SUMO in Drosophila Development

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Abstract

The ubiquitin-like protein SUMO is conjugated covalently to hundreds of target proteins in organisms throughout the eukaryotic domain. Genetic and biochemical studies using the model organism *Drosophila melanogaster* are beginning to reveal many essential functions for SUMO in cell biology and development. For example, SUMO regulates multiple signaling pathways such as the Ras/MAPK, Dpp, and JNK pathways. In addition, SUMO regulates transcription through conjugation to many transcriptional regulatory proteins, including Bicoid, Spalt, Scm, and Groucho. In some cases, conjugation of SUMO to a target protein inhibits its normal activity, while in other cases SUMO conjugation stimulates target protein activity. SUMO often modulates a biological process by altering the subcellular localization of a target protein. The ability of SUMO and other ubiquitin-like proteins to diversify protein function may be critical to the evolution of developmental complexity.

Keywords

SUMO • Ubc9 • Ubiquitin-like proteins • Ras/MAPK signaling • Dpp • Medea • Drosophila development • Scm • Groucho • Bicoid • Spalt

15.1 The SUMO Pathway

Small Ubiquitin-related Modifier (SUMO) is one of many ubiquitin-like proteins with diverse functions in cell biology and development. SUMO has a structure very similar to that of ubiquitin, and like ubiquitin, is covalently conjugated to a large variety of target proteins. Sumoylation and ubiquitylation are homologous processes catalyzed by homologous enzymes

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(Bayer et al. 1998; Smith et al. 2012). Sumoylation is reversible and like many post-translational modifications (e.g., phosphorylation, acetylation, methylation, etc.) functions as a switch to modulate target protein activity. Depending on the target, sumoylation can alter protein function in different ways, often by regulating target protein subcellular localization, interactions with other proteins, and protein stability (Seeler and Dejean 2003; Smith et al. 2012).

SUMO is conserved throughout the eukaryotic domain. The single SUMO family protein in Drosophila is encoded by the *smt3* gene, while the human genome encodes four SUMO family proteins, SUMO1, SUMO2, SUMO3, and SUMO4 (Huang et al. 1998; Smith et al. 2004, 2012). Drosophila SUMO is more closely related to human SUMO2 and SUMO3 than to the other human SUMO family members (Smith et al. 2012). *smt3* is an essential gene that is required both maternally and zygotically (Nie et al. 2009).

Sumoylation of a target protein requires three steps, which are catalyzed by enzymes generally termed E1 (the activating enzyme), E2 (the conjugating enzyme), and E3 (the ligase) (Fig. 15.1). Drosophila SUMO is first expressed in an immature form, containing a two-amino acid C-terminal extension, which is removed by one of the ubiquitin-like proteases, Ulp1 or Ulp2, to expose a required C-terminal Gly-Gly motif (Smith et al. 2004). Coupled to the hydrolysis of ATP to AMP and pyrophosphate, SUMO becomes covalently attached to the E1 enzyme, a heterodimer consisting of SUMO Activating Enzyme 1 (SAE1) and SUMO Activating Enzyme 2 (SAE2) subunits, via a thioester linkage between a cysteine residue in SAE1 and the C-terminal carboxyl group of SUMO. SUMO is then transferred to a cysteine residue in the E2 enzyme Ubc9 (Long and Griffith 2000). While ubiquitylation employs multiple alternative conjugating enzymes, Ubc9 is the only known

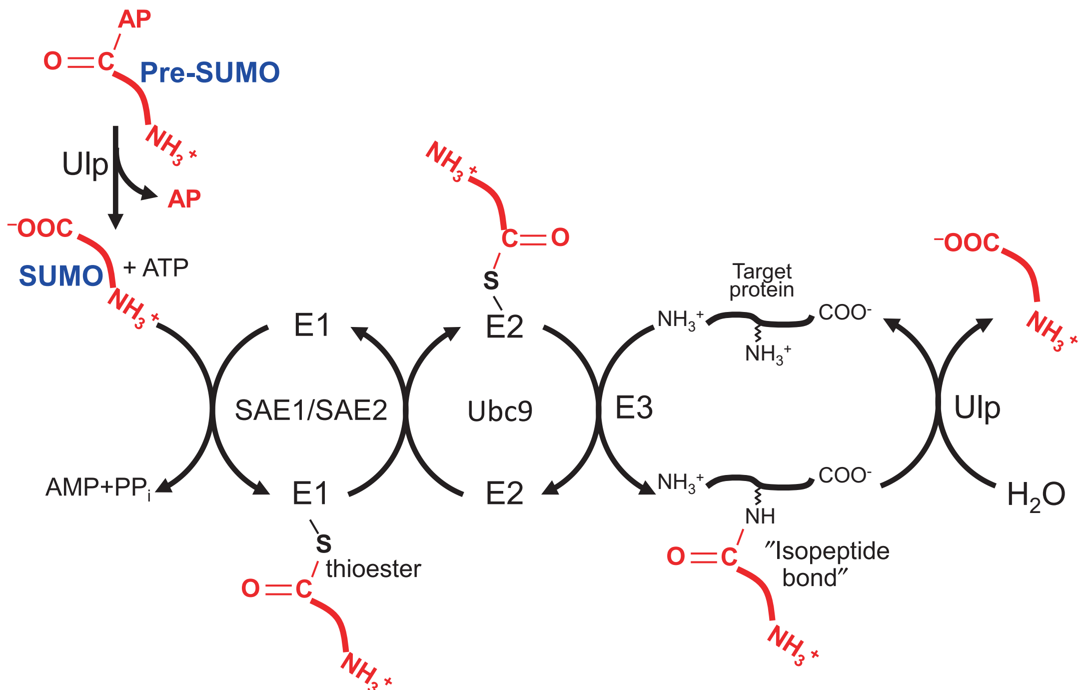

Fig. 15.1 *SUMO conjugation and deconjugation.* SUMO is initially synthesized as a pre-protein with a two amino acid C-terminal extension (AP). The AP is cleaved off by a Ulp family protease to generate mature SUMO. SUMO is then attached to a target protein via a three-step pathway involving the E1 activating enzyme SAE1/SAE2, the E2 conjugating enzyme Ubc9, and a ligation step, which may or may not require an E3 enzyme. The resulting isopeptide bond between the target protein and the C-terminus of SUMO can be hydrolyzed by a Ulp family protease

conjugating enzyme in the SUMO pathway. SUMO is then ligated to an acceptor lysine residue in the target protein. This residue frequently falls within a sequence with similarity to a ΨKXE consensus motif (Ψ is any hydrophobic amino acid and X is any amino acid) (Rodriguez et al. 2001). Unlike ubiquitin conjugation, which has an obligate requirement for an E3 ligase to catalyze the transfer of ubiquitin from the E2 to the target, there is no absolute requirement for a ligase in the catalysis of SUMO conjugation. However, E3 ligases often help Ubc9 select its target and a number of proteins have been found that have SUMO ligase activity, including the PIAS family proteins, RanBP2, and Pc2 (Agrawal and Banerjee 2008; Pichler et al. 2002; Schmidt and Muller 2002; Smith et al. 2012). SUMO modification can be reversed by either Ulp1 or Ulp2, both of which catalyze the hydrolysis of the isopeptide (amide) linkage between SUMO and the lysine side chain in the target protein (Smith et al. 2004).

SUMO-modified proteins are able to interact non-covalently with other proteins through SUMO interaction motifs (SIMs). These motifs possess a hydrophobic core with the consensus sequence V/I-V/I-X-V/I (X is any amino acid) (Hecker et al. 2006; Song et al. 2004). The SIM forms a β strand that interacts with the β2 strand of SUMO in either a parallel or an anti-parallel orientation (Baba et al. 2005; Kerscher 2007). Serine and threonine residues adjacent to the SIM hydrophobic core can be phosphorylated, and the phosphate group forms a salt bridge to a conserved lysine residue within SUMO (Hecker et al. 2006).

15.2 SUMO and Drosophila Development

The remainder of this review will focus on a few of the many roles of SUMO in regulating embryogenesis and imaginal development in *Drosophila melanogaster*. Several of the signaling pathways required for oocyte and embryonic patterning as well as imaginal disc patterning, such as the Ras/MAPK pathway, the Decapentaplegic (Dpp) pathway, and the Jun N-terminal Kinase (JNK) pathway, are regulated by SUMO. In addition, multiple spatially regulated sequence-specific transcription factors that control Drosophila development, such as the maternal morphogen Bicoid and the wing determinant Spalt, are regulated by SUMO. Finally, important ubiquitously localized transcriptional corepressors, including the Polycomb group (PcG) protein Scm and Groucho (Gro), are also regulated by SUMO.

It may be that SUMO most often acts as a negative regulator of target protein activity, e.g., it negatively regulates JNK signaling, Dpp signaling, Gro function, and Scm function. However, SUMO is also sometimes used to enhance pathway activity, e.g., in the case of the Ras/MAPK signaling, Bicoid function, and Spalt function.

15.2.1 Regulation of Signal Transduction by SUMO

15.2.1.1 Ras/MAPK Signaling

The Ras/MAPK signal transduction pathway is required to pattern the follicle cell epithelium during egg chamber development (Reeves and Stathopoulos 2009). This requires the secretion of the TGF-α-like protein Gurken from the presumptive dorsal side of the oocyte, and the binding of Gurken to the Torpedo receptor tyrosine kinase (RTK) in the membranes of the overlying follicle cells. Subsequent dimerization and cytoplasmic autophosphorylation of Torpedo leads to the formation of a docking site for the adaptor protein DRK (Pawson and Gish 1992; Simon et al. 1991, 1993). DRK, in turn, recruits the GTP exchange factor Son of Sevenless (SoS) for Ras activation through the exchange of GDP for GTP in the membrane tethered Ras protein (Bonfini et al. 1992). Ras then stimulates a phosphorylation cascade involving the sequential activation of three Ser/Thr kinases, Raf, MEK, and MAPK (Leevers et al. 1994; McCubrey et al. 2007; Wellbrock et al. 2004), thus triggering the adoption of a dorsal follicle cell fate. These follicle cells then secrete dorsal eggshell structures such

as the pair of dorsal appendages that act as respiratory filaments (Brand and Perrimon 1994; Hsu and Perrimon 1994; Schnorr and Berg 1996).

Early evidence that SUMO has a role in Ras signaling came from a study demonstrating that reduction of *smt3* gene dosage enhanced the eggshell pattering defect resulting from a hypomorphic *ras* mutation. Specifically, mothers homozygous for a weak *ras* allele and heterozygous for an *smt3* P-allele exhibited fused dorsal appendages, which is indicative of a ventralized egg chamber (i.e., the partial loss of the dorsal follicle cell fate) (Schnorr et al. 2001). This is consistent with a requirement for SUMO in Ras signaling. Subsequently, a number of proteins known to influence Ras/MAPK signaling were found to be SUMO-conjugation targets (Nie et al. 2009). Furthermore, RNAi knock down of SUMO in S2 cells revealed that SUMO is required for robust Ras/MAPK signaling in response to the RTK ligands insulin and Spitz. SUMO knockdown led to reduced levels of activated MEK and MAPK in the stimulated cells indicating that SUMO likely acts upstream of MEK and downstream of the RTK in the pathway. Several of the Ras pathway SUMO conjugation targets in the early embryo, including protein phosphatase 2A, and 14-3-3 family proteins are known to function via the Raf protein (Abraham et al. 2000; Light et al. 2002; Rommel et al. 1996, 1997; Wassarman et al. 1996), suggesting that SUMO may influence Ras signaling at the level of Raf.

15.2.1.2 Dpp Signaling

Dpp signaling, which is required for many developmental pathways, including embryonic dorsoventral patterning and imaginal disc patterning, is also regulated by SUMO. In this case, SUMO appears to interfere with signaling in contrast to its role in enhancing Ras/MAPK signaling. Dpp is a member of the BMP subfamily of TGFβ family ligands and signals through a heteromeric receptor consisting of a type I subunit (Saxophone or Thickveins) and a type II subunit (Punt) (Shimmi et al. 2005). Both type I and type II subunits possess Ser/Thr kinase activity. After activation of the receptor by Dpp, the type II subunit phosphorylates the type I subunit, and then the type I subunit phosphorylates the Smad family transcription factor Mothers against Dpp (Mad). Phospho-Mad then interacts with the co-Smad Medea (Med) and activates downstream targets at the level of transcription (Affolter et al. 2001).

A yeast two-hybrid screen uncovered an interaction between Med and Ubc9, and tissue culture experiments using S2 cells demonstrated Med sumoylation (Miles et al. 2008). Furthermore, overexpression of SUMO in the embryo inhibited the transcription of the Medea target genes *Ance* and *ush*, while expression of the two targets increased upon expression of a Med mutant containing a defective SUMO acceptor site. Fluorescent Recovery After Photobleaching (FRAP) studies showed that sumoylation of Med occurs in the nucleus and allows for the shuttling of Med out of the nucleus, thus explaining how Med sumoylation interferes with Dpp signaling.

15.2.1.3 Jun N-Terminal Kinase Signaling

The Jun-N-terminal Kinase (JNK) pathway, another highly conserved MAPK signaling pathway, regulates multiple processes during Drosophila development, including dorsal closure in embryos, thorax closure in pupae, and stress induced apoptosis (Etter et al. 2005; Igaki 2009; Luo et al. 2007). Intrinsic and external

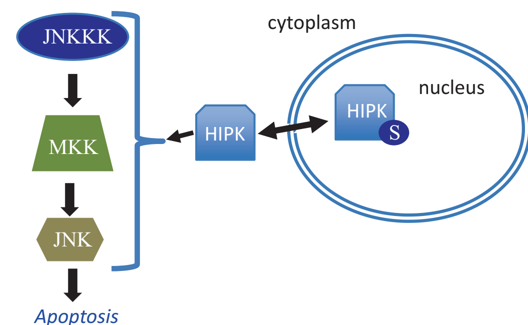

Fig. 15.2 *Regulation of apoptosis by SUMO.* Apoptosis can be triggered by activation of the JNK pathway, which involves the sequential action of JNKKK, MKK, and JNK. This pathway is activated at an unknown step by HIPK. Sumoylation (S) of HIPK prevents excess apoptosis by sequestering HIPK in the nucleus

stimuli triggers the pathway by activating JNK Kinase Kinase (JNKKK), which then phosphorylates MAPK Kinase (MKK) for the subsequent phosphorylation and activation of JNK (Biteau et al. 2011).

As mentioned above, the JNK pathway upregulates apoptosis and SUMO antagonizes this process since SUMO knockdown by RNAi led to increased apoptosis in the wing disc (Huang et al. 2011). However, when SUMO and JNK were knocked down at the same time, increased apoptosis was not observed. In addition, SUMO knockdown in the wing disc led to increased expression of the JNK target genes *puckered* and *matrix metalloproteinase I*.

Further genetic analysis suggests that SUMO may regulate JNK activity via homeodomain-interacting protein kinase (HIPK) (Huang et al. 2011) (Fig. 15.2). HIPK knockdown attenuates SUMO knockdown-induced apoptosis. Furthermore, HIPK is a SUMO conjugation target and SUMO is required for retention of HIPK in the nucleus. Apparently, when cells are depleted of SUMO, HIPK enters the cytoplasm where it encounters and activates the JNK pathway leading to increased apoptosis.

15.2.2 Regulation by SUMO of Spatially Restricted Sequence-Specific Transcription Factors

15.2.2.1 Bicoid

Lesswright (*lwr*), the gene encoding Ubc9, was independently discovered for its role in anterior patterning. Hence, an alternative name for *lwr* is *semushi*, which means "hunchback" in Japanese, reflecting the similarity between the *semushi* phenotype and that of the gap gene *hunchback* (*hb*). Loss-of-function mutations in either gene perturb segmentation of the anterior portion of the early embryo (Epps and Tanda 1998). Further examination of the *semushi* mutants revealed reduced expression of *hb*.

SUMO may mediate anterior patterning by controlling the function of the transcription factor Bicoid, a classical morphogen that is distributed in an anteroposterior gradient in the early embryo and that functions as an activator of *hb*. In particular, Ubc9 function may be required for the nuclear translocation of Bicoid (Epps and Tanda 1998). Paradoxically, however, a cell culture assay using an *hb* enhancer element to drive reporter gene expression revealed that sumoylation of Bicoid inhibits its ability to activate transcription (Liu and Ma 2012).

15.2.2.2 Spalt

Spalt (Sal) and Spalt-related (Salr) are highly conserved zinc-finger transcription factors that regulate wing vein formation and the expression of *knirps* during wing development (Barrio and de Celis 2004; de Celis and Barrio 2000; de Celis et al. 1996). Both proteins contain two SUMO-acceptor lysine residues and mutations in the genes encoding SUMO and Ubc9 enhance the ectopic wing vein phenotype observed in flies heterozygous for a small deficiency that removes both *sal* and *salr* (Sanchez et al. 2010). Wild-type Sal overexpression results in ectopic vein formation, while expression of Sal containing mutations in the SUMO acceptor sites does not, thus suggesting that SUMO conjugation is required for Sal activity. In contrast, mutagenesis of the SUMO acceptor lysine residues in Salr enhanced the wing venation defect due to overexpression suggesting that sumoylation of Salr interferes with its activity. These contrasting effects of the mutations in Sal and Salr on wing venation were paralleled by contrasting effects on *knirps* expression. Mutagenesis of the SUMO acceptor sites in Sal interfered with its ability to up-regulate *knirps*, while mutagenesis of the acceptor sites in Salr enhanced its ability to up-regulates *knirps*.

The mechanism by which SUMO influences Sal and Salr function may be related to the ability of SUMO to control the subnuclear localization of these two proteins. For example, while Sal exhibits diffuse nuclear localization in wild-type wing discs, reduced levels of Ubc9 (presumably leading to reduced levels of sumoylation) result in the appearance of large punctate Sal-containing nuclear bodies.

15.2.3 Regulation by SUMO of Co-repressors

15.2.3.1 The Polycomb Group Protein Scm

While the spatially regulated transcription factors (i.e., the products of the gap and pair rule genes) that initiate homeotic gene expression are only present in the early embryo, the spatially restricted patterns of homeotic gene expression are somehow maintained throughout embryonic and imaginal development. This cellular memory is thought to be provided by two groups of genes termed the Polycomb Group (PcG) and the Trithorax Group (TrxG), with the former being required for epigenetic stability of the repressed state, while the latter is required for epigenetic stability of the active state.

Many of the PcG proteins are members of one of three different complexes, the Pleiohomeotic Repressive Complex (PhoRC), Polycomb Repressive Complex 1 (PRC1), and Polycomb Repressive Complex 2 (PRC2) (Schwartz and Pirrotta 2013). PhoRC, which contains Pleiohomeotic (Pho) and Scm-related gene containing four MBT domains (Sfmbt), binds to cis-regulatory elements in the homeotic gene complex termed polycomb response elements (PREs), where they are thought to recruit PRC2. This complex contains Enhancer of zeste (E(z)), a SET family histone methyltransferase, which catalyzes the trimethylation of lysine 27 on histone H3 (H3K27me3). H3K27me3 then serves as a docking site for PRC1. This complex ubiquitinates histone H2A and directs the compaction of chromatin, with this latter function serving to reduce the accessibility of associated genes to a TrxG-encoded chromatin remodeling complex that opens up the chromatin allowing the transcriptional machinery to gain access to the DNA template. An additional PcG gene product that is essential for PcG function is Sex combs on midleg (Scm), which may be a peripheral component of PRC1 (Fig. 15.3).

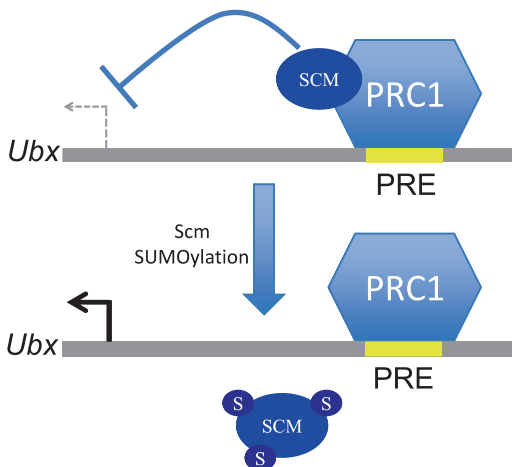

Fig. 15.3 *Regulation of Scm-mediated repression by SUMO.* Scm, a peripheral component of PRC1, is an essential of Polycomb group protein. Polycomb group-mediated repression of genes such as *Ubx* requires the recruitment of PRC1 along with Scm to the PRE. Sumoylation (S) of Scm results in the release of Scm from the PRE and the loss of repression

Like its *C. elegans* homolog SOP-2 (Zhang et al. 2004), Drosophila Scm is regulated by SUMO. Knockdown of SUMO in S2 cells was found to increase association of Scm with a PRE in the homeotic gene complex and to result in the derepression of the homeotic gene *Ultrabithorax* (*Ubx*). Conversely, knockdown of the SUMO deconjugating enzyme Ulp1 was found to decrease Scm association with the PRE. These findings are consistent with the idea that SUMO acts to negatively regulate Scm activity and, through Scm, to alleviate PcG-mediated repression. In support of this idea, mutagenesis of three consensus SUMO acceptor sites in Scm significantly reduced Scm sumoylation and led to increased association of Scm with the PRE (Smith et al. 2011). Consistent with the idea that SUMO negatively regulates Scm activity and therefore positively regulates *Ubx* expression, knockdown of SUMO in developing haltere discs results in an *Ubx*-like phenotype, i.e., a partial haltere-to-wing transformation.

The mechanism by which SUMO controls Scm and therefore polycomb group activity is unclear. Both Scm and the PRC1 component Polyhomeotic contain sterile alpha motif (SAM) domains, which are capable of mediating the formation of long protein filaments, and that may be required for chromatin compaction (Boettiger et al. 2016; Peterson et al. 2004). The functions

of the Scm SAM domain are complex: it is required for recruitment of Ubc9 and thus sumoylation, but it also appears to have an independent requirement in the recruitment of Scm to the PRE (Smith et al. 2011). We speculate that Scm sumoylation could modulate PcG function by modulating the role of the SAM domain in such processes as Scm recruitment, filament formation, and chromatin compaction.

15.2.3.2 Groucho

Groucho is a transcriptional co-repressor required for function of many of the transcriptional repressors that act throughout Drosophila development, including, the Hairy-Enhancer of split family factors, the Runt family factors, Engrailed, Dorsal, Capicua, and Brinker (Dubnicoff et al. 1997; Hasson et al. 2001; Jimenez et al. 1997; Paroush et al. 1994). Groucho functions, in part, by mediating the recruitment of Histone Deacetylase 1 (HDAC1) to its target genes (Turki-Judeh and Courey 2012).

Groucho is a sumoylation target (Nie et al. 2009). In mammalian cells, SUMO appears to positively regulate Groucho function by helping to mediate the recruitment of HDAC1 through a SIM in HDAC1 (Ahn et al. 2009). On the other hand, work in Drosophila suggests that SUMO antagonizes Groucho-mediated repression. Degringolade (Dgrn) a SUMO Targeted Ubiquitin Ligase (STUbL) appears to bind Groucho in a SUMO dependent manner leading to the sequestration and therefore inactivation of Groucho. Thus in the absence of SUMO, sequestration does not occur allowing for Groucho-mediated repression (Abed et al. 2011).

15.3 Conclusion

SUMO acts as a genetic switch that targets hundreds or thousands of proteins to regulate a wide variety of essential cellular and developmental processes. Illuminating its biological roles is as challenging as trying to arrive at a comprehensive understanding of the roles of other common protein modifications, such as phosphorylation, acetylation, and glycosylation (Lomeli and Vazquez 2011). Due to the pleiotropic functions of SUMO in development, global disruption of sumoylation is not usually instructive. Therefore, approaches such as mapping and mutating individual SUMO acceptor sites, SUMO-substrate fusions, and tissue-specific overexpression or knockdown of SUMO pathway components must be utilized to dissect specific SUMO functions from one another.

Another challenge to understanding the many biological roles of SUMO is the so-called “SUMO enigma” (Hay 2005). In most cases, it appears that only a small fraction of any given sumoylation target is conjugated to SUMO at any one time. Paradoxically, however, sumoylation of proteins such Scm, Sal, Groucho, HIPK, and Med often leads to near quantitative effects on the activity or subcellular localization of these proteins. While this enigma remains unresolved, two speculative non-mutually exclusive explanations are as follows. First, it is possible that cyclic rounds of conjugation and deconjugation are required for progress through a pathway. Second, perhaps deconjugation leaves behind a protein that still retains the memory of being sumoylated. For example, sumoylation could be required to overcome a kinetic barrier to the formation of a protein complex that remains stable after deconjugation has occurred.

The ease with which the Drosophila genome can be manipulated has allowed us to overcome the challenges described above. Since pathways regulated by SUMO are highly conserved across the eukaryotic domain, studies of sumoylation in Drosophila may provide insight into how SUMO leads to increased developmental complexity by diversifying protein function.

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