Sumoylation modulates 20-hydroxyecdysone signaling by maintaining USP protein levels in *Drosophila*

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The nuclear receptor complex for the insect steroid hormone, 20-hydroxyecdysone (20E), is a heterodimer of EcR and USP. It has been shown that *Drosophila* EcR and USP can be sumoylated in mammalian cells, but it is unknown whether EcR-USP sumoylation naturally occurs in *Drosophila*. In *Drosophila* cells, USP, but not EcR, was sumoylated by Smt3, the only *Drosophila* SUMO protein. The presence of EcR enhanced USP sumoylation, which is further enhanced by 20E treatment. In addition to the Lys20 sumoylation site, five potential acceptor lysine residues in USP were predicted and verified. Mutation of the USP sumoylation sites or reduction of smt3 expression by RNAi attenuated 20E-induced reporter activity. Moreover, in the salivary glands, reducing smt3 expression by RNAi decreased 20E-induced reporter activity, gene expression, and autolysosome formation. Importantly, at least partially, the sumt3 RNAi-mediated reduction in 20E signaling resulted from decreased protein levels of USP. In conclusion, sumoylation modulates 20E signaling by maintaining USP protein levels in *Drosophila*.

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1. Introduction

Nuclear receptors form a large, conserved superfamily of ligand-activated transcription factors that are crucial for cell growth, development, reproduction, homeostasis, and metabolism. In insects, the nuclear receptor complex for the steroid hormone, 20-hydroxyecdysone (20E, the molting hormone), is a heterodimer of the ecdysone receptor (EcR) and ultraspiracle protein (USP) (Koelle et al., 1991; Yao et al., 1993). Insect EcR and USP are orthologs of the mammalian nuclear receptors farnesoid X receptor/liver X receptor receptors is mainly governed by ligand binding. Accumulating evidence in mammals has suggested that nuclear receptor transcriptional activity is also regulated by post-translational modifications, including phosphorylation, ubiquitination, and sumoylation (Faus and Haendler, 2006; Anbalagan et al., 2012). However, relatively little is known regarding how the transcriptional activity of EcR-USP and other insect nuclear receptors is regulated by post-translational modifications. Previous studies have shown that both EcR and USP are phosphoproteins in a number of insect species and have suggested that protein kinase C (PKC) might modulate 20E signaling by phosphorylating EcR and USP (Song and Gilbert, 1998, 2003; Rauch et al., 1998; Nicolaï et al., 2000). Importantly, Ser35 of *Drosophila* USP is a PKC phosphorylation site involved in PKC-modulated 20E signaling (Wang et al., 2012). An EcR isoform in *Drosophila*, EcR-A, is subjected to ubiquitin-mediated protein degradation, indicating that 20E signaling is regulated by isoform-specific ubiquitination of EcR (Gradilla et al., 2011). It was recently reported that *Drosophila* EcR and USP can be covalently modified by mammalian SUMO-1 and SUMO-3 in human HEK293 cells using the Ubc9 fusion-directed sumoylation (UFDS) method.

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2.2. Flies and genetics

In eukaryotic cells, the small ubiquitin-like modifier (SUMO) proteins are covalently attached to and detached from various substrate proteins in many pathways, thereby influencing the molecular actions of these target proteins. The cycle of dynamic and reversible sumoylation is well conserved among all eukaryotes. Prior to the first conjugation cycle, a nascent SUMO is proteolytically cleaved to expose the C-terminal glycine–glycine (GG) motif, which is bound to and activated by an E1 enzyme (SUMO activating enzyme). SUMO is then transferred to the catalytic cysteine of the E2 enzyme (SUMO conjugating enzyme, Ubc9). Ubc9 catalyzes the formation of an isopeptide bond between the C-terminal glycine of SUMO and a lysine (K) residue in the consensus sumoylation motif (ΨKXE/D) of the substrate protein; this typically occurs in conjunction with a specific SUMO E3 ligase. Sumoylation is reversible because SUMO-specific proteases can cleave the isopeptide bond and release SUMO for inclusion in additional cycles (Flotho and Melchior, 2013). Sumoylation is involved in various cellular processes, such as transcriptional regulation, protein stability, and nuclear-cytosolic transport. For example, by altering protein stability and/or subcellular localization, sumoylation regulates the transcriptional activity of numerous mammalian nuclear receptors (Knutson and Lange, 2013), including the androgen receptor, progesterone receptor, peroxisome proliferator-activated receptor-γ, steroidogenic factor 1, estrogen receptor, retinoid X receptor-α, RAR-related orphan receptor-α, and thyroid receptor.

Recently, we determined that PKC-mediated USP phosphorylation at Ser35 modulates 20E signaling in Drosophila (Wang et al., 2012). During the preparation of this manuscript, two subsequent reports showed that Drosophila USP (Bielska et al., 2012) and EcR (Seliga et al., 2013) could be covalently modified by mammalian SUMO proteins in human HEK293 cells. Using bioinformatic prediction, mass spectrum analysis, and PCR-based site-directed mutagenesis, six potential sumoylation sites in USP proved to be possible sumoylation acceptor lysine residues (Bielska et al., 2012). In this study, we aimed to characterize the role of sumoylation in regulating the transcriptional activity of EcR-USP in Drosophila. We first showed that USP, but not EcR, was sumoylated in Drosophila cells. We further demonstrated that sumoylation modulated 20E signaling by maintaining USP protein levels in Drosophila cells and the salivary glands (SGs).

2.3. Cell culture

Drosophila Kc and S2 cells were cultured at 27 °C in Schneider's Drosophila medium (Invitrogen, USA) supplemented with 10% fetal bovine serum (Hyclone, USA). Transient transfection assays were performed for 48 h using Effectene (Qiagen, Germany). The final DNA concentration was 2 μg/ml and the DNA:Effectene ratio was 1:25. In certain experiments, cells were treated with 1 μM 20E (dissolved in DMSO; Sigma–Aldrich, USA) for the indicated period of time (Wang et al., 2012).

2.4. UFDS

The low levels of sumoylation and its dynamic and reversible nature in cells hinder the detection of target protein sumoylation. The UFDS method (Jakobs et al., 2007), in which a potential target protein is fused to Ubc9 to improve its sumoylation, was employed to detect EcR-USP sumoylation in Drosophila cells. Drosophila Ubc9 was fused to the 5’-end of either EcR or USP and cloned into pAc5.1 (Invitrogen, USA) to construct Ubc9-EcR and Ubc9-USP, respectively. In certain experiments, a V5 tag was fused to Ubc9-EcR and to Ubc9-USP to form Ubc9-EcR V5 and Ubc9-USP V5, respectively. Using PCR-based site-directed mutagenesis (Life Technologies, USA), the six possible sumoylated lysines in USP were individually mutated to arginines to generate Ubc9-USP16R, Ubc9-USP20R, Ubc9-USP37R, Ubc9-USP170R, Ubc9-USP379R, and Ubc9-USP506R. In addition, the six possible sumoylated lysines in USP were simultaneously mutated to arginines to generate Ubc9-USP16-506. To facilitate the detection of sumoylated EcR-USP, we fused GFP to the 5’-end of smt3, the only Drosophila SUMO gene, to create GFP-smt3. Transient transfection assays were performed as described above.

2.5. Quantitative real-time PCR and Western blotting

Quantitative real-time PCR (qPCR) and Western blotting were performed as previously described (Wang et al., 2012). The primary antibodies used in this study were AB11 USP (a kind gift from Dr. Kafatos), Ag10.2 EcR-common (Developmental Studies Hybridoma Bank, USA), 25E9.D7 Br-core (Developmental Studies Hybridoma Bank), E10 V5 tag (Abcam, UK), GFP rabbit polyclonal antibody (Beyotime Institute of Biotechnology, China), and B512 a-tubulin mouse monoclonal antibody (Beyotime).

2.6. Dual luciferase assay

The reporter pGL3 vector (Promega, USA) containing four repeated 20E response element (EcRE) sequences (GACAA GGGTTCAATGCACTTGTC) and the hsp27 mini promoter was used as the firefly luciferase reporter (EcRE-Luc) (Wang et al., 2012). The reference pRL vector (Promega) expresses Renilla luciferase driven by the actin3 promoter. S2 cells were seeded in 24-well plates and were transfected as described above with 300 ng of plasmid mixture in 150 μl of medium per well. In some experiments, 100 ng of HA-EcR-B1, 100 ng of Ubc9-USP or Ubc9-USP 16-506, 90 ng of EcRE-Luc, and 10 ng of pRL were co-transfected into the cells. After 24 h, the medium was replaced with fresh medium containing 1 μM 20E for another 24 h. Dual luciferase assays were performed using the Dual Luciferase Assay System (Promega) and a Modulus Luminometer (Turner BioSystems, USA). The relative luciferase activity was calculated by normalizing the firefly luciferase to the Renilla luciferase (Wang et al., 2012; Tian et al., 2013; He et al., 2014).

In certain experiments, smt3 and GFP (control) dsRNAs were generated using the T7-RiboMAX™ Large Scale RNA Production Kit.

(Bielska et al., 2012; Seliga et al., 2013). However, whether EcR-USP sumoylation naturally occurs in Drosophila is unknown; moreover, the biological significance of EcR-USP sumoylation is unknown.

Possible sumoylation sites in Drosophila EcR and USP were predicted using three bioinformatics tools: SUMOPlot™ (www.abgent.com/doc/sumoplott2), SUMOsplot 2.0 (sumosplot.biocuckoo.org/prediction.php), and UbPred (http://www.ubpred.org/).

2.2. Flies and genetics

All the flies were grown on a standard cornmeal/molasses/agar medium at 25 °C. FKH-GAL4 (an SG-specific GAL4) flies were presented by Dr. D. J. Andrew. The wild type w1118 (stock number 3605), UAS-smt3ΔdRNA (36125), and EcR–lacZ (4516) strains were obtained from the Bloomington Drosophila stock center. UAS-smt3ΔdRNA, EcRE–lacZ flies were obtained by genetic recombination. For the GAL4/UAS experiments, the FKH-GAL4 strain was crossed with the UAS-smt3ΔdRNA, UAS-smt3ΔdRNA::EcRE–lacZ, and w1118 strains.
3. Results

3.1. USP sumoylation in Drosophila cells

In this study, we first investigated whether USP was sumoylated in Drosophila Kc cells using the UFDS method. Ubc9-USP V5 and/or GFP-smt3 were transfected into Kc cells, and then Western blotting was performed to detect USP sumoylation using the USP, V5, and GFP antibodies. Sumoylation of endogenous USP was not detected using the USP and GFP antibodies, even upon overexpression of GFP-smt3 (Fig. 1A, the first lanes in the left and right panels). Upon overexpression of Ubc9-USP V5, Ubc9-USP-V5 and its SUMO-modified form by the endogenous Smt3 (Smt3-Ubc9-USP-V5) were detected by both the USP and V5 antibodies (Fig. 1A, the middle lanes in the left and middle panels). Moreover, upon co-overexpression of Ubc9-USP V5 and GFP-smt3, Ubc9-USP V5, Smt3-Ubc9-USP V5, and an additional band corresponding to GFP-Smt3-Ubc9-USP V5 (showing Ubc9-USP V5 is sumoylated by GFP-Smt3) were detected by all the USP, V5, and GFP antibodies (Fig. 1A, the third lanes in all three panels).

When Ubc9-USP V5 is overexpressed in S2 cells, USP sumoylation was also detected (Fig. 1B). Notably, less endogenous USP protein was detected in S2 cells than in Kc cells (Fig. 1B), which is consistent with our previous observation that Kc cells are more sensitive to 20E treatment than S2 cells (Wang et al., 2012). The results in human HEK293 cells (Bielska et al., 2012) and Drosophila cells (Fig. 1) demonstrated that USP can be sumoylated.

3.2. Verification of USP sumoylation by the trans-sumoylation system

Although Drosophila EcR can be sumoylated in HEK293 cells (Seliga et al., 2013), we failed to detect EcR sumoylation in Drosophila Kc (Fig. 2A) or S2 cells (data not shown). No matter how to adjust the transfection system and the Western blotting conditions, EcR sumoylation was never detected suggesting that EcR sumoylation might be comparatively weaker than USP sumoylation in Drosophila cells.

It has been reported that Drosophila EcR and muristerone A do not influence the sumoylation patterns of USP in HEK293 cells (Bielska et al., 2012). As shown above (Fig. 1A), GFP-Smt3-USP was not detected upon co-overexpression of GFP-smt3 and USP in S2 cells (Fig. 2B, the first lane in the left panel). By contrast, using the trans-sumoylation system that monitors protein–protein interactions (Srivastav et al., 2011), GFP-Smt3-USP appeared upon co-overexpression of GFP-smt3, USP, and Ubc9-EcR B1 V5 (Fig. 2B, the second lane in the left panel). It is necessary to note that sumoylation of Ubc-EcR B1 V5 was not detected upon co-overexpression of GFP-smt3, USP, and Ubc9-EcR B1 V5 (Fig. 2B, the second lane in the left panel). These trans-sumoylation results indicated that the protein–protein interaction between Ubc9-EcR B1 and USP (heterodimerization) enhanced USP but not EcR sumoylation in Drosophila cells.

Importantly, USP sumoylation was further enhanced by 20E after co-overexpression of GFP-smt3, USP, and Ubc9-EcR B1 V5 in S2 cells (Fig. 2B, the middle and right panels; Fig. 2B). Again, sumoylation of Ubc-EcR B1 V5 was not detected upon co-overexpression of GFP-smt3, USP, and Ubc9-EcR B1 V5 and treatment with 20E (Fig. 2B, the right panel). These trans-sumoylation results suggested that the conformational change in the Ubc9-EcR/USP heterodimer induced by 20E binding enhanced the USP proximity to Ubc9 and further facilitated USP sumoylation. By contrast, EcR sumoylation was never detected in the presence of USP and 20E using the trans-sumoylation system, supporting the above observation that EcR sumoylation is weaker than USP sumoylation in Drosophila cells.
To strengthen the above results that the presence of EcR and 20E enhance USP sumoylation, we further employed the CheckMate™ mammalian two-hybrid system (Fields and Song, 1989). Upon co-overexpression of pBIND-USP, pACT-smt3, pcDNA3.1-EcR.B1, pG5luc, and pRL in HEK293 cells, muristerone A significantly increased the relative luciferase activity, indicating that 20E enhanced the USP-Smt3 interaction in the presence of EcR.B1 (Fig. 2C). However, without pBIND-USP, pACT-smt3, or pcDNA3.1-EcR.B1, the relative luciferase activity is very low and 20E does not enhance the luciferase activity (data not shown). The data described above determined that USP, but not EcR, was sumoylated in Drosophila cells and that USP sumoylation was enhanced by EcR and 20E.

3.3. USP sumoylation and the SUMO pathway are required for optimal 20E signaling in Drosophila cells

In the previous report (Bielska et al., 2012) six possible sumoylation sites at lysines 16, 20, 37, 170, 379, and 506 in USP were predicted and verified in HEK293 cells. The same results were also obtained using the three above mentioned bioinformatics tools. To confirm whether these residues were also sumoylated in Drosophila cells, the six lysines in USP were mutated to arginines either individually or simultaneously using PCR-based site-directed mutagenesis. Ubc9-USP, Ubc9-USP<sup>PS20R</sup>, Ubc9-USP<sup>PS506R</sup>, Ubc9-USP<sup>PS170K</sup>, Ubc9-USP<sup>PS379K</sup>, and Ubc9-USP<sup>PS506R</sup> were individually transfected into S2 cells. As determined by Western blotting using the USP antibody (Fig. 3A and 3A'), USP sumoylation became undetectable when all six lysines were mutated to arginines. The detection of USP sumoylation on multiple acceptor lysine residues in both HEK293 cells (Bielska et al., 2012) and Drosophila cells (Fig. 3A and A') implied that USP sumoylation might play a physiological role, presumably in regulating 20E signaling in Drosophila.

Because numerous mammalian studies have revealed that the sumoylation of nuclear receptors regulates their transcriptional activity (Knutson and Lange, 2013), we investigated whether USP sumoylation was involved in regulating the transcriptional activity of EcR-USP, and thus 20E signaling, in Drosophila. S2 cells were co-transfected with HA-EcR-B1, Ubc9-USP or Ubc9-USP<sup>PS6m</sup>, EcRE-Luc, and pRL and then treated with 20E. Dual luciferase assays revealed that the 20E-induced reporter activity was significantly lower in EcR.B1/Ubc9-USP<sup>PS6m</sup> cells compared with EcR.B1/Ubc9-USP cells (Fig. 3B). Next, we examined the possible regulatory role of the SUMO pathway in 20E signaling by reducing Smt3 expression using RNAi in Kc cells, which were subsequently co-transfected with HA-EcR-B1, Ubc9-USP or Ubc9-USP<sup>PS6m</sup>, EcRE-Luc, and pRL and treated with 20E. Dual luciferase assays indicated that smt3 RNAi significantly reduced 20E-induced reporter activity compared with the GFP RNAi control (Fig. 3C). Meanwhile, smt3 RNAi did not affect the mRNA levels of either EcR or USP (data not shown). The experimental data demonstrated that USP sumoylation and the SUMO pathway were required for optimal 20E signaling in Drosophila cells.

3.4. The SUMO pathway is required for optimal 20E signaling in Drosophila SGs

We further examined whether the SUMO pathway was required for 20E signaling in Drosophila SGs by performing in vivo GAL4/UAS
with pBIND-luciferase assays were subsequently performed. 

Fig. 2. USP sumoylation is enhanced by EcR and 20E. (A) Drosophila Kc cells were transfected with Ubc9-EcR.B1.V5 and/or GFP-smt3 for 48 h, and then Western blotting was performed to detect EcR.B1 sumoylation in the cell lysates using V5 and GFP antibodies. (B–B′) The trans-sumoylation system. Drosophila S2 cells were transfected with GFP-smt3, USP, and/or Ubc9-EcR.B1.V5 for 24 h and then treated with 20E or DMSO (control) for another 24 h. Western blotting was performed to detect USP sumoylation in the cell lysates using the USP (B) and V5 (B′) antibodies. (B′) Quantification of the GFP-Smt3-USP protein levels in the middle panel in (B). (C) The CheckMate™ mammalian two-hybrid system. Human HEK293 cells were transfected with pBIND-ISP, pACT-smt3, pCDNA3-EcR.B1, pG5 luc, and pRI for 24 h and then treated with muristerone A (a 20E agonist) or DMSO (control) for another 24 h; dual luciferase assays were subsequently performed.

Second, we directly measured the changes in the mRNA levels of key genes in the 20E-triggered transcriptional cascade in response to Smt3 RNAi in the SGs at WPP. The EcR, USP, and Br-C mRNA levels in the FKh-GAL4>UAS-smt3dsRNA animals were not apparently affected; however, E75B and E93 expression was significantly reduced in the FKh-GAL4>UAS-smt3dsRNA animals compared with the control FKh-GAL4 animals (Fig. 4B).

Third, we measured the effect of smt3 RNAi on 20E-induced Atg genes, apoptotic genes, and autophagosome formation (Yin and Thummel, 2005; Ryoo and Baehrecke, 2010; Tian et al., 2013; Liu et al., 2013, 2014) in the SGs at WPP. Both autophagy and caspas, which function in parallel, contribute to autophagic cell death in dying SGs, but autophagy plays a more important role than caspas (Jiang et al., 2000; Berry and Baehrecke, 2007; Scott et al., 2007). Except for Hid mRNA expression, the mRNA levels of all the measured Atg genes (Atg1, Atg2, Atg5, Atg7, Atg8a, and Atg12) and the key apoptotic genes (the initiator caspase gene Drice, the effector caspase gene Drice, and the death activator gene Reaper) were significantly reduced in the FKh-GAL4>UAS-smt3dsRNA animals compared with the control FKh-GAL4 animals (Fig. 4C). Moreover, as revealed by TEM, fewer autophagosomes were observed in the FKh-GAL4>UAS-smt3dsRNA animals compared with the control FKh-GAL4 animals (Fig. 4D). Reducing smt3 expression in Drosophila SGs decreased 20E-induced reporter activity, gene expression, and autolysosome formation, demonstrating that the SUMO pathway was required for optimal 20E signaling in vivo.

3.5. The SUMO pathway is required for maintaining USP protein levels

Sumoylation might regulate the transcriptional activity of nuclear reporters by modulating protein stability and nucleocytoplasmic transport (Knutson and Lange, 2013). We then investigated the molecular mechanism by which the SUMO pathway regulates 20E signaling after reducing smt3 expression by RNAi in Drosophila Kc cells and SGs at WPP. As determined by Western blotting, smt3 RNAi decreased USP protein levels by approximately 40% in both Kc cells (Fig. 5A and 5A′) and SGs (Fig. 5B and B′). Notably, EcR protein levels were not apparently affected by smt3 RNAi in the SGs, whereas Br-C protein levels were significantly reduced (Fig. 5B). As determined by immunohistochemistry, USP protein levels were significantly reduced in the SGs, but its subcellular localization was not obviously affected (Fig. 5C).

The Western blotting and immunohistochemistry results illustrated that the SUMO pathway regulates EcR-USP transcriptional activity and thus 20E signaling at least partially by maintaining USP protein levels rather than by altering its subcellular localization in Drosophila. Taken together, we concluded that sumoylation modulated 20E signaling by maintaining USP protein levels in Drosophila.

4. Discussion

4.1. EcR-USP sumoylation differently occurs in Drosophila and mammalian cells

In human HEK293 cells (Bieliska et al., 2012) and Drosophila cells (Figs. 1–3), USP is sumoylated on multiple acceptor lysine residues, with Lys20 being the primary sumoylation site. In contrast, EcR is
only sumoylated in human HEK293 cells (Seliga et al., 2013), not in Drosophila cells as revealed by both the UFDS method (Fig. 2A) and the trans-sumoylation system (Fig. 2B and B'). It is likely that EcR sumoylation is weaker than USP sumoylation in Drosophila cells. Alternatively, the SUMO system in human HEK293 cells might be more robust than that in Drosophila cells. There are three SUMO proteins (SUMO-1 to SUMO-3) in mammals. Drosophila EcR was more efficiently sumoylated by SUMO-1 than by SUMO-3 (Seliga et al., 2013), highlighting the different substrate preferences of the three mammalian SUMO proteins. In Drosophila, there is only one SUMO protein (Smt3), which is the ortholog of the mammalian SUMO-3, supporting the observation that EcR sumoylation is undetectable in Drosophila cells.

In the previous report (Bielska et al., 2012), HR38 (but not EcR and muristerone A) was used as an interacting partner in the trans-sumoylation system to enhance USP sumoylation. Using the same trans-sumoylation system, we have clearly showed that Ubc9-EcR.B1 enhanced USP sumoylation in Drosophila cells and that the level of USP sumoylation was further enhanced by 20E in the presence of EcR (Fig. 2B). The hypothesis that Ubc9-EcR/USP heterodimerization induced by 20E binding enhanced USP-Smt3 interaction and further facilitated USP sumoylation was verified using the CheckMate™ mammalian two-hybrid system in human HEK293 cells (Fig. 2C). The opposite results obtained in the two studies might be due to use of different methods and different cell lines. Considering the high efficiency of the UFDS system in HEK293 cells, it might be difficult to detect the small changes on Ubc9-USP caused by EcR and 20E (Bielska et al., 2012). However, with the trans-sumoylation system in Drosophila cells, subtle effects of Ubc9, EcR, and 20E on USP sumoylation were observed (Fig. 2B–B’).
4.2. **USP sumoylation modulates 20E signaling in Drosophila**

Sumoylation is a key regulatory protein modification that plays important roles in health and disease (Flotho and Melchior, 2013). In *Drosophila*, Smt3 is required for the synthesis of ecdysone (the immediate precursor of 20E) and the maintenance of the lipid content in the prothoracic gland (PG) during the larval–pupal transition (Talamillo et al., 2008). Reducing smt3 expression with RNAi in the PG decreased βFtz-f1 protein levels, resulting in the downregulation of the Halloween genes that are responsible for ecdysone synthesis (Parvy et al., 2005; Talamillo et al., 2008) and the scavenger receptor genes that mediate lipid uptake (Talamillo et al., 2013). Moreover, the sumoylation of βFtz-f1, another insect nuclear receptor, regulates its transcriptional activity (Talamillo et al., 2013).

βFtz-f1 sumoylation modulates ecdysone synthesis in the PG (Talamillo et al., 2013); here, we demonstrated that USP sumoylation directly modulated 20E signaling in both *Drosophila* cells and SGs (Figs. 3 and 4). Moreover, we determined that sumoylation modulated 20E signaling in *Drosophila* at least partially by maintaining USP protein levels, but not by altering its subcellular localization (Fig. 5). Therefore, the molecular mechanism by which the SUMO system regulates the transcriptional activity of nuclear receptors (Knutson and Lange, 2013) is generally conserved from *Drosophila* to humans.

Using mass spectrum analysis in a previous study (Wang et al., 2012), we determined that Ser35 of *Drosophila* USP is a PKC phosphorylation site, but we could not identify the phosphorylation sites in *Drosophila* EcR, confirming that USP phosphorylation is more evident than EcR phosphorylation in *Drosophila* (Song and...
Gilbert, 1998; Song et al., 2003). Similarly, this study demonstrated that USP sumoylation was more pronounced than EcR sumoylation in Drosophila cells (Figs. 1 and 2). We propose that the transcriptional activity of EcR, a ligand-activated nuclear receptor, is mainly governed by 20E binding; however, as an EcR binding partner and an orphan nuclear receptor, USP is more flexible and is subjected to multiple post-translational modifications, including phosphorylation and sumoylation.

**Authors’ contributions**

SL conceived and designed the experiments. JW and SW performed research and analyzed the data. SL, SW, and JW wrote the paper. All authors read and approved the final manuscript.

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