SUMO regulates somatic cyst stem cell maintenance and directly targets the Hedgehog pathway in adult Drosophila testis

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ABSTRACT
SUMO (Small ubiquitin-related modifier) modification (SUMOylation) is a highly dynamic post-translational modification (PTM) that plays important roles in tissue development and disease progression. However, its function in adult stem cell maintenance is largely unknown. Here, we report the function of SUMOylation in somatic cyst stem cell (CySC) self-renewal in adult Drosophila testis. The SUMO pathway cell-autonomously regulates CySC maintenance. Reduction of SUMOylation promotes premature differentiation of CySCs and impedes the proliferation of CySCs, which leads to a reduction in the number of CySCs. Consistent with this, CySC clones carrying a mutation of the SUMO-conjugating enzyme are rapidly lost. Furthermore, inhibition of the SUMO pathway phenocopies disruption of the Hedgehog (Hh) pathway, and can block the proliferation of CySCs induced by Hh activation. Importantly, the SUMO pathway directly regulates the SUMOylation of Hh pathway transcription factor Cubitus interruptus (Ci), which is required for promoting CySC proliferation. Thus, we conclude that SUMO directly targets the Hh pathway and regulates CySC maintenance in adult Drosophila testis.

KEY WORDS: SUMOylation, Hedgehog, Adult stem cell, Testis, Drosophila

INTRODUCTION
PTMs regulate diverse cellular processes, including transcription, replication, and DNA repair (Flatto and Melchior, 2013). SUMOylation, first identified in the 1990s (Mahajan et al., 1997; Matunis et al., 1996), is a ubiquitin-like PTM (Hannoun et al., 2010; Smith et al., 2012). Through an enzymatic cascade involving E1-activating enzyme, E2-conjugating enzyme and E3 ligase, SUMO is finally attached to the substrate acceptor lysine (Lys, K) residue in vivo (Gareau and Lima, 2010; Hickey et al., 2012). Disruption of the SUMO pathway during embryogenesis is lethal in many species from Drosophila to mouse (Fig. 1A). Several signaling pathways have been reported to regulate CySCs, including the JAK-STAT pathway (Issigonis et al., 2009; Kiger et al., 2001; Leatherman and DiNardo, 2010; Singh et al., 2010; Tulina and Matunis, 2001), the EGFR pathway (Eun et al., 2014; Kiger et al., 2000; Tran et al., 2000), the Hpo pathway (Amoyel et al., 2014) and the Hh pathway (Amoyel et al., 2013; Michel et al., 2012; Zhang et al., 2013b). Although these pathways govern stem cell behavior, the function of PTMs during this process is rarely reported.

RESULTS AND DISCUSSION
The SUMO pathway is autonomously required for CySC maintenance
In order to identify novel factors that are autonomously required for CySC maintenance, we conducted a genetic screen for PTM regulators because PTMs regulate diverse cellular processes and might play important roles in CySCs. Genes were knocked down specifically in adult somatic cyst cells, by RNAi using c587-Gal4 and a temperature-sensitive allele of Gal80 (Gal80ts, a Gal4 inhibitor). Adult flies (0 to 3 days old) were shifted from 18°C to 29°C for 5 days to inactivate Gal80 and permit Gal4 to drive dsRNA expression. A zinc finger homeodomain 1 (Zfh1) antibody was used to mark CySCs and their direct daughters (Leatherman and DiNardo, 2008). As shown in Fig. 1AC, cells with strong Zfh1 staining adjacent to the hub are CySCs, and those with lower Zfh1 staining level around the hub are newly formed cyst cells in wild-type (WT) testes. Interestingly, knocking down Su(var)2-10 (Suppressor of variegation 2-10), a SUMO E3 ligase, significantly decreased the number of Zfh1+ cells (Fig. 1C, C′, E, E′) as early as 3 days after RNAi induction (Fig. 1B). To ascertain the function of the SUMO pathway, we knocked down hvr (lesswright, the only known SUMO E2 in Drosophila) in the same system, and observed a similar phenotype (Fig. 1B-D′).

To confirm the phenotypes from the knockdown assays, we utilized the mosaic analysis with a repressible cell marker (MARCM) system (Lee and Luo, 2001) to generate clones for the hypomorphic allele hvr±-3 (Apionishev et al., 2001). At 3 days after clone induction (ACI), GFP+ clones with strong Zfh1 staining (termed CySC clones) could be recovered in both control and hvr±-3 testes (Fig. 1G-H′). At 5 days ACI, CySC clones could still be recovered in control testes (Fig. 1I-I′), whereas few hvr±-3 testes contained CySC clones (Fig. 1J-J′). We then counted the percentage of testes with at least one CySC clone at 3, 5 or 7 days ACI to evaluate the stemness of clonal CySCs. Such fractions were comparable between control and hvr±-3 mutants at 3 days ACI (Fig. 1F), suggesting comparable abilities to generate CySC clones.
At 7 days, few testes contained detectable lwr mutant CySC clones, whereas CySC clones still existed in the control testes (Fig. 1F). Combining the results from RNAi assays with those from the MARCM assays, we conclude that SUMO pathway is autonomously required for maintaining the stemness of CySCs.

We also investigated the primary function of SUMOylation in GSCs maintenance. However, no obvious abnormality was observed even after Su(var)2-10 or lwr was specifically knocked down in adult germline cells for 5 days (Fig. S1). Thus, we focused our further investigation on CySCs.
Reduction of SUMOylation induces differentiation and impedes proliferation of CySCs

Because CySCs were poorly maintained when the SUMO pathway was disrupted, we asked whether these phenotypes were caused by premature differentiation or impeded proliferation of CySCs. Eyes absent (Eya) was used to mark differentiated cyst cells, and it is normally undetectable in CySCs or their immediate daughter cyst cells in WT testis (Fabrizio et al., 2003) (Fig. 2A). Yet we found several Eya+ cells locate very close, even adjacent to, the hub in hwr or Su(var)2-10 RNAi tests (Fig. 2B,C), suggesting that those cells around the hub initiated differentiation. We then measured the distance between the hub and the Eya+ cells, and found that when hwr was knocked down the percentage of testes with shorter distance was increased (Fig. 2D) and the distance between the hub and the

Fig. 2. The SUMO pathway inhibits CySC differentiation and promotes CySC proliferation. (A–C) Representative testes showing the distances (yellow brackets) between the closest differentiated cyst cell (Eya, yellow arrowhead) and the hub (FasIII, red arrowhead) after RNAi induction for 3 days. Scale bars: 25 μm. (D) The percentage of testes showing indicated distance between the hub and the closest Eya+ cell. (E) The distance between the hub and the three closest Eya+ cells. Data are presented as individual values and mean±s.e.m. *P<0.05. (F–H″) Representative testes showing Zfh1 (green) and EdU (red) staining, after RNAi induction for 3 days. Asterisks indicate the hub. Scale bars: 10 μm. (I) The numbers of mitotic Zfh1+ cells (EdU+Zfh1+ cells). Data are presented as individual values and mean±s.e.m. ***P<0.001.
three nearest Eya’ cells became shorter (Fig. 2E). These results indicate that the SUMO pathway is required for inhibition of CySC differentiation.

Next, we conducted 5-ethyl-2′-deoxyuridine (EdU) incorporation and it showed that dividing CySCs were detected with lower frequency in lwr RNAi and Su(var)2-10 RNAi tested than in controls (Fig. 2F-I; Fig. S2A), implying that the proliferation capacity of CySCs was also reduced.

The SUMO pathway genetically interacts with the Hh pathway

The JAK-STAT pathway, the EGFR pathway, the Hpo pathway and the Hh pathway are the major players currently identified in regulating CySCs (Amoyel et al., 2014; Kiger et al., 2000, 2001; Michel et al., 2012; Tran et al., 2000; Tulina and Matunis, 2001). Are the phenotypes induced by SUMO pathway suppression mediated by one of them? We monitored the activities of these pathways in CySCs upon lwr or Su(var)2-10 suppression driven by c587-Gal4. Patched (Ptc) expression has been used as readout of the Hh pathway (Amoyel et al., 2013; Michel et al., 2012). We found that the level of Ptc was reduced in lwr RNAi tested compared with control (Fig. 3A-B′), implying that the SUMO pathway positively regulates Hh signaling activity in CySCs, although we cannot rule out the possibility that the change of Ptc staining might simply reflect a change in the number of CySCs, rather than a direct modification of Hh pathway activity.

The JAK-STAT, EGFR and Hpo signaling activities in CySCs of lwr or Su(var)2-10 RNAi tested were indistinguishable from those in control tested as revealed by the intensity of staining for phosphorylated STAT (pSTAT; Stat92E – FlyBase) (Zhang et al., 2013a), the dual phosphorylated form of MAP kinase (dpERK; also known as Rolled) (Gabay et al., 1997; Kiger et al., 2000) and expanded-lacZ (ex-lacZ) (Amoyel et al., 2014), general reporters of these three pathways, respectively (Fig. S2B-G). Using a flip-out technique, we generated lwr RNAi clones and found that these readouts were also indistinguishable from nearby control cells (Fig. 3C-E′′′), suggesting that the decreased number of Zfh1+ cells in 3-day-old lwr/Su(var)2-10 RNAi tested is unlikely to be mediated by blockage of the JAK-STAT, EGFR or Hpo pathways although we cannot entirely rule out this possibility.

To test further whether the Hh pathway mediates the function of the SUMO pathway on CySC maintenance, we performed genetic epistasis assays. Knockdown of ptc (an inhibitor of the Hh pathway; Beachy et al., 2004; Huang et al., 2013; Jiang and Hui, 2008; Jiang et al., 2016) increased the number of Zfh1+ cells (Fig. 3F,G,I). Importantly, this increase could be blocked by knockdown of lwr (Fig. 3F-I). To confirm this genetic relationship, we used MARCM assays to monitor three different aspects of stemness of CySCs: the clone size (number of clonal cells; Fig. 3J), the ratio of EdU incorporation (Fig. 3K), and the capacity for maintaining stemness at the niche (Fig. 3L). These data together showed that the effect of ptc RNAi on CySCs could be disrupted by muting lwr. Taken together, we conclude that the SUMO pathway is indispensable for CySC maintenance promoted by Hh signaling.

The SUMO pathway directly modifies Ci

Based on the genetic interaction assays, we speculated that the SUMO pathway might directly modify some key component(s) of the Hh pathway to regulate CySC maintenance. Considering that SUMO substrates are highly enriched for transcription factors (Hendriks et al., 2014), a likely candidate is Ci, a transcription factor of the Hh pathway (Hui and Angers, 2011; Li et al., 2014; Motzny and Holmgren, 1995). Ci can interact directly with Lwr (Fig. 4A), implying a potential for Ci to be SUMOylated. To test whether Ci could be SUMOylated, and to map the potential SUMOylation sites in Ci, we truncated full-length Ci into two fragments, named as CiN and CiC (Fig. S4A). We then generated an anti-SUMO antibody (Fig. S3) and performed a bacterial SUMOylation assay (Mencia and de Lorenzo, 2004; Nie et al., 2009; Uchimura et al., 2004). We did detect SUMOylated forms of both CiN and CiC (Fig. S4B).

To map further the SUMOylation sites in Ci, we truncated CiN and CiC into two smaller fragments: CiN-K782, CiN-K782R, and CiC-K782, CiC-K782R (Fig. S4A). CiN-K782 and CiC-K782 could be SUMOylated, whereas CiN-K782R could not (Fig. S4C), indicating multiple SUMOylation sites in Ci. To map the precise SUMOylation sites, we prepared SUMOylated CiN and CiC for liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Fig. S4D,E). LC-MS/MS recovered nine potential SUMOylation sites in CiN, and one potential SUMOylation site in CiC (Fig. S4A, asterisk). One representative LC-MS/MS map is shown in Fig. S4F.

We then mutated all nine K residues in CiN to generate CiN-K9R, and mutated K782 in CiN to generate CiN-K782R. A subsequent SUMOylation assay showed that SUMOylation levels of CiN-K9R and CiC-K782R were significantly reduced compared with CiN and CiC, respectively (Fig. 4B,C), indicating that these sites are real SUMOylation sites.

SUMO conjugation promotes Ci activity in terms of CySC proliferation

To investigate the role of Ci SUMOylation in CySCs, we generated transgenic flies for WT or different mutants of Ci. Mutating all ten K residues mentioned above to arginine (Arg; R) generated Ci10KR. Overexpression of WT Ci, but not Ci10KR, increased the number of Zfh1+ cells (Fig. 4D), implying that SUMO modification is indispensable for Ci activity in promoting CySC self-renewal. We then observed a crucial function of K782 as CiK782R did not increase the number of Zfh1+ cells (Fig. 4E-H′′′). Importantly, artificial fusion of SUMO protein to CiK782R can restore the function of CiK782R in promoting CySC self-renewal similar to wild-type Ci (Fig. 4E-I′). Taken together, we conclude that accurate SUMOylation of Ci is essential for CySC proliferation in adult Drosophila testes.

Considering that the conserved Hh signaling pathway (Briscoe and Thérond, 2013) has been reported to regulate the testis stem cell in mammals (Bitgood et al., 1996; Makela et al., 2011; Petrova and Joyner, 2014; Yao et al., 2002) and that the biological functions of the SUMO pathway in embryogenesis are highly conserved from Drosophila to mammals (Flotho and Melchior, 2013), it is intriguing to test whether the SUMO pathway is also required for mammalian adult testis by targeting the Hh signaling pathway. Actually, Gli proteins, the homologs of Ci in mammals, can be SUMOylated, as reported by two groups (Cox et al., 2010; Han and Thérond, 2013) has been reported to regulate the testis stem cell in mammals (Bitgood et al., 1996; Makela et al., 2011; Petrova and Joyner, 2014; Yao et al., 2002) and that the biological functions of the SUMO pathway in embryogenesis are highly conserved from Drosophila to mammals (Flotho and Melchior, 2013), it is intriguing to test whether the SUMO pathway is also required for mammalian adult testis by targeting the Hh signaling pathway. Actually, Gli proteins, the homologs of Ci in mammals, can be SUMOylated, as reported by two groups (Cox et al., 2010; Han et al., 2012), further supporting a conserved modification of the conserved transcription factors. However, their conclusions about the function of SUMOylation on Hh signaling activity are controversial. It is possible that different Gli proteins might be differently regulated by SUMOylation. Based on our study in adult CySCs, we favor the hypothesis that Ci SUMOylation promotes Hh signaling activity in maintaining CySCs, which might function in a context-dependent manner.

In this study, we identified the involvement of a SUMO E3 ligase in CySC maintenance through a genetic screen. Combining a targeted gene knockdown approach with a MARCM system generating a homozygous mutant allele, we demonstrated that SUMOylation inhibits differentiation as well as promotes proliferation of CySCs under physiological conditions.
Fig. 3. The SUMO pathway genetically interacts with the Hh pathway. (A–B′) Representative testes showing Ptc (green) and DAPI (purple) staining after RNAi induction for 3 days. Asterisks indicate the hub. Scale bars: 10 μm. (C–D′) Representative testes showing Zfh1 (red), and pSTAT (C) or ex-lacZ (detected by staining with anti-β-galactosidase antibody; D) (blue) staining after flp-out clone induction for 3 days. Yellow arrows indicate lwr RNAi CySC clones, and red arrows indicate control CySCs. Asterisks indicate the hub. White circles outline the cells. Scale bars: 10 μm. (E–E‴) Representative testes showing dpERK (red), Vasa (blue) and DAPI (white) staining, after RNAi induction for 3 days. Yellow arrows indicate an lwr RNAi CySC clone, and red arrows indicate a control CySC. Asterisks indicate the hub. Scale bar: 25 μm. (I) The numbers of Zfh1+ cells per testis after RNAi induction for 3 days. Data are presented as mean±s.e.m., ***P<0.001, n>15. (J) The numbers of clonal CySCs (GFP+ cells with strong Zfh1 staining) per testis after clone induction (ACI) for 5 days. Data are presented as individual values and mean±s.e.m. **P<0.01. (K) The fraction of mitotic CySC clones. Each point indicates the ratio of EdU+ CySC clones to all CySC clones in each testis. Data are presented as individual values and mean±s.e.m. **P<0.01. (L) The fraction of testes with at least one CySC clone, at 3, 4 and 5 days ACI.
Mechanistically, we found that the SUMO pathway genetically interacts with the Hh pathway, and directly catalyzes Ci SUMOylation, which is required for promoting CySC proliferation, although the function of SUMOylation in keeping CySCs stemness might involve other unknown factor(s). In summary, we identified that the SUMO pathway/SUMOylation directly targets the Hh pathway and regulates CySC maintenance in adult Drosophila testis (Fig. S4G).

Fig. 4. SUMOylation directly targets Ci and promotes Ci activity in terms of CySC proliferation. (A) Western blot assay showing that GST-Ci can directly interact with His-Lwr in a GST-fusion protein pull-down assay. Black arrowheads point to the GST or GST-Ci in lanes 1 and 2, respectively. (B,C) Western blot assay showing the SUMOylation status of different Ci fragments. Red arrowheads point to the SUMOylated Ci fragments and green arrowheads point to the un-SUMOylated Ci fragments. Asterisk indicates a non-specific band. (D,E) The numbers of Zfh1+ cells per testis, after transgene induction for 7 days. Data are presented as mean±s.e.m. ***P<0.001; n.s., not significant. n>20. (F-I′) Representative testes showing Zfh1 (green), Ci (red) and DAPI (blue) staining, after transgene induction for 7 days. Asterisks indicate the hub. Scale bars: 10 μm.
MATERIALS AND METHODS

Fly stocks
Flies were raised on standard yeast/molasses medium at 25°C unless otherwise stated. Fly stocks used are detailed in supplementary Materials and Methods.

RNAi and overexpression studies
All RNAi and overexpression-related experiments were performed in the Gal4-Gal80ts system (Kawase et al., 2004; Melcher and Xu, 2001; Suster et al., 2004). Except where specifically mentioned, all the assays were carried out using c587-Gal4.

Generation of clones using the MARCM system
Adult male flies were collected 0-3 days after eclosion and heat shocked for two rounds of 1 h heat shock at 37°C and 1 h rest at 25°C. After the final heat shock, they were returned to 25°C.

Immunostaining of testes, quantification of GSCs and EdU incorporation
Immunostaining of testes and quantification of GSCs were carried out as previously described (Zhang et al., 2013b). Antibodies used in this study: mouse anti-FasIII (DSHB, 7G10, 1:1000), mouse anti-Ptc (DSHB, Apa1, 1:100), mouse anti-Eya (DSHB, 10H6, 1:100), mouse anti-Hts (DSHB, 1B1-C, 1:1000), rabbit anti-Vasa (Santa Cruz, sc-26877, 1:200), rabbit anti-Zfh1 [a gift from Dr. Ruth Lehmann (School of Medicine, New York University, 1:5000)], rat anti-Ci (DSHB, 2A1, 1:200), mouse anti-pSTAT [a gift from Dr. Xinhua Lin (Cincinnati Children’s Hospital Medical Center), 1:500], rabbit anti-dpERK (Cell Signaling, 4370P, 1:100). EdU incorporation was performed with standard protocol following the manufacturer’s instructions (Invitrogen). See supplementary Materials and Methods for details.

DNA constructs and transgenes
DNA constructs and transgenes are described in supplementary Materials and Methods.

Bacterial SUMOylation assay, GST protein pull-down assay and western blot analysis
Bacterial SUMOylation assay, GST protein pull-down assay and western blot analysis were carried out as previously described (Nie et al., 2009; Shi et al., 2013). See supplementary Materials and Methods for details.

Statistical analysis
Data are presented as mean±s.e.m. Statistical significance was calculated using two-tailed Student’s t-tests. P-values are indicated in figures or in figure legends.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
X.L. and C.P. conceived the study, designed and performed experiments, analyzed data and prepared the manuscript; Z.Z., Y.X., H.C., S.Z., T.G. and H.H. contributed reagents/materials/analysis tools; H.S. and L.Z. analyzed the data; Y.Z. supervised and conceived the study, analyzed data and edited the manuscript.

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References


