Neuron, Volume 61

Supplemental Data

Pias3-Dependent SUMOylation Directs Rod Photoreceptor Development

Akishi Onishi, Guang-Hua Peng, Chengda Hsu, Uel Alexis, Shiming Chen, and Seth Blackshaw

Supplemental materials and methods

Double chromatin immunoprecipitation assays (double ChIP):

For each reaction, six P14 mouse retinas were dissected and treated with 1 % formaldehyde for 15 min at room temperature to cross-link chromatin binding proteins. After cell lysis, the chromatin was fragmented to an average length of about 600 bp by sonication, and subjected to immunoprecipitation with 2 μg of the first IP antibody or a control IgG in the presence of Protein A or G beads. The immunoprecipitated protein–DNA complexes were washed as previously described (Chen et al., 2005), and eluted from the beads with 300 μl of an Elution Buffer (50 mM $NaHCO_3$, 1% SDS). 1/3 volumes of the elutes were reserved as single ChIP samples for PCR analysis later, while the rest were used for the second immunoprecipitation. After being diluted to 600 μl with IP dilution buffer (167 mM NaCl, 16.7 mM Tris-HCl pH8.1, 1.2 mM EDTA, 1.1%Triton X-100, 0.01% SDS), each sample was divided into two equal aliquots and immunoprecipitated using 1.5 μg of the second IP antibody and control IgG, respectively, in the presence of Protein A or G beads. After washing and elution steps, all the samples were heated at 67 °C for 4–5 h to reverse the cross-links. The DNA in the complexes was then purified and analyzed, together with the single ChIP samples, by PCR with candidate gene specific primers. Quantitative chromatin immunoprecipitation was performed as previously described (Peng and Chen, 2007).

Cell based co-IP and SUMOylation assays:

HEK293 cells were cultured on 100 mm plates and co-transfected with a total of 6 μg of recombinant DNA using calcium phosphate method (Wang et al., 2002). Each

transfection includes 2 μg of each of the following recombinant protein expression vectors or the respective empty vector controls: wild-type or mutant forms of pCAG-Nr2e3, wild-type or mutant forms of pCAG-Pias3 and pTag-FLAG-SUMO1. Each transfection was done in duplicate, and at least three independent experiments were performed. Whole cell extracts were prepared in a 3-fold volume of RIPA buffer [50 mM Tris-HCl (pH 7.4), 300 mM NaCl, 1% NP 40, 0.25% Na-deaxycholate, 1mM EDTA] in the presence of 20mM N-ethylmaleimide (NEM, Sigma), an inhibitor of de-SUMOylation enzyme, and a mixture of protease inhibitors (Sigma). 200 μl of the cell lysate were subject to immunoprecipitation with 1 μg of an IP antibody coupled to Protein A (for rabbit antibody) or G beads (for mouse antibody, Sigma) for overnight at 4 C. After being washed five times with the wash buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% Triton X-100), the bound proteins were eluted and analyzed by SDS-PAGE and immunoblotting using antibodies listed in table ST2. The blotting membrane was treated with a Tris stripping buffer containing 100 mM β-mercaptoethanol and 2% SDS before the second and third staining.

Luciferase analysis:

Luciferase analysis was performed essentially as previously described for both the rhodopsin promoter and the 4x optimal Nr2e3 binding site (Chen et al., 2005; Peng et al., 2005) using HEK293T cells 48 hrs post-transfection. For the rhodopsin promoter, however, roughly 2kb of the bovine rhodopsin promoter (Matsuda and Cepko, 2004) was used instead of the 225 bp proximal rhodopsin promoter used in the original study (Peng et al., 2005). Since 293T cells express low levels of endogenous Pias3 (data not shown), 10ng of an shRNA construct selectively targeting human but not mouse Pias3 was included in each transfection to eliminate confounding effects of endogenous Pias3.

Supplemental Figures:

Figure S1: Controls for Pias3 antibody and Pias3 shRNA.
(A) Immunohistochemistry with anti-Pias3 antibody to confirm the specificity in transfected HEK293 cells.
(B) Immunohistochemistry of the P7 dissociated retinas electroporated *in vivo* with U6-cotrol and U6-Pias3. Immunolocalization signals of anti-Pias3 (red) were down-regulated in electroporated cells with U6-sPias3.
(C) Fraction of Pias3-positive electroporated cells shown in (B), indicating significant reduction of Pias3-positive cells electroporated with U6-Pias3 compared with U6-control. Data are represented as mean +/- SD. Double asterisk, $p < 0.005$ by Student's t-test ($n = 3$).
(D) Fraction of Rho4D2-positive electroporated cells counted from the P14 dissociated retinas electroporated *in vivo* at P0 with human PIAS3, which is resistant to the mouse U6-Pias3, and U6-control/U6-Pias3. The fraction of Rho4D2/GFP-positive cells was upregulated even in the presence of U6-Pias3, indicating that human PIAS3 can rescue mouse Pias3 function. Mutant forms of human PIAS3 that lack SUMOylase activity, however, cannot rescue the reduction in rhodopsin expression seen following Pias3 knockdown. Data are represented as mean +/- SD ($n = 3$).

Figure S2: Pias3 differentially regulates Nr2e3-dependent transcriptional activation and repression.
(A) SUMOylation-independent activation of rhodopsin expression by Nr2e3 in conjunction with Pias3. HEK293T cells were transiently transfected with 100 ng of pGL2 luciferase expressing vector expressed from the bovine rhodopsin promoter (2174 bp) (Matsuda and Cepko, 2007), along with a combination of the indicated vectors expressing Nrl, Crx, wildtype Nr2e3 and Nr2e3 with K to R mutations at K187, K325, and K332 (triple mutant), wildtype Pias3 and SUMOylase-deficient Pias3 (Pias3 ΔSUMO). '+' and '++' represent 15 ng and 30 ng, respectively, of the indicated vector, whereas '–' represents 50~200 ng of the empty vector, keeping the total amounts of DNA constant.

(B) SUMOylation-dependent enhancement of Nr2e3-dependent repression by Pias3. HEK293T cells were transiently transfected with 100 ng pTK-Luc vector expressed from four repeats of the Nr2e3 optimal binding sequence in combination with a minimal promoter (Chen, et al. 2005), along with indicated combinations of vectors expressing wildtype Nr2e3, Nr2e3 triple mutant, Nr2e3 fused with SUMO1 (Nr2e3-SUMO1), wildtype Pias3 and SUMOylase-deficient Pias3. '+' and '++' represent 5 ng and 10 ng, respectively, of the indicated vector, whereas '–' represents 5~20 ng of the empty vector, keeping the total amounts of DNA constant. Data are represented as mean +/- SD.

Figure S3: Pias3 overexpression/knockdown in *rd7/rd7* mice.
(A) Section immunohistochemistry of the P14 *rd7/rd7*mouse retinas electroporated *in vivo* at P0 with CAG-GFP, CAG-Pias3, U6-control and U6-Pias3 labeled with Gtγ2 antibody.
(B) Fraction of the GFP-positive cells localized at OONL, ONL and INL observed in *rd7/rd7* mice (adapted from S3A) and in wildtype retinas (adapted from Figure 1H). Pias3 overexpression and knockdown do not repress the generation of INL cells with Muller-like morphology in *rd7/rd7* retinas, and the fraction of cells showing cone-like position and morphology following Pias3 knockdown is substantially reduced relative to wildtype animals. Data are represented as mean +/- SD. Double asterisk, $p < 0.005$ by Student's t-test ($n = 6$).

Figure S4: Pias3 preferentially occupies photoreceptor-specific promoters: additional data.
(A) Quantitative PCR for the promoters for M-opsin (Mop), S-opsin (Sop), Rhodopsin (Rho) and metabotropic glutamate receptor 6 (mGluR6) to test the immunoprecipitation efficiency of the antibodies used in Figure 3. Relative IP versus Input ratio is presented as percent of Input, which is calculated based on a formula: $(2^{-\Delta Ct}) \times 100\%$, where ΔCt=Ct [IP] – Ct [Input]. For each Input to calculate the ratio, the chromatins recovered by each round of ChIP are used. Data are presented as mean ± SD from three trails.
(B) Pias3 preferentially occupies photoreceptor gene promoters independent of either Crx or Nr2e3. ChIP assays were performed using P10 retina from wild-type, $Crx^{-/-}$ and *rd7/rd7* mice as described for Figure 3.

Figure S5: Pias3 interacts with Nr2e3 using its RING and N-terminal LXXLL domains. Co-immunoprecipitation of the indicated fragments of Pias3 (^{35}S-labeled) with full-length Nr2e3 (without ^{35}S-labeling) or its empty vector control (lane C, shown with full-length Pias3 only) was performed using *in vitro* translated proteins and anti-Nr2e3 (p183). Radiolabeled proteins are detected using autoradiography. Input controls for each translated proteins were displayed in the lower panel.

Figure S6. Quality controls for assays using the anti-SUMO1 antibody.
(A) Specificity tests for a mouse monoclonal anti-SUMO1 antibody (α-SUMO1). HEK293T cells cultured on 100mm plates were transfected with 4 micrograms of either a Flag-Sumo1 expression vector (+) or the empty vector control (-). The whole cell extracts were prepared and subject to immunoprecipitation with α-SUMO1 in the presence of protein A/G beads. After extensive washes, the immunoprecipitated proteins were eluted from the beads and analyzed along with the input controls by SDS-PAGE (11% gel) and immunoblotting with anti-Flag M2 monoclonal antibody (α-Flag) and α-SUMO1. The results showed that α-SUMO1 can specifically immunoprecipitate Flag-SUMO1 modified proteins, which are readily identifiable using immunoblotting with either α-Flag or α-SUMO1. Negative controls without SUMO1 overexpression did not yield positive results, demonstrating the specificity/quality of the α-SUMO1 antibody used in the ChIP and co-immunoprecipitation assays.
(B) Quantitative PCR for the promoters for M-opsin (Mop), S-opsin (Sop), Rhodopsin (Rho) and metabotropic glutamate receptor 6 (mGluR6) using immunoprecipitated DNAs with antibodies for Pias3 and Sumo1 to test immunoprecipitation efficiency of the antibodies used in Figure 6A. Relative IP versus Input ratio is calculated as in Figure S4A. Data are presented as mean ± SD from three trails.

References:

Chen, J., Rattner, A., and Nathans, J. (2005). The rod photoreceptor-specific nuclear receptor Nr2e3 represses transcription of multiple cone-specific genes. J Neurosci *25*, 118-129.

Matsuda, T., and Cepko, C.L. (2004). Electroporation and RNA interference in the rodent retina in vivo and in vitro. Proceedings of the National Academy of Sciences of the United States of America *101*, 16-22.

Matsuda, T., and Cepko, C.L. (2007). Controlled expression of transgenes introduced by in vivo electroporation. Proceedings of the National Academy of Sciences of the United States of America *104*, 1027-1032.

Peng, G.H., Ahmad, O., Ahmad, F., Liu, J., and Chen, S. (2005). The photoreceptor-specific nuclear receptor Nr2e3 interacts with Crx and exerts opposing effects on the transcription of rod versus cone genes. Hum Mol Genet *14*, 747-764.

Peng, G.H., and Chen, S. (2007). Crx activates opsin transcription by recruiting HAT-containing co-activators and promoting histone acetylation. Hum Mol Genet *16*, 2433-2452.

Wang, X., Xu, S., Rivolta, C., Li, L.Y., Peng, G.H., Swain, P.K., Sung, C.H., Swaroop, A., Berson, E.L., Dryja, T.P., and Chen, S. (2002). Barrier to autointegration factor interacts with the cone-rod homeobox and represses its transactivation function. J Biol Chem *277*, 43288-43300.

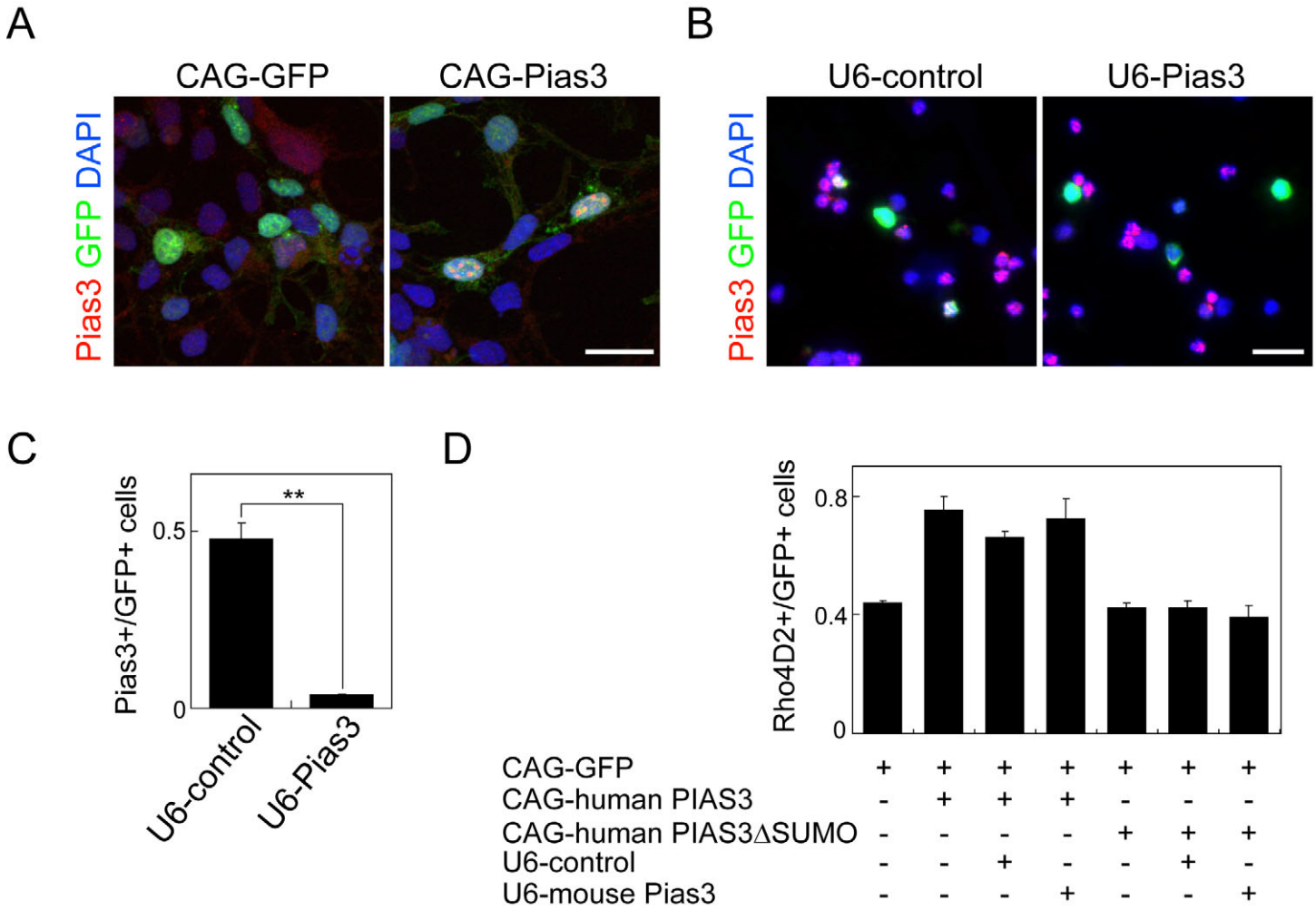
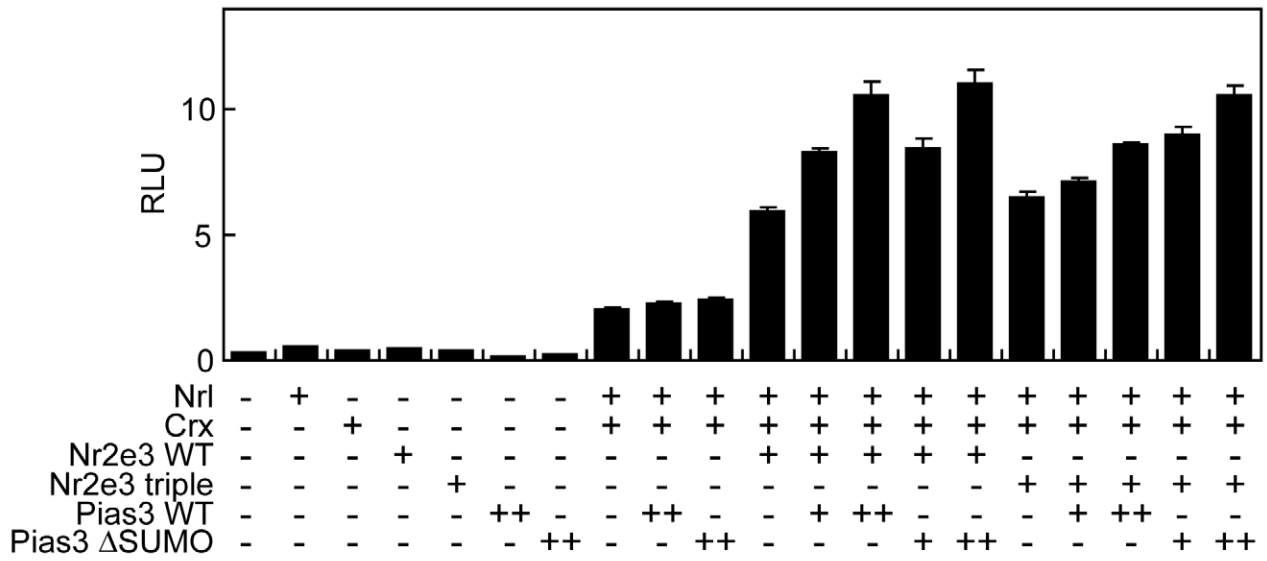


Figure S1 Onishi et al.

A. Rhodopsin -2174bp



B. 4x optimal Nr2e3-binding site

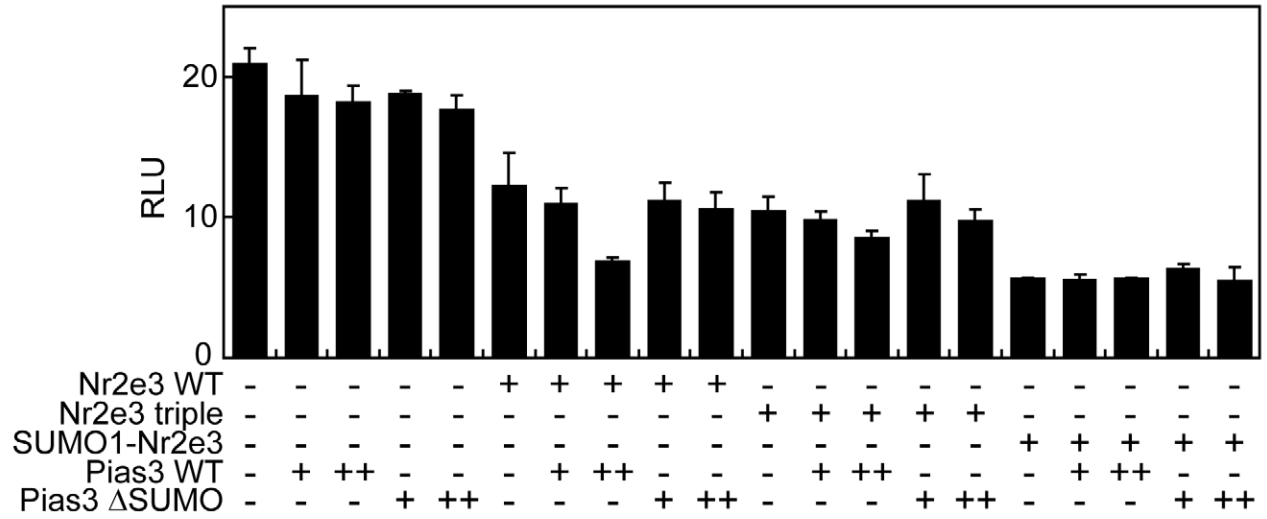
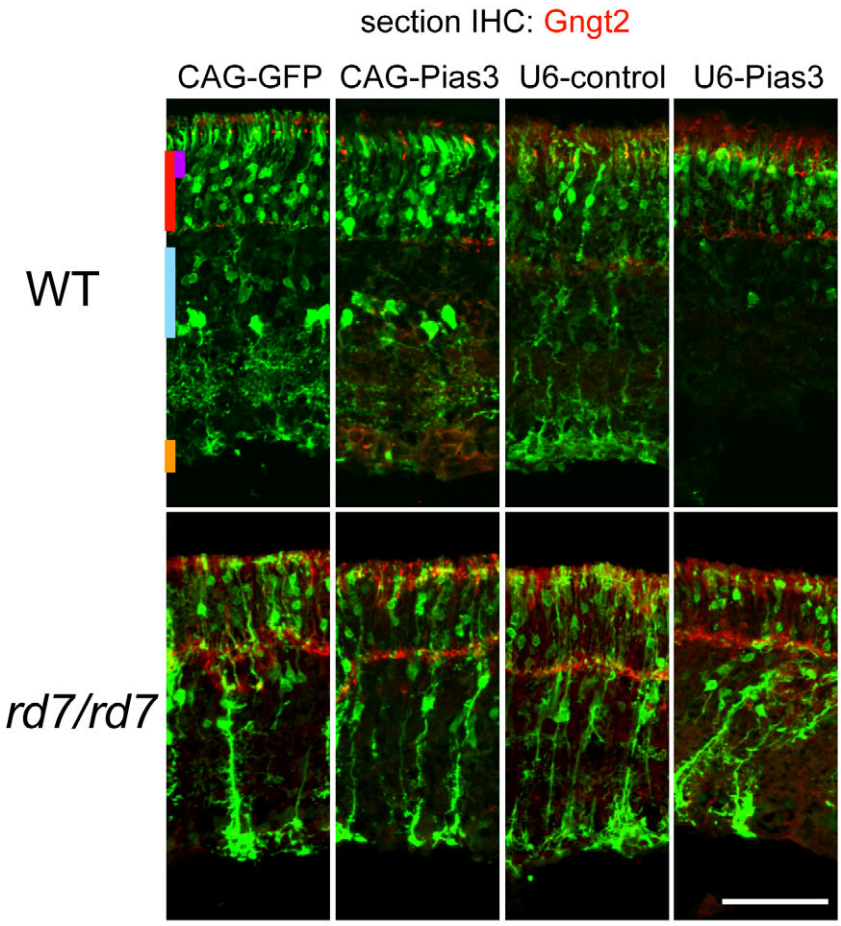


Figure S2 Onishi et al.

A



B

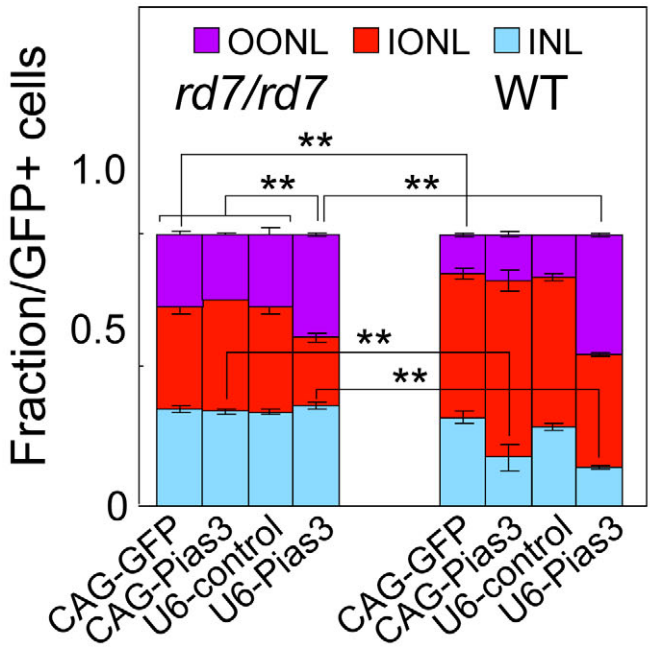
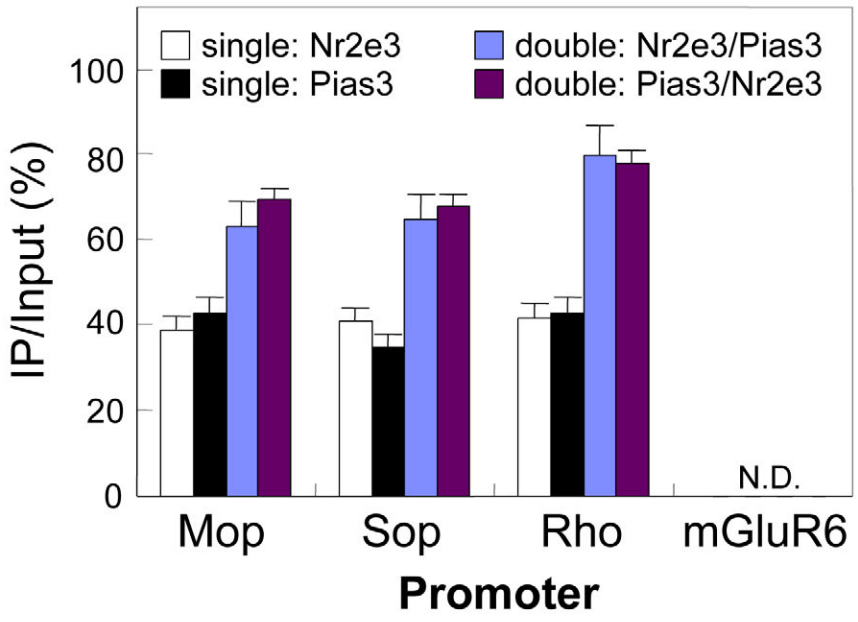


Figure S3 Onishi et al.

A



B

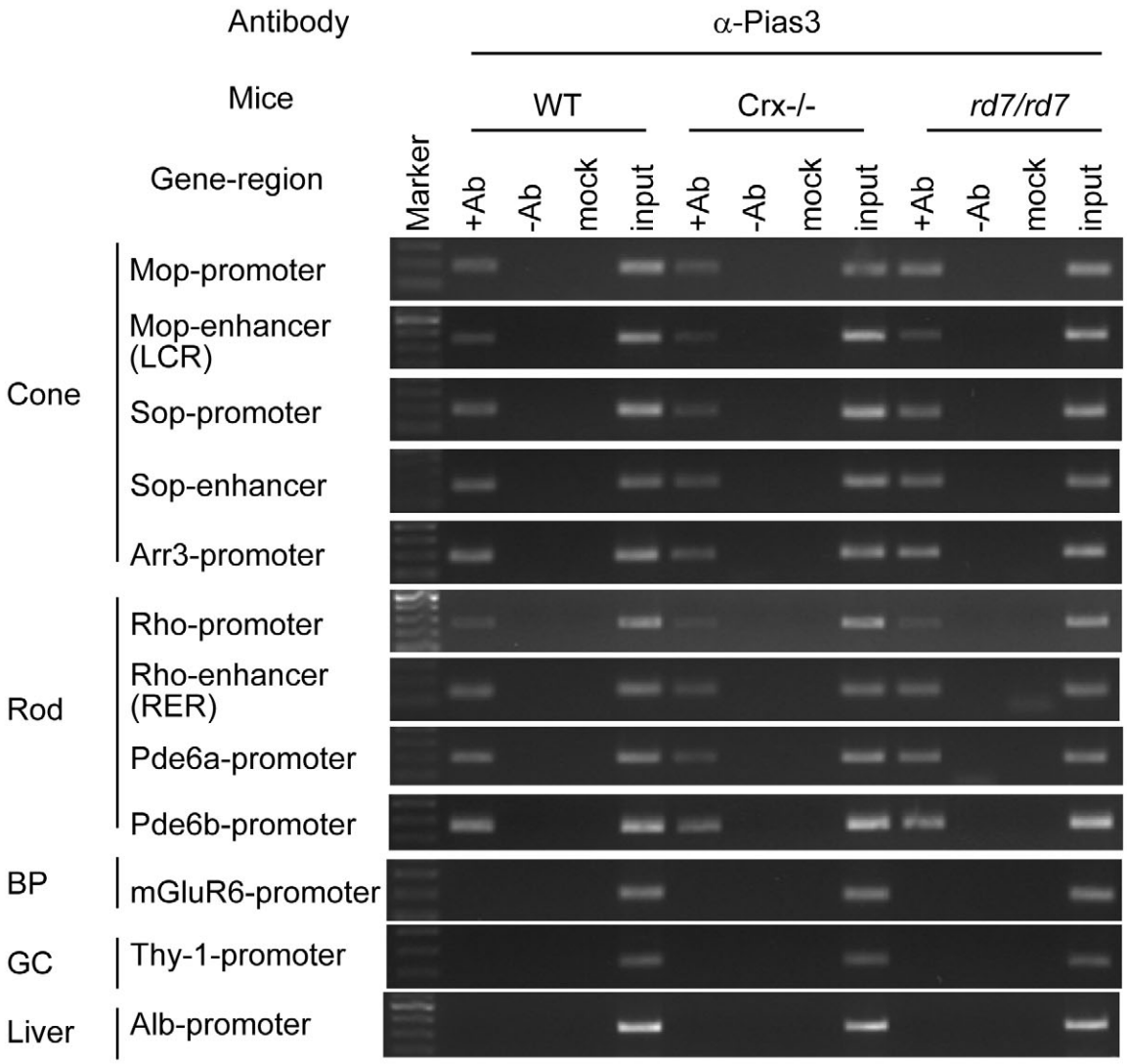


Figure S4 Onishi et al.

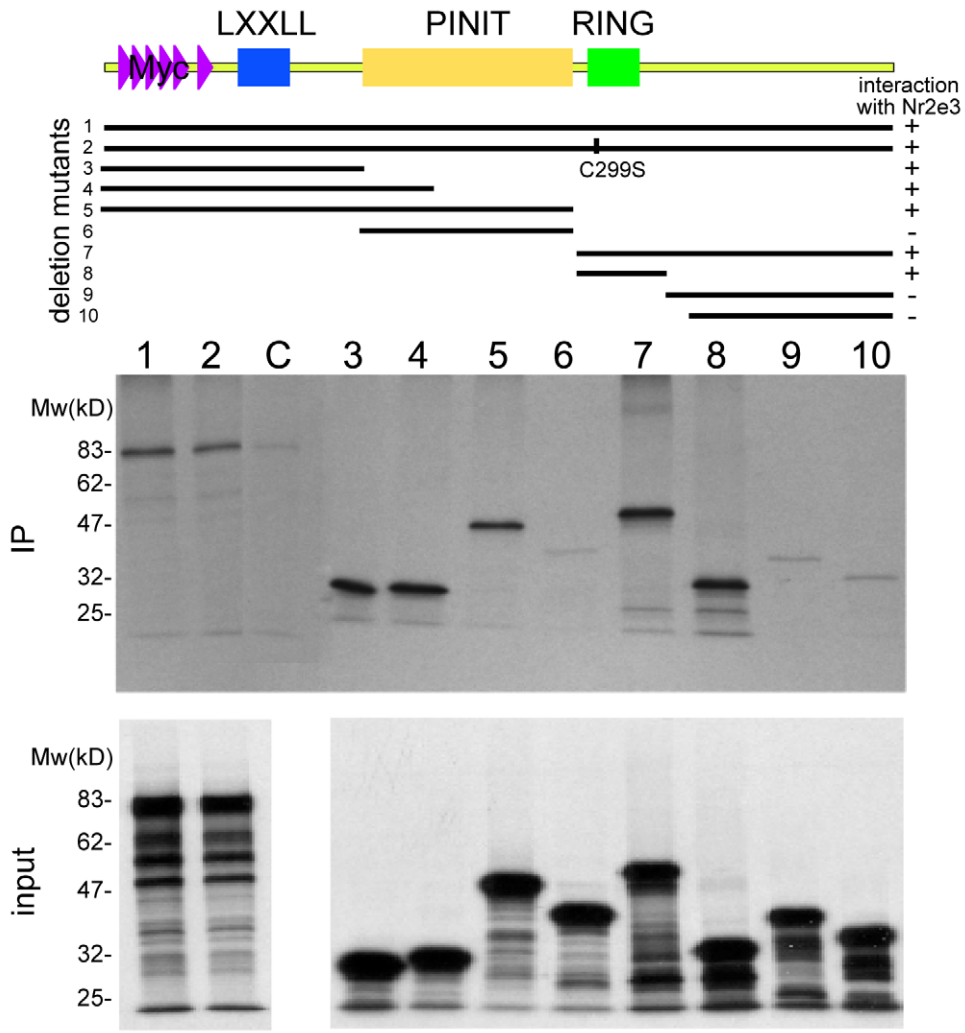
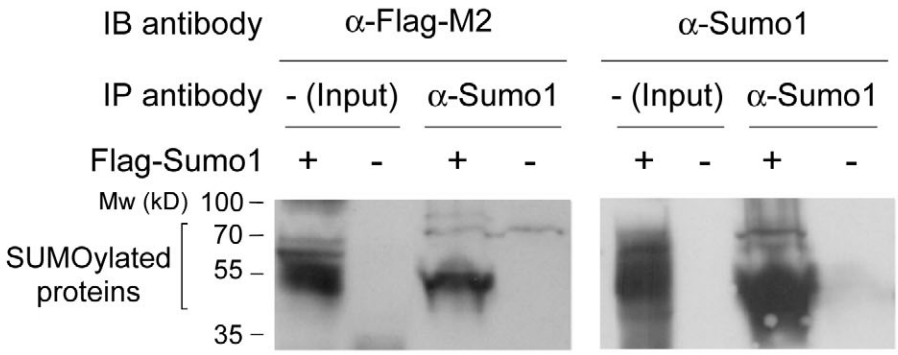


Figure S5 Onishi et al.

A



B

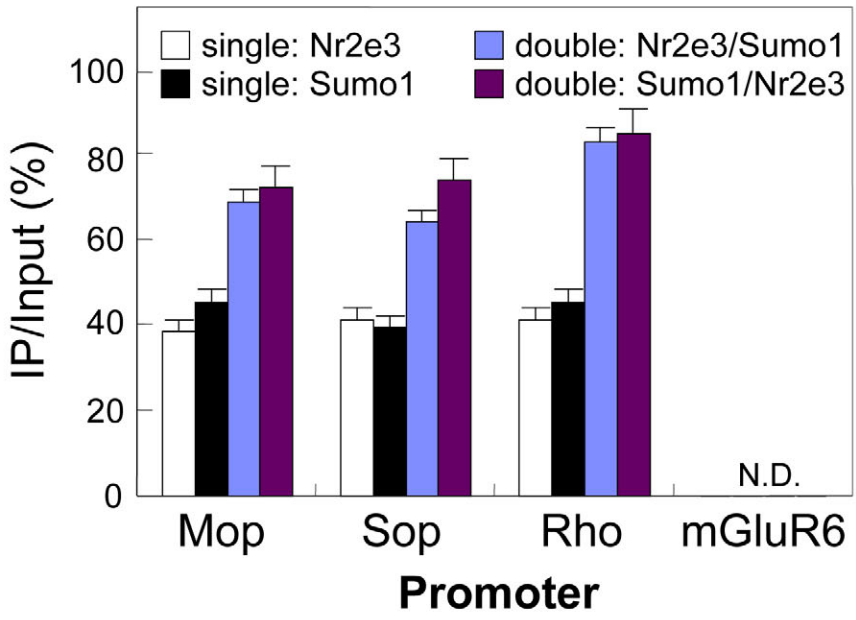


Figure S6 Onishi et al.