Supplementary Information

Protein Microarray Characterization of the *S*-Nitrosoproteome

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SI Material and Methods

GST purification. Invitrogen entry clone plasmids of the 300 highest-significance *S*-nitrosylated proteins were shuttled to the GST-purification plasmid desti-15 (Invitrogen) using the Clonase-based Gateway cloning system (Invitrogen).

TOP10 *E. coli* cells transformed with the GST plasmids were grown overnight in

2 mL of ampicillin-containing LB broth in a 37°C shaking incubator. The cells were transferred to 20 mL of ampicillin-containing LB broth and grown for 1 h before treatment with 1 mM IPTG for 5 h. The cells were then pelleted via centrifugation at 4000 rpm for 10 min. The cells were lysed in PBS (1 mg/mL lysozyme, proteasome inhibitor cocktail) using a sonifier at 4°C. Triton X-100 was added at 1% and the samples were mixed for 30 min at RT. Samples were then centrifuged at 12000 g for 10 min at 4°C. Supernatants were retrieved and treated with 50% glutathione beads and incubated for 30 min at RT with shaking. Beads were pelleted via centrifugation at 500 g for 5 min. Beads were washed with PBS twice via centrifugation at 500 g for 5 min each time. Bound protein was eluted from beads by treatment with glutathione elution buffer containing 0.154 g free glutathione per 50 mL elution buffer. Samples were shaken for 10 min at RT, then beads were pelleted via centrifugation at 500 g for 5 min. Supernatant containing purified protein was collected and protein concentration was assayed via the Bradford method as well as Coomassie blue staining after running on SDS-PAGE.

Biotin switch method *in vitro*. GST-Recombinant proteins were treated with 100 μM GSH or GSNO in HEN buffer (250 mM Hepes, 1 mM EDTA, 0.1 mM Neocupoine, 0.5% NP-40). GSH or GSNO was removed from solution by passing the solution through G25 Sephadex spin columns (Bio-Rad) via centrifugation at 3500 rpm for 4 min. The samples were then incubated with 20 mM methyl

methanethiosulfonate (MMTS) (Sigma) at 50°C for 30 min. Excess MMTS was removed by passing the samples through the spin columns 3 times via centrifugation at 3500 rpm for 1 min. The samples were incubated with 50 mM ascorbate (Sigma) and 0.4 mM biotin-HPDP (Pierce) for 1 h at RT with shaking. Unreacted biotin-HPDP was then removed by treatment with -20°C acetone and centrifugation at 2600 g for 10 min at 4°C. The pellet was suspended in HENS buffer (HEN, 1% SDS) and neutralization buffer (20 mM Hepes-NaOH (pH 7.7), 100 mM NaCl, 1 mM EDTA, 0.5% Triton X-100). Samples were incubated with 50 µl of Neutravidin-agarose beads (Thermo Scientific) for 12 h. The beads were washed 5 times with high salt neutralization buffer (20 mM Hepes-NaOH (pH 7.7), 600 mM NaCl, 1 mM EDTA, 0.5% Triton X-100) and then eluted by elution buffer (20 mM Hepes-NaOH (pH 7.7), 100 mM NaCl, 1 mM EDTA, 100 mM 2-ME) and Laemmli sample buffer including 2-ME to recover the bound protein and subjected to immunoblot analysis.

SI Figure Legends

Figure S1 Expression of proteins on chip. (**A**) The same representative blocks shown in Figure 2A probed with anti-GST antibody in the presence of GSH or GSNO.

Figure S2 Confirmation of the predicted motif. S-nitrosylation of mutating a site that is not predicted to be the motif as a negative control.

Figure S3 The effect of 100 μM GSNO on (**A**) Parkin and (**B**) XIAP E3 ligase ubiquitination activity.

Figure S4 Screening of UbcH E2 enzymes for RNF11, RNF41 and RNF141. (*A*) no E3 ligase, (*B*) RNF11, (*C*) RNF41 and (*D*) RNF141 *in vitro* ubiquitination assay in the presence of different UbcH E2 enzymes. Assays were assessed by immunoblot analysis with anti-ubiquitin and anti-GST antibodies.

Figure S5 The effect of WT and C175S mutant RNF141 by 100 *μ*M GSNO. (*A*) *In vitro* and (*B*) *in vivo* ubiquitination activity was analyzed by immunoblot with anti-ubiquitin and anti-GST antibodies, anti-HA and anti-V5 antibodies

respectively in presence or absence of GSNO. Actin and HA-ubiquitin expression were monitored as loading controls.

SI Datasets

Dataset S1 A-D. The list of all 16,368 proteins on the microarray chip. The list includes the gene name, clone ID, description, various accession numbers, and t and *p* values. Transcriptional variants are included on Gene Ontology annotation as unique proteins. The list is separated into four parts to minimize file size and access issues.

Dataset S2. The list of 834 *S*-nitrosylated proteins identified by protein chip microarray. The list includes the gene name, SNO/GSH normalization ratio, short description, IOH # (Invitrogen), gene symbol, various accession numbers, alternative gene names, molecular class, biological process, cellular compartment, MS identified cysteine sites, acknowledgement of previously known NO targets, the identity of functional or non-functional cysteines, a listing of the cysteine-containing domains in the proteins, and the site of cysteines in proteins that only contain one cysteine. Gene annotation was compiled from the Invitrogen Ultimate ORF browser and the human protein reference database (HPRD) (1). Under the list of 834 *S*-nitrosylated proteins containing a cysteine

are listed the 23 proteins identified as being significantly *S*-nitrosylated which do not contain any cysteines, thus representing the false positives.

Dataset S3. The list of 138 identified cysteines in 131 peptides from 95 proteins. The list includes the gene name, protein name, Uniprot accession number, molecular weight, citation of previously reported sites, the identification of domains the identified cysteines are part of (HPRD), and the identified cysteines. Previously reported sites were compiled from various reports.

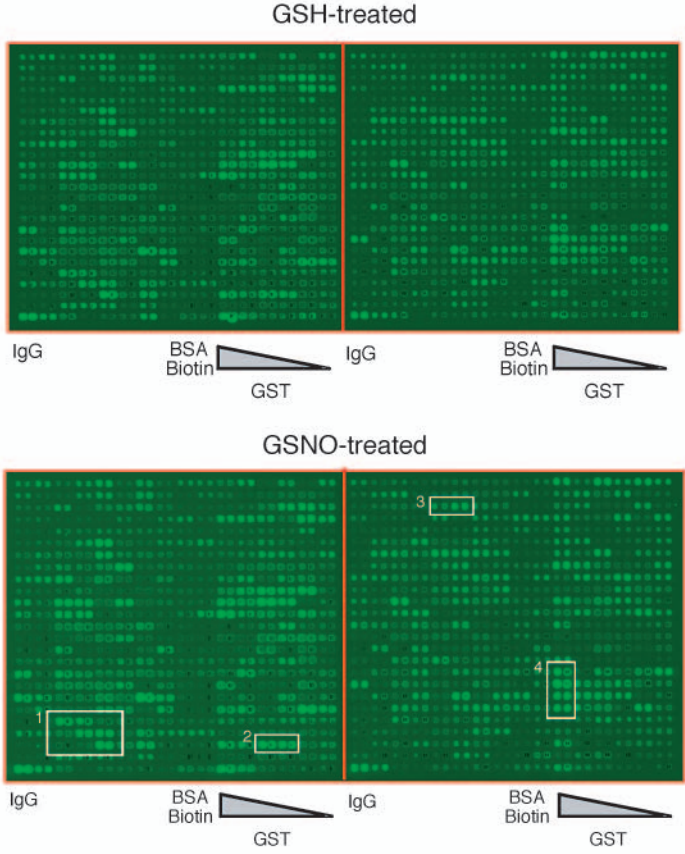
Dataset S4. Peptide sequences including deviations from expected cleavage specificity, precursor charge and mass/charge (m/z) for each assignment and peptide identification scores.

Dataset S5. MS/MS Spectra

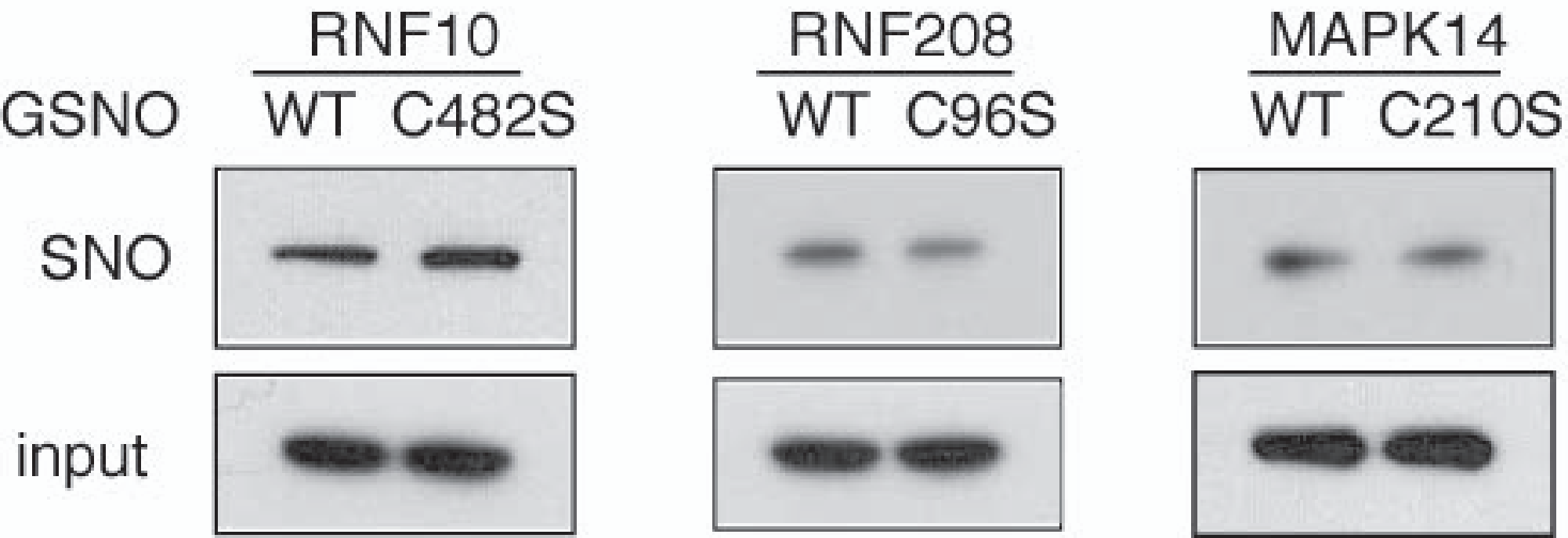
References

1. Prasad, T. S. K., Goel, R., Kandasamy, K., Keerthikumar, S., Kumar, S., Mathivanan, S., Telikicherla, D., Raju, R., Shafreen, B., Venugopal, A., Balakrishnan, L., Marimuthu, A., Banerjee, S., Somanathan, D. S., Sebastian, A., Rani, S., Ray, S., Kishore, C. J. H., Kanth, S., Ahmed, M., Kashyap, M., Mohmood, R., Ramachandra, Y. L., Krishna, V., Rahiman, A. B., Mohan, S., Ranganathan, P., Ramabadran, S., Chaerkady, R. and Pandey, A. (2009) Human Protein Reference Database - 2009 update. *Nucleic acids research* 37, D767-D772.

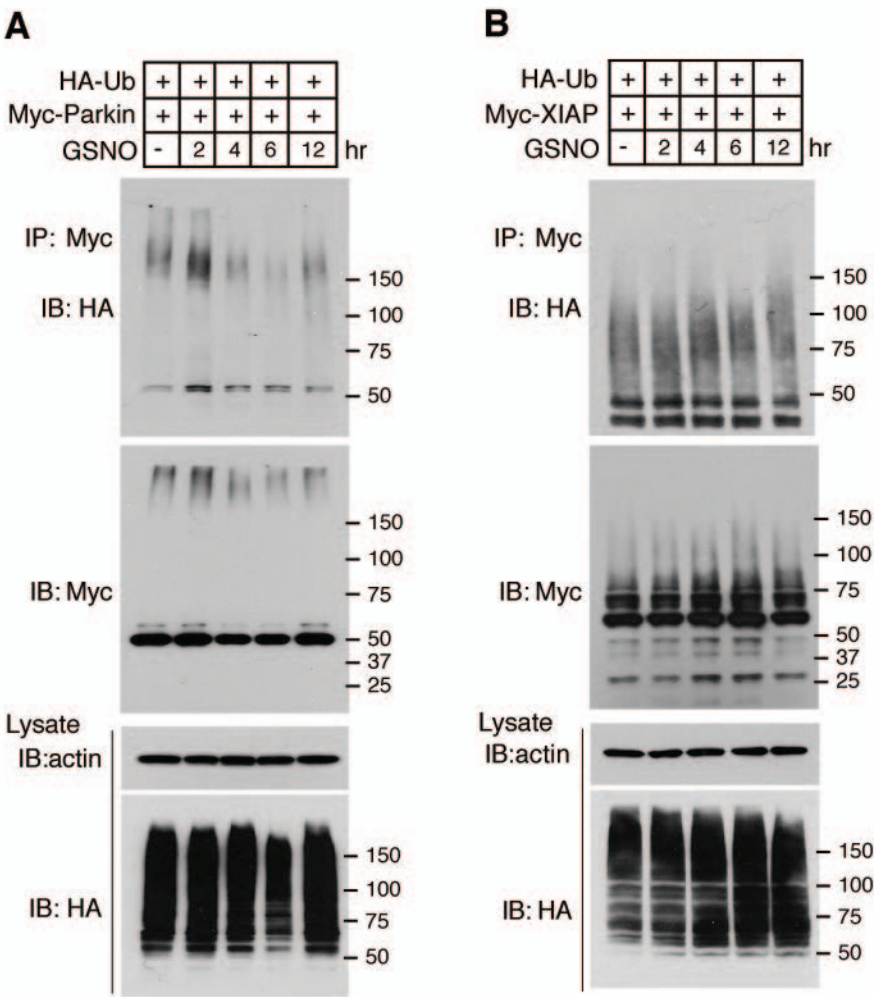
Supplementary Figure 1



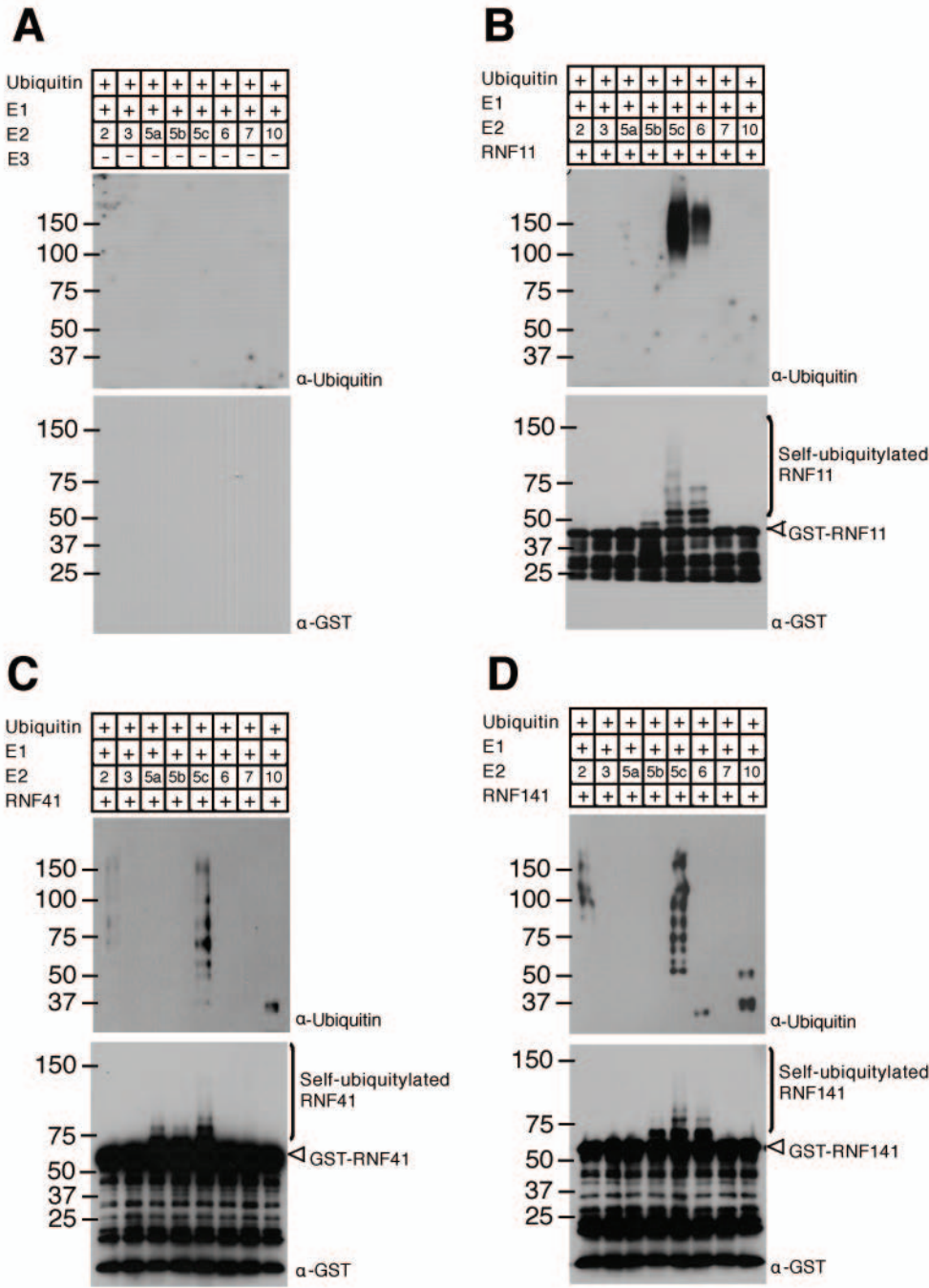
Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4



Supplementary Figure 5