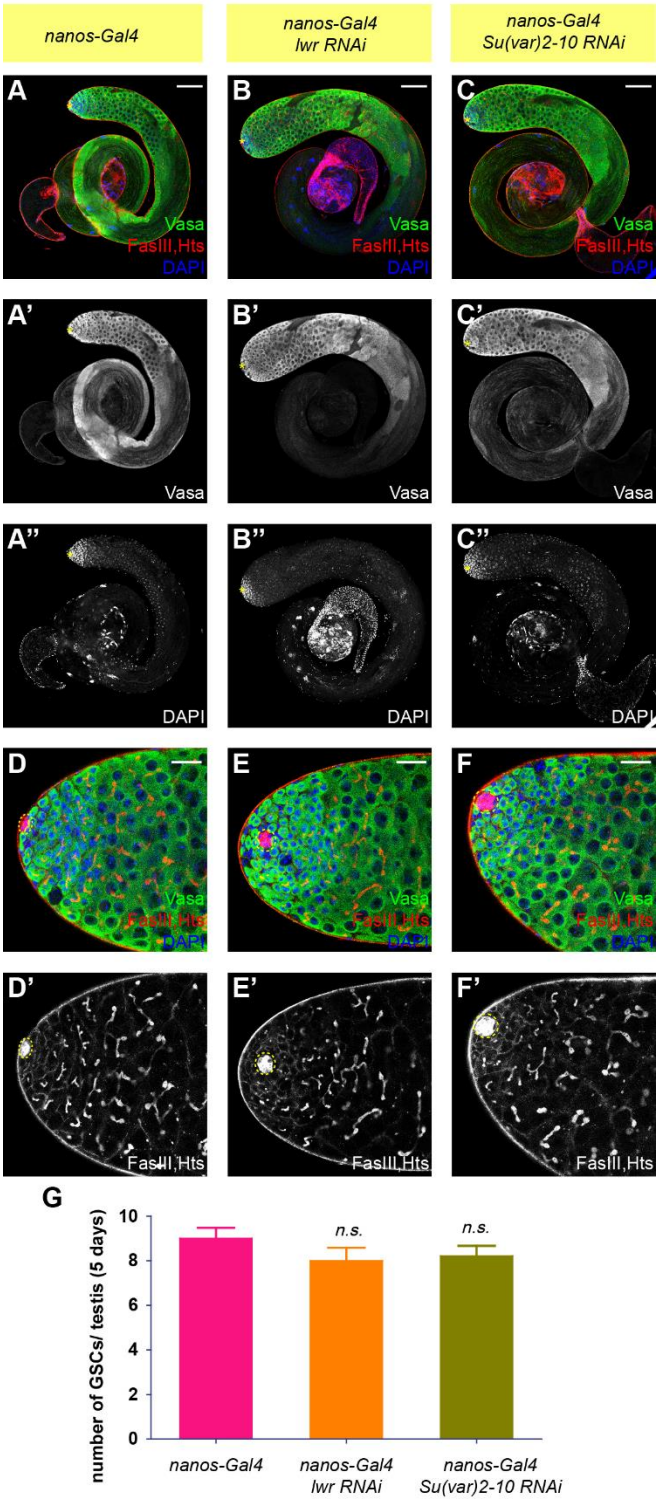

Fig. S1. SUMO pathway is not autonomously required for GSCs maintenance

(A-C'') Whole testes of indicated genotypes showing Vasa (green), FasIII with Hts (red), and DAPI (blue) staining. Asterisks indicate the hub. Compared with control (A-A''), germline cells (Vasa positive) appear

normal in *lwr RNAi* (B-B'') and *Su(var)2–10 RNAi* testes (C-C''), after RNAi induction by *nanos*-Gal4 for 5 days. Scale bars, 100 μm.

(D-F') Enlarged testis apex of indicated genotypes showing Vasa (green), FasIII with Hts (red), and DAPI (blue) staining. Yellow circles indicate the hub. In *lwr RNAi* (E and E') and *Su(var)2–10 RNAi* testes (F and F'), Hts staining appears dots in GSCs and gonialblasts, while becomes branched in spermatogonial cells, which is indistinguishable from control (D and D'), after induction RNAi by *nanos*-Gal4 for 5 days. Scale bars, 25 μm.

(G) The numbers of GSCs in testes, after RNAi induction for 5 days. Data are presented as individual values and mean ± s.e.m. n.s., not significant, n > 10.

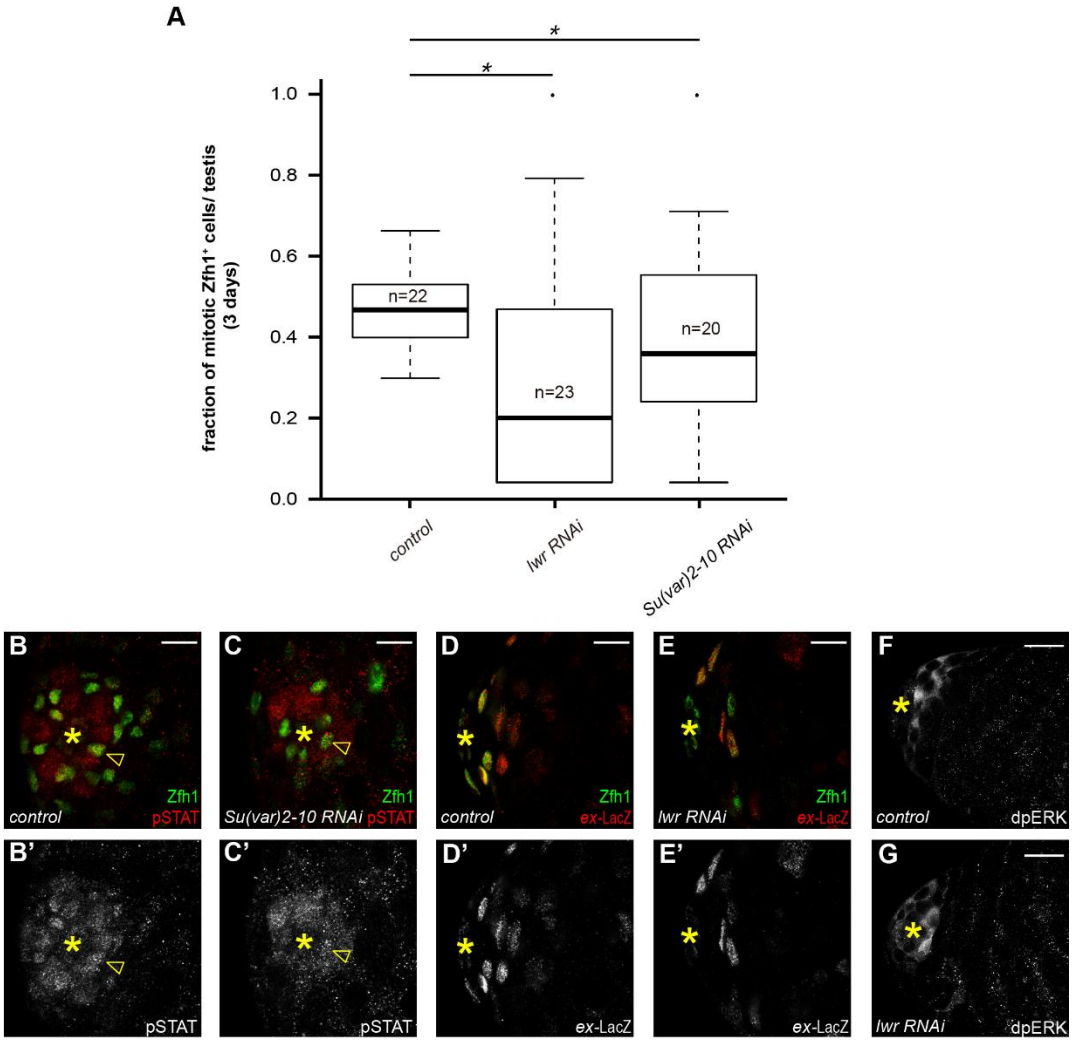

Fig. S2. Influence of SUMO pathway on different CySC self-renewal factors

(A) The fraction of mitotic Zfh1$^+$ cells (ratio of EdU$^+$Zfh1$^+$ cells to all Zfh1$^+$ cells) in testes, after RNAi induction for 3 days. Data are presented as individual values and mean ± s.e.m. *, $P < 0.05$.

(B-C') Representative testes showing Zfh1 (green) and pSTAT (red) staining, after RNAi induction for 3 days. Asterisks indicate the hub. Arrowheads indicate certain CySCs. Scale bars, 10 μm.

(D-E') Representative testes showing Zfh1 (green) and *ex*-LacZ (red, detected by anti-LacZ (β-galactosidase) antibody) staining, after RNAi induction for 3 days. Asterisks indicate the hub. Scale bars, 10 μm.

(F and G) Representative testes showing dpERK staining, after RNAi induction for 3 days. Asterisks indicate the hub. Scale bars, 25 μm.

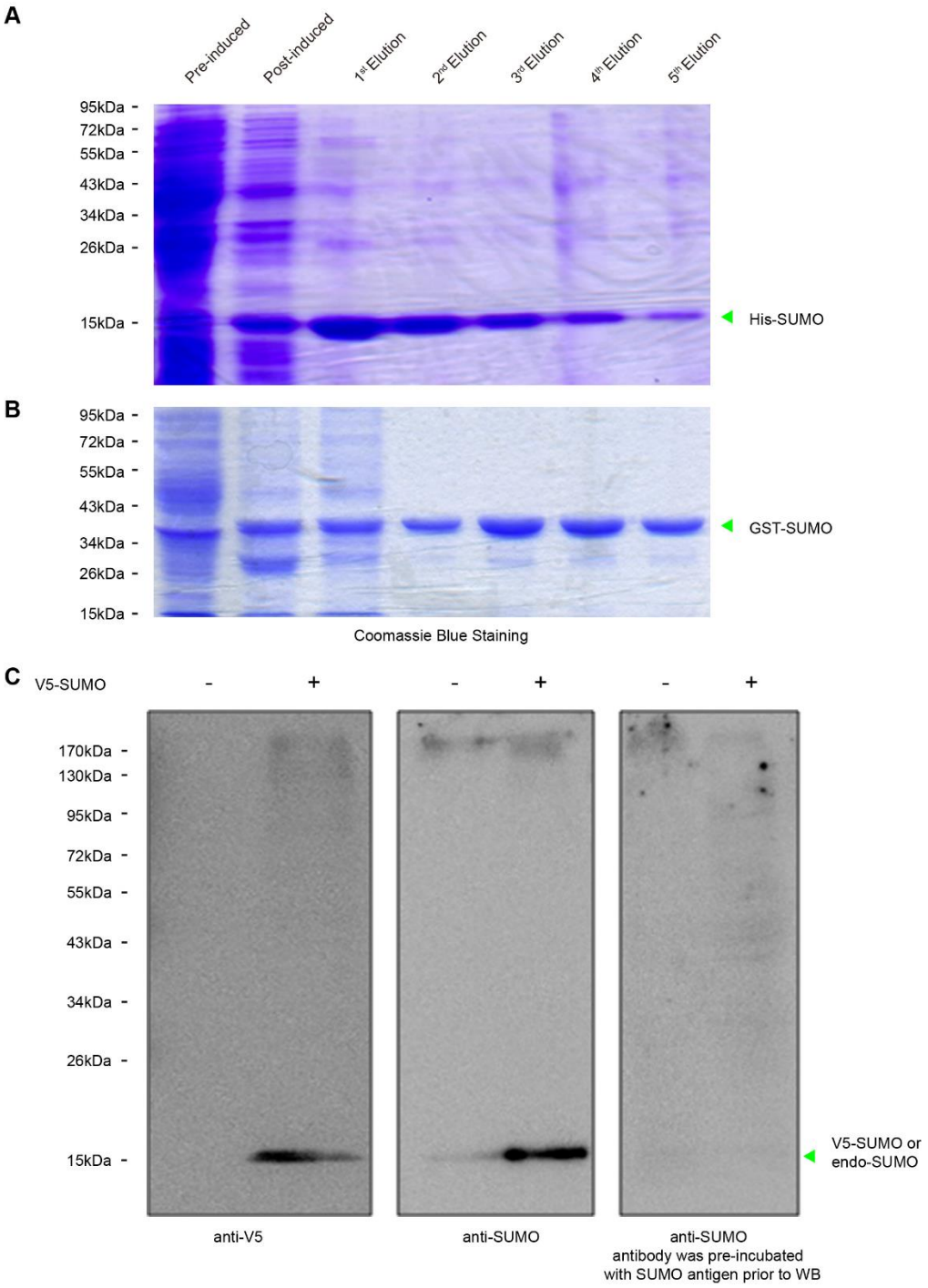

Fig. S3. Generation and characterization of the anti-SUMO antibody

(A) Coomassie Brilliant Blue staining showing the His-tagged SUMO proteins (A, green arrowhead), purified for immunizing rabbits to generate anti-SUMO antibody.

(B) Coomassie Brilliant Blue staining showing the GST-tagged SUMO proteins (B, green arrowhead), purified for purification of the specific anti-SUMO antibody generated in rabbits.

(C) The western blot assay showing that the specificity of the anti-SUMO antibody generated in this study. Control vectors or plasmids containing V5-SUMO ORF were transfected into S2 cells. Cells were collected and lysed in SDS loading buffer for WB 48hrs later. Anti-V5 antibody can only recognize the exogenous V5-SUMO (left panel), while the anti-SUMO antibody can recognize both the endogenous and exogenous SUMO proteins (middle panel). However, if the anti-SUMO antibody was incubated with purified SUMO proteins prior to the immunoblot assay, no obvious bands were detected (right panel), suggesting the anti-SUMO antibody generated in this study specifically recognizes SUMO proteins.

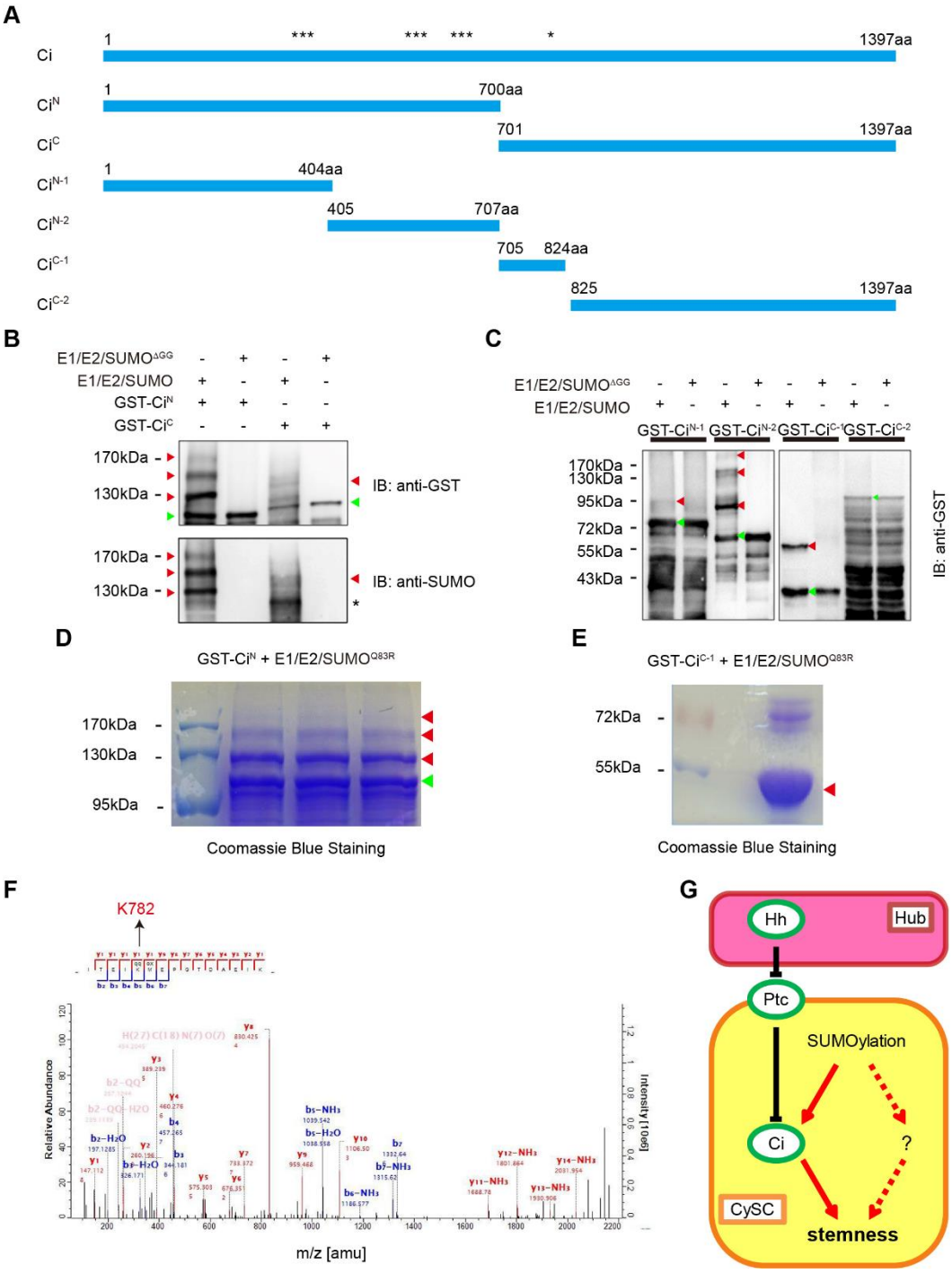

Fig. S4. Identification of the SUMO acceptor sites in Ci

(A) A diagram showing different fragments of Ci used for mapping the SUMO sites (B -E). Asterisks indicate ten potential SUMOylation sites (K351, K360, K364, K535, K545, K561, K611, K614, K662, and K782) identified in the LC-MS/MS.

(B) The western blot assay showing SUMOylation status of different Ci fragments. SUMOylation assay was carried out as described in methods. Red arrowheads point to the SUMOylated Ci fragments recognized by both anti-GST and anti-SUMO antibodies while the green arrowheads point to the un-SUMOylated Ci fragments only recognized by the anti-GST antibody. Asterisk indicates nonspecific band. $SUMO^{\Delta GG}$ is a conjugation-defective form of SUMO lacking the C-terminal di-glycine motif.

(C) The SUMOylation assay was carried out as described in methods. The red arrowheads point to the extra SUMOylated Ci fragments detected only in lanes 1, 3 and 5, compared with control (lanes 2, 4, 6 and 8), while the green arrowheads point to the un-SUMOylated Ci fragments detected in all lanes.

(D and E) Coomassie Brilliant Blue staining showing the SUMOylation products of Ci^{N} (D) or $Ci^{C\text{-}1}$ (E) used for LC-MS/MS. The red arrowheads point to the SUMOylated Ci fragments while the green arrowheads point to the un-SUMOylated Ci fragments.

(F) LC-MS/MS result showing one representative SUMO acceptor site identified in this assay.

(G) A model for the function of SUMOylation in CySCs. SUMOylation functions cell-autonomously to promote the proliferation and inhibit the differentiation of CySCs. Such regulatory role of SUMOylation is partially mediated by the Hh pathway via site specific SUMOylation of Ci. Without proper SUMOylation, Ci can't promote CySCs proliferation. Besides, unknown factor(s) might work in parallel with Ci to mediate the function of SUMOylation in maintaining CySCs stemness. See text for details.

Supplementary Materials and Methods

Fly stocks

Flies were raised on standard yeast/molasses medium at 25°C unless otherwise stated. *Su(var)2–10* RNAi (VDRC, #30709), *lwr* RNAi (VDRC, #33685), *ptc* RNAi (NIG, #2411R-1), *tubGal80ts* (II) (Bloomington, #7019), *tubGal80ts* (III) (Bloomington, #7017), *FRT40 Lwr*$^{4-3}$ (Bloomington, #9321) were obtained from VDRC, NIG or Bloomington. *c587*-Gal4 (X) and *nanos*-Gal4 (III) were gifts from Drs. Dahua Chen and Xun Huang. The *attp-Myc-Ci/Ci*^{10KR}*/Ci*^{K782R}*/SUMO-Ci*^{K782R} transgenic flies were generated with a P element-mediated insertion at the 75B site of the third chromosome in this study. All the forms of Ci were inserted in 75B site of the third chromosome so that both the genetic backgrounds and the expression levels of these transgenes should be the same.

Fly stocks used for the MARCM assays are listed below.

yw hsflp/Y; FRT40/FRT40 tubGal80; tubGal4 uas-GFP,

yw hsflp/Y; FRT40/FRT40 tubGal80; uas-ptc RNAi/tubGal4 uas-GFP,

yw hsflp/Y; FRT40 lwr$^{4-3}$*/FRT40 tubGal80; uas-ptc RNAi/tubGal4 uas-GFP.*

Immunostaining of testes

Testes of adult male flies were dissected and fixed in freshly made 4% formaldehyde in PBS buffer at room temperature for 30min, then rinsed with buffer PBT (PBS, 0.1% Triton X-100) and washed four times with buffer PBTA (PBS, 0.1% Triton X-100, 1%

BSA). Testes were incubated with primary antibody diluted in PBTA for overnight at 4ºC, then washed with PBT and incubated with secondary antibody diluted in PBTA for 2hrs at room temperature. After wash, testes were mounted in 40% glycerol. Leica LAS SP8 confocal microscope was employed to take immunostaining images.

Quantification of GSCs

Serial confocal reconstructions of the entire testis apex were used for quantification (Issigonis et al., 2009). GSCs were identified by Vasa positive cells attaching the hub and with round fusomes. Data were presented as Mean ± SEM, n>10. P-values were obtained by student's t-test between two groups.

EdU incorporation assay

Testes of adult male flies were dissected in S2 medium, and incubated in 100μg/ml EdU (Invitrogen, Click-iT® EdU Alexa Fluor® 647 Imaging Kit) in S2 medium for 30min at 25°C. Samples were then rinsed with PBS. For staining with other primary antibodies, subsequent immunostaining procedures were as described above. Before mounting, EdU was labelled following Click-iT® EdU Imaging Kits Protocol.

DNA constructs and transgenes

To construct *pET28a-His-Lwr*, full length *Lwr* cDNA was made from *Drosophila Melanogaster* embryonic RNA using RT-PCR and then inserted into the *pET28a* vector.

To construct *pGEX-4T-1-GST-Ci^{N}/Ci^{C}/Ci^{N-1}/Ci^{N-2}/Ci^{C-1}/Ci^{C-2}*, different fragments of Ci were got by PCR with *pGEX-4T-1-Ci* as the template (Zhang et al., 2013), and then inserted into the *pGEX-4T-1* vector. PCR-based site-directed mutagenesis was used to mutate the nine lysines (K351, K360, K364, K535, K545, K561, K611, K614 and K662) in Ci^{N} and the K782 in Ci^{C} to generate the *pGEX-4T-1-GST-Ci$^{N\text{-}9KR}$/Ci$^{C\text{-}K782R}$*. The single vector (Q^{SUMO} and Q$^{\Delta GG}$) encoding all four polypeptides required for SUMOylation, i.e., SUMO^{WT} or SUMO$^{\Delta GG}$, Lwr, SAE2 and SAE1, which can reduce variation in expression levels in *E.coli* were kind gifts from Dr. Albert J. Courey (Nie et al., 2009). To shorten the SUMO branched peptide generated after tryptic digestion for LC-MS/MS, Q^{Q83R} was generated by mutating the Q83 of *Drosophila* SUMO to R as reported previously (Matic et al., 2010). PCR-based site-directed mutagenesis was used to mutate the ten K or the K782 mentioned above to R to generate the *pUAST-attB-Myc-Ci^{10KR}/Ci^{K782R}*. SUMO was fused with *Myc-Ci^{K782R}* to generate *pUAST-attB-SUMO- Myc-Ci^{K782R}*.

The bacterial SUMOylation assay

As reported by Dr. Courey's lab (Nie et al., 2009), the vector encoding specific GST tagged Ci fragment (WT or mutant, ampicillin resistance) was co-transformed into *Escherichia coli* (*E.coli*) BL21 cells with Q^{SUMO}, Q$^{\Delta GG}$ or Q^{Q83R} vector (kanamycin resistance). Transformed colony with double resistance was picked for IPTG induction at 37ºC for 4hrs. GST fusion proteins were purified with glutathione agarose beads (GE) for western blot analysis.

GST fusion protein pull-down assay and western blot analysis

As reported previously (Zhang et al., 2013), GST and His fusion proteins were produced in *E. coli BL21* and purified with glutathione agarose beads (GE Healthcare) or Ni^{2+} NTA column (QIAGEN) respectively. GST fusion protein-loaded beads were incubated with purified His-Lwr in GST pull-down lysis buffer (20 mM Tris-Cl, pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.5% NP-40, and PMSF) at 4ºC for 1hr. The beads were washed three times with lysis buffer, and subjected for western blot analysis. Western blot assay was performed according to standard protocols as previously reported (Shi et al., 2013).

Generation of anti-SUMO antibody

His-tagged SUMO proteins was purified from *E.coli* BL21 strain. Rabbits were immunizd with purified His-tagged SUMO proteins for four times, with the interval of 10, 20, 20 days. Serum was collected two months later, and anti-SUMO antibody was purified with GST-tagged SUMO proteins with standard protocol (Dodson et al., 2007). Validation of the antibody was carried out as detailed in supplementary Fig. S3C.

Supplementary References

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