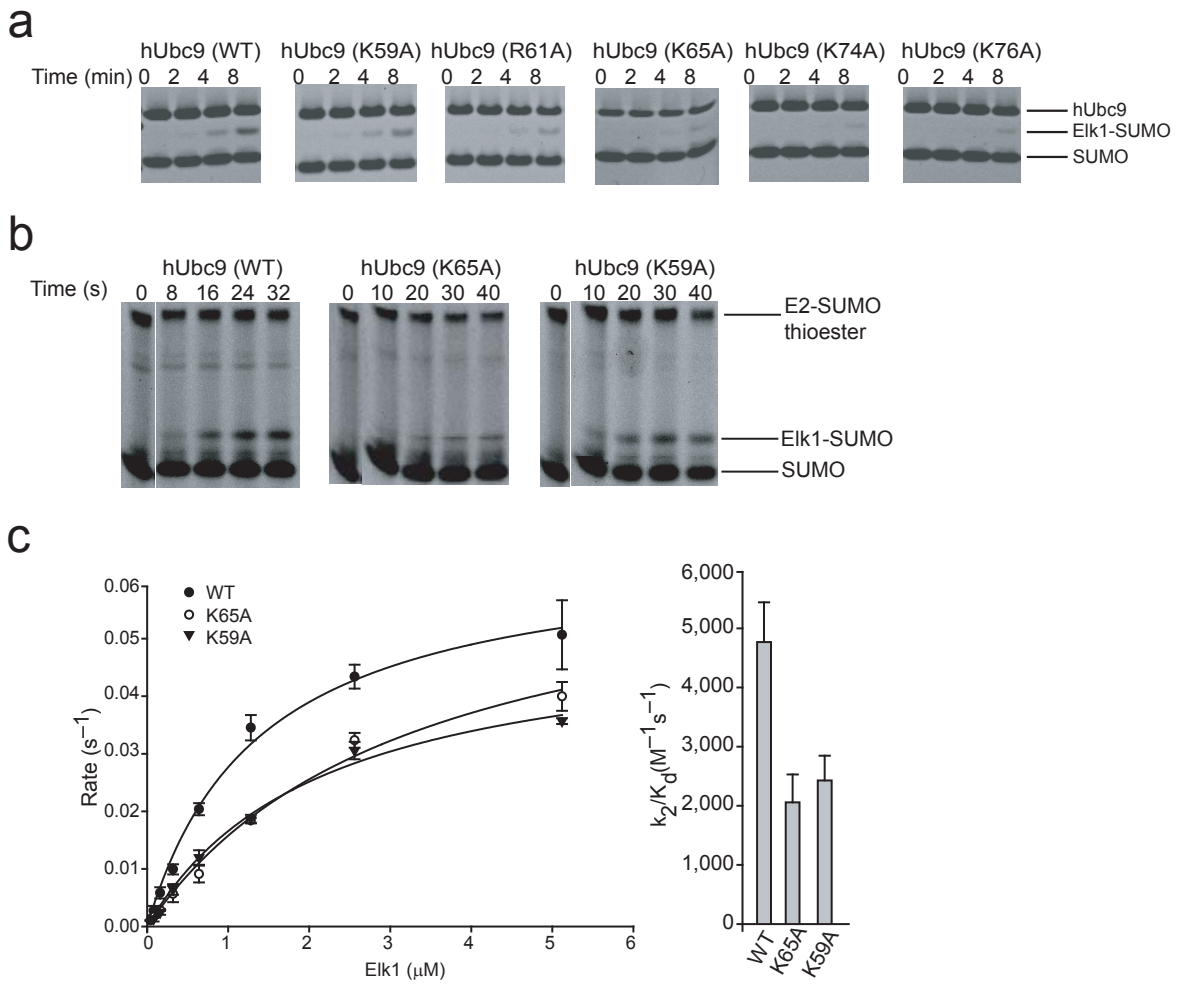
Supplementary Figure 4. Normalized chemical shift analysis. (a) Bar graph indicating normalized chemical shifts (Δ^2) for the 1:8 molar ratio between Ubc9 and MEF2. (b) Bar graph indicating normalized chemical shifts (Δ^2) for the 1:8 molar ratio between Ubc9 and MEF2P. The value (Δ^2) is calculated as described in the Methods. (c) Bar graph depicting values obtained by taking the absolute value of differences between normalized chemical shifts obtained from solutions containing Ubc9:MEF2P and Ubc9:MEF2 at a 1:8 molar ratio. Several of the amino acid residues that are specifically discussed in the text are indicated by three letter code and amino acid position within the linear sequence of Ubc9. Axes are labeled. The y-axis indicates the magnitude of the chemical shift perturbation while the x-axis indicates the amino acid position within the Ubc9 sequence. Proline residues or positions that could not be assigned in the spectra do not have a corresponding bar above the indicated sequence position.



Supplementary Figure 5. Structure of human Ubc9 and NMR chemical shift perturbation analysis. Amino acid positions are colored according to the normalized chemical shifts from NMR spectra obtained from the 1:8 Ubc9:substrate ratios for the (a) non-phosphorylated and (b) phosphorylated MEF2 peptide. (c) Structure of human Ubc9 colored by the value of the absolute difference between normalized chemical shifts. a-c depict Ubc9 in ribbon and cartoon with arrows indicating β-strands and wide ribbons indicating α-helices. Several amino acid positions specifically discussed in the text are indicated by three letter code and number within the Ubc9 sequence. The position for a ψ-K-x-E consensus site is labeled and indicated by dashed lines above the Ubc9 diagram for reference. The color bars at the lower left of each panel denote chemical shifts as a relative color spectrum from lowest (blue) to highest (red) as mapped on the Ubc9 structure.



Supplementary Figure 6. Two Ubc9 residues previously shown to mediate NDSM recognition are not required for PDSM discrimination. (a) Ribbon representation for the Ubc9 structure indicating the approximate distances between the glutamate Cα position within the ψ-K-x-E SUMO consensus motif and Lys59 and Arg61, two basic residues previously shown to be important for NDSM recognition. Lys59, Arg61 and the structure of the ψ-K-x-E SUMO consensus motif are shown in stick representation. The Cα-Cα distance (11Å) between glutamate and hydrophobic residues within the SUMO consensus motif is indicated for perspective. (b) Close-up of Lys59 and Arg61 as in a but now including Asn39, Glu41, and Glu78 in stick representation. Potential hydrogen bond or salt bridging interactions are indicated by a dashed line. (c) Upper panel; insets showing examples of SDS-PAGE analysis and fluorescent detection for Ubc9-R61A mediated SUMO conjugation to $MEF2_P$ and MEF2 at substrate concentrations of 80 μM. Lower panel; plot on the left indicates initial rates versus substrate concentration, the middle bar chart indicates the individual specificity constants (k_2/K_d) for Ubc9-KR61A mediated SUMO conjugation to $MEF2_P$ (red) and MEF2 (black). The bar chart (right) indicates the fold preference for $MEF2_P$ (gray) expressed as $MEF2_P(k_2/K_d)/MEF2(k_2/K_d)$.



Supplementary Figure 7. Kinetic analysis and detection of products in SUMO conjugation reactions under conditions of multiple and single turnover for Elk1. (a) Insets representing SDS-PAGE analysis for reactions obtained under conditions of multiple turnover for SUMO conjugation assays using Elk1 substrates for wild-type Ubc9 and indicated Ubc9 mutants at an Elk-1 concentration of 50 μM. Time points are indicated in minutes. The relative positions are indicated at the right for Ubc9, SUMO, and the Elk1-SUMO product. Elk1 is not visible in the gel. (b) Insets representing SDS-PAGE analysis for reactions obtained under conditions of single turnover for SUMO conjugation using Elk1 substrates mediated by wild-type Ubc9, Ubc9-K65A, and Ubc9-K59A in the presence of the SUMO E3 Nup358/RanBP2 IR1* at an Elk1 concentration of 6.4 μM. Time points are indicated in seconds. The relative positions for the E2-SUMO thioester, the Elk1-SUMO product, and SUMO are indicated at the right. (c) Plots depicting the kinetic analysis for single turnover reactions using Ubc9 and Ubc9 mutant isoforms in b by varying concentrations of the Elk1 substrate. Rate is depicted on the y-axis and Elk1 concentration on the x-axis. On the right, comparative bar graphs indicating the relative catalytic and specificity constants calculated for wild-type Ubc9, Ubc9-K65A and Ubc9-K59A using the Elk1 substrate. Error bars (±1 s.d) were obtained by performing at least three independent reactions.