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The Topors protein is a tumor suppressor in human that associates with and regulates a number of cell cycle regulators that including topoisomerase I and p53. It possesses both ubiquitin and SUMO ligase activity and its mutation or downregulation has been associated with some human cancers and diseases. The Drosophila homologue, dTopors, is an ubiquitin E3 ligase. We have investigated the role of Dtopors in sumoylating proteins in the male germ line. Although nuclear lamin localization is disrupted in dtopors mutants, we find no evidence of lamin modification by Dtopors. We observe an increase in the overall sumoylation of testis proteins and a corresponding decrease in the pool of free SUMO in homozygous *dtopors* versus heterozygous *dtopors* flies. Based on this result, we propose a model explaining the role of dtopors in altering germline sumoylation. We have constructed a SUMO-GFP transgenic protein to investigate how dtopors is altering the pool of SUMO in the cell. We demonstrate that this transgenic construct can rescue *smt3* mutant flies and is expressed in spermatocytes. This tool will allow one to measure SUMO synthesis and processing, and how these aspects of SUMO dynamics are affected by *dtopors* during meiosis in *D. melanogaster* males.

REGULATION OF SUMOYLATION BY *DTOPORS* DURING MALE MEIOSIS IN *DROSOPHILA MELANOGASTER*

By

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Approved by

_____
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To

My Grandmother

Support, Motivation and Love

APPROVAL PAGE

This thesis has been approved by the following committee of the Faculty of The Graduate School at The University of North Carolina at Greensboro.

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CHAPTER I

INTRODUCTION

Protein function is regulated by post-translational modifications, and these modifications are essential for cell viability. Ubiquitin (Ubi) and ubiquitin-like proteins (Ubls), including SUMO (Small Ubiquitin-like Modifier), are a subset of post-translational modifications in which a small peptide is conjugated to the target protein, altering its fate or activity. Ubiquitinconjugation, or ubiquitination, is a major pathway for regulated degradation of intracellular proteins in eukaryotes. Ubiquitin-directed proteolysis is a two-step process in which a ubiquitin molecule is first covalently attached to the target protein in a reaction catalyzed by three enzymes (E1 activating, E2 conjugating and E3 ligase) working in concert. Subsequently, the 26S proteosome recognizes the ubiquitin-tagged target proteins and degrades them into small peptides. The ubiquitin is released and is reusable (Wilkinson 1995; Goldberg 2003).

SUMO was originally identified as *smt3* in *Saccharomyces cerevisiae* a suppressor of a mutation in the centromeric protein MiF2 (Meluh and Koshland 1995). Yeast two hybrid assays showed that human SUMO is a binding partner of the double-strand break repair proteins RAD-51 and RAD-52 (Shen, Cloud et al. 1996), the apoptosis-inducing factor Fas/APO-1(Okura, Gong et al. 1996) and PML (Boddy, Howe et al. 1996). A cellular consequence of covalent attachment of SUMO (sumoylation) was first demonstrated in a study of the RanGTPase-activating protein RanGAP1.

Sumoylation was found to modulate its partitioning between the cytosol and the nuclear pore complex (Matunis, Coutavas et al. 1996). These studies indicated that sumoylation is reversible and can alter localization of the modified target by altering protein interactions. At the molecular level, sumoylation may alter the exposure of protein surfaces and thereby influence interactions with other macromolecules.

The three dimensional structure of SUMO resembles that of ubiquitin, but the two proteins share less than 20% amino acid sequence identity (Bayer, Arndt et al. 1998; Mossessova and Lima 2000; Bernier-Villamor, Sampson et al. 2002). SUMO proteins are ubiquitously expressed throughout the eukaryotic kingdom. Some organisms like yeast (Lapenta, Chiurazzi et al. 1997), the roundworm *C. elegans* (Jones and Candido 1993) and the fly *D. melanogaster* (Francois L, 2000) have a single SUMO gene (*smt3*), whereas many plants and vertebrates have several SUMO genes. For example, the human genome encodes four SUMO proteins (SUMO1-4) (Li, Guo et al. 2005) and Arabidopsis encodes eight paralogs (Kurepa, Walker et al. 2003; Lois, Lima et al. 2003).

In most cases ubiquitination leads to protein degradation but sumoylation does not. In contrast, sumoylation can stabilize the target protein by competitively blocking ubiquitination of a particular lysine residue, as in the case of modification of K21 on IkappaBalpha (Desterro, Rodriguez et al. 1998). Before the sumoylation pathway is initiated SUMO isopeptidases cleave the C-terminal end of SUMO to deglycinate it and make it ready for the three-step sumoylation process to follow (Li and Hochstrasser 2000). SUMO is first covalently linked to the SUMO E1-activating enzyme, and then

transferred to the SUMO E2-conjugating enzyme, which carries out target modification with the aid of a SUMO E3 ligase. Deconjugation to release free SUMO from the target is mediated by SUMO isopeptidases (Desterro, Rodriguez et al. 1998; Gong, Li et al. 1999; Okuma, Honda et al. 1999; Li and Hochstrasser 2000). The largest group of enzymes identified in sumoylation pathway is the SUMO E3 ligases. Some of the E3 ligases that have been discovered are PIAS1 (Hochstrasser 2001), Siz1 and Siz2 (Johnson and Gupta 2001), Mms21(Zhao and Blobel 2005), RanBP2 (Pichler, Gast et al. 2002), Pc2 (Kagey, Melhuish et al. 2003) and Topors (Weger, Hammer et al. 2005).

There is no simple way to predict the consequence of sumoylation on protein function as it depends on the target proteins. Genetic studies of sumoylation in different model organisms indicate a role for SUMO conjugation in higher order chromatin structure and in chromosome segregation, but the molecular basis of these effects is largely unknown. Mutations in the SUMO protease *ulp2* in *S. cerevisiae* show many phenotypes indicating genomic instability and defective targeting of the condensin complex, required for chromosome condensation of rDNA repeats during meiosis (Li and Hochstrasser 2000) (Strunnikov, Aravind et al. 2001). In *D. melanogaster*, mutation of the putative PIAS family SUMO E3 ligase Su(var)2-10 leads to chromosome segregation defects, enhanced minichromosome loss and abnormal telomere clustering and defects in telomere nuclear lamina associations (Hari, Cook et al. 2001). A mutation in the Ubc9 homolog *lesswright* (an E2 SUMO conjugating enzyme) perturbs disjunction of homologs in meiosis I in Drosophila females (Apionishev, Malhotra et al. 2001). The *lesswright* mutation was originally identified via a second site suppression screen of a

mutation in *no distributive disjunction* (*nod*), a protein involved in segregating achiasmate homologs. The researchers found that *lesswright* partially suppresses several mutations that affect chromosome segregation in Drosophila female meiosis (Apionishev, Malhotra et al. 2001). A more specific meiotic role of sumoylation has been identified in the assembly of the synaptonemal complex (SC). The SC is a zipper-like structure connecting paired homologous chromosomes during meiosis I prophase (von Wettstein 1984 ; Page and Hawley 2004). Cheng, Lo et al.(2006) first established the relationship between SC formation and Ubc9-mediated sumoylation. They showed that Zip3, a SUMO E3 ligase, is involved in initiation of SC formation. In a *zip3* null mutant, polycomplex forms in place of SC. Their result suggested that Zip1, a "building block" of SC, binds to SUMO-conjugated proteins. This data suggests a role of SUMO-conjugated products in SC polymerization via Zip3-dependent SUMO modifications (Cheng, Lo et al. 2006). Sumoylation has been detected to be an important modification of all cohesion/condensin SMC complexes in yeast (Lee and O'Connell 2006). A non-SMC component of cohesion complex, Mms21, is reported to be a SUMO ligase which can recognize its own complex as a substrate. Mms21-mediated sumoylation most likely controls chromosome localization and may affect the role of Mms21 in recombination during meiosis (Lee and O'Connell 2006).

An additional role of sumoylation in meiosis may be the formation of the mammalian sex body, a specialized transcriptionally repressed chromatin domain occupied by the sex chromosomes during meiotic prophase in spermatogenesis (Solari 1974)(Handel 2004). For proper spermatogenesis, the timely proliferation of

spermatogonia, meiotic division of spermatocytes and post-meiotic maturation of spermatids is essential (La Salle, Sun et al. 2008). Indirect immunofluorescence studies using anti-SUMO antibodies in mouse and human testis show that SUMO is concentrated on the sex body during chromatin condensation, when it could facilitate synapsis between the X and Y chromosomes (Vigodner and Morris 2005; Vigodner, Ishikawa et al. 2006).

It is presently unknown which aspects of these SUMO localizations and functions may be widely conserved. In humans during meiotic prophase, SUMO1 has been reported to localize additionally to centromeric and pericentromeric heterochromatin, a pattern that is not observed in mouse (Vigodner, Ishikawa et al. 2006). Dejean and colleagues investigated a Ubc9 knockout mouse and studied the effects of its mutation on the SUMO-conjugating system (Dejean, 2005). Embryos deficient for Ubc9 die shortly after the blastocyst stage (Di Bacco and Gill 2006). Cells subjected to RNAi knockdown of SUMO proteases like SNEP5 display impaired growth, binucleated cells and dumbell-shaped or multi-lobed nuclei, suggesting a balance between sumoylation and desumoylation is important for progression of the cell cycle (Di Bacco and Gill 2006). Recent studies have further reported that developmental control of sumoylation pathway proteins is required for meiotic progression in mouse (La Salle, Sun et al. 2008).

Alternate achiasmate meiotic pathways have been described in some taxonomic groups such as Lepidopteran females and Dipteran males (Wolf 1994), and it is currently unclear if these systems also require sumoylation. The best characterized example of an achiasmate pathway is in *D. melanogaster* males, where there is no formation of SC

(Meyer, Hess et al. 1961). The male and female meiosis in Drosophila differ in that only males lack recombination (Meyer, Hess et al. 1961) (Rasmussen 1973). The *lesswright* mutation disrupts female meiosis but it is not known whether it has any effect in males. There are a number of genes that have been identified that affect male meiosis in Drosophila (Wakimoto, Lindsley et al. 2004). One of particular interest with respect to sumoylation is *dtopors*. The human homolog, Topors, can act as both an ubiquitin and SUMO ligase.

A discussion of the possible role(s) of *dtopors* in male meiosis first requires a brief description of this unusual system. To understand the dynamics of homolog pairing in male Drosophila, Vazquez, Belmont et al. (2002) designed an experiment that allowed them to follow specific chromosomal loci in living spermatocytes throughout all stages of meiosis, using a GFP-Lac repressor (GFP-Lac I)/lac operator(LacO) system as a tag. In this experiment, an array of LacO sequences were inserted at specific chromosomal locations and labeled using a green fluorescent-lacI fusion protein expressed in the male germ line. The chromosomes were then tracked live from mitotically dividing spermatogonia to mature spermatocytes, and through meiosis. Their results showed that most homologs are already paired before entering meiosis, the pairing frequency increases as cells transit from spermatogonia to spermatocytes and this pairing is observed for 13 different euchromatic lacO inserts tested. The pairing is sustained until mid-prophase I. At this stage, chromosomes reorganize and bivalents separate into nuclear territories (Cenci, Bonaccorsi et al. 1994). Shortly after the bivalents have separated into territories, both homologous pairing and sister chromatid cohesion appear

to be released all along the euchromatic regions (Vazquez, Belmont et al. 2002).. While maintenance of homologous pairing in male Drosophila is still not well understood, models suggest that it may be mediated by homolog chromosome entanglements (Duplantier, Jannink et al. 1995 ;Vazquez, Belmont et al. 2002), via cohesion in the heterochromatin or by the establishment of chromosome domains in spermatocyte nucleus (Vazquez, Belmont et al. 2002).

Although it is unclear how pairing is first established, genetic studies have identified a number of genes involved in regulating pairing maintenance. Tomkiel et al. (2000) have genetically and cytologically characterized a gene, *teflon*, specifically involved in the maintenance of autosome pairing. Analysis of four ethyl methanesulfonate (EMS)-induced mutations in this gene revealed autosomal nondisjunction in meiosis I specifically in male flies. They did not see a measurable effect on sex chromosomes, which suggests that sex chromosome and autosome segregation are at least in part controlled by different genetic pathways. These results led to the conclusion that *teflon* is involved in mediating or regulating the maintenance of autosomal homolog pairing in Drosophila male meiosis I (Tomkiel, Wakimoto et al. 2001).

In another study, Thomas et al. (2005) found two genes *stromalin in meiosis* (*snm*) and *modifier of mdg4 in meiosis* (*mnm*) that were subsequently shown to be involved in maintenance of homolog pairing in male Drosophila (Thomas, Soltani-Bejnood et al. 2005). The two gene products co-localize to sex chromosomes during prophase I and metaphase I, but are undetectable at anaphase I. Mutations in either gene

result in both sex and autosome nondisjunction, leading to the conclusion that they function in stabilizing homolog pairing (Thomas, Soltani-Bejnood et al. 2005). Thomas et al. (2005) also found that Teflon is required for MNM localization to autosomes, and presumably for its function in autosome pairing. They found that mutations in *teflon* do not affect the localization of MNM and SNM on sex chromosomes while they eliminate the localization of MNM on autosomes. A genetic screen for *teflon modifiers* identified *mnm* mutations, but not *snm* mutations, as enhancers of *teflon* (Thomas et al., 2007). This supports the model that Teflon recruits and stabilizes MNM to paired autosomes where the two proteins may interact to secure the connection between the autosomal bivalents (Thomas, Soltani-Bejnood et al. 2005), while a different factor may be required to recruit and stabilize SNM and MNM to paired sex chromosomes. This study did not indicate whether SNM and MNM have any role in establishing homolog pairing, but rather they proposed that these proteins help to maintain pairing in the absence of crossovers during meiosis in Drosophila males.

The strongest suggestion of a role of sumoylation in fly male meiosis comes from the phenotype of mutations in *dtopors*, the fly homolog of human Topors (topoisomerase I interacting RS rich), a nuclear protein that was first identified as a topoisomerase I-binding protein (Haluska, Saleem et al. 1999). Topors is the only identified protein possessing dual ligase activities, acting as both a ubiquitin and SUMO ligase, (Rajendra, Malegaonkar et al. 2004) (Pungaliya, Kulkarni et al. 2007). Homozygous *dtopors* mutant males produce aneuploid progeny in which meiosis I segregation of both autosomes and sex chromosomes is disrupted. Mutations in *dtopors* also disrupt the nuclear lamina in

male germline cells. During meiosis in Drosophila males the paired homologs move in to separate domains in the nucleus and are in close association with the nuclear lamina. Vazquez, Belmont et al. (2002) in their study speculated that at this stage maintenance of pairing between homologs may involve tethering to the nuclear lamina (Vazquez, 2002). A condensation defect observed by Matsui, Sharma and Tomkiel (unpublished) in their study of *dtopors* may be a consequence of lamina disruption. Nuclear blebbing is also observed in spermatocytes of *dtopors* mutants (Wakimoto, Lindsley et al. 2004). In this phenotype the integrity of the nuclear structure is abrogated resulting in one or more protrusions or blebs emanating from the nucleus. In addition, in some cells, the perpendicular centriole components of centrosomes appear to precociously separate at meiosis I (Matsui, Sharma & Tomkiel unpublished data).

Human Topors has been studied extensively, as there are suggestions that it may act as a tumor suppressor, and it has also been identified as a cause of retinitis pigmentosum. The interaction between Topors and Topoismomerase I has been found to occur through the N-terminal 250 amino acids of human Topoisomerase I (TopoI), and was detected in a combination of yeast two-hybrid and *in vitro* binding assays (Haluska, Saleem et al. 1999). This interacting region in Topo I has many significant binding partners like nucleolin (Bharti, Olson et al. 1996), TATA-binding protein (Merino, Madden et al. 1993) and SV40 T antigen (Haluska, Saleem et al. 1999). This suggests that Topors may be involved in regulating the interaction of TopoI with its binding partners. Topors is also found to interact with p53 (Zhou, Wen et al. 1999), a putative prostate tumor suppressor NKX3.1 (Guan, Pungaliya et al. 2008), DJ-1 (Shinbo, Taira et

al. 2005), a protein highly expressed in normal human lung (LUN) (Chu, Kakazu et al. 2001) and regulatory proteins of adeno-associated virus type-2 (AVV-2) (Weger, Hammer et al. 2002). Topors has also been shown to increase viral gene expression (Weger, Hammer et al. 2002). Topors is the only protein to date known to have both E3 ubiquitin and SUMO-1 ligase activity (Saleem, Dutta et al. 2004; Weger, Hammer et al. 2005).

Several studies suggest that Topors may act as tumor suppressor by negatively regulating cell growth. A study on human colon adenocarcinomas showed that the expression of *topors* mRNA is downregulated in human colon adenocarcinomas, along with an increase in methylation of a CpG island in the *topors* gene promoter (Saleem, Dutta et al. 2004). The further study of the publicly available cDNA microarray database Oncomine by Rhodes and Kalyana-Sundaram et al. (2007) reveals that Topors mRNA is also downregulated in seminomas and ovarian cancers, while in bladder, myeloma, endometrial, prostate and salivary gland tumors it is found to be overexpressed (Rhodes, Kalyana-Sundaram et al. 2007). Depending on the cellular context Topors may be both oncogenic and tumor suppressive.

More than 50% of human cancers contain mutations in the p53 gene (Levine, Chang et al. 1994). p53 is a human tumor suppressor that is universally expressed in vertebrates and has important functions in cell cycle control, cell differentiation, apoptosis, gene regulation and tumor suppression. p53 induces gene expression in response to DNA damage which ultimately leads to either cell cycle arrest or apoptosis

(Zhou, Wen et al. 1999). *In vitro* ubiquitination assays show that in concert with E1 and E2 enzymes, Topors induces the formation of polyubiquitin chains. Topors *in vitro* directs the ubiquitination of p53. The *in vivo* assays revealed that Topors also directs p53 ubiquitination when overexpressed in both $MDM2^{+}$ and $MDM2^{-}$ cell lines. This suggests an analogy between Topors-p53 and MDM2-p53 relationship. Topors also decreases the protein levels of p53 in human osteosarcoma cell lines, confirming that Topors triggers the degradation of p53 *in vivo* (Rajendra, Malegaonkar et al. 2004).

The NK class homeobox NKX3.1 is the most extensively studied transcription factor that acts as a putative tumor suppressor in prostate cancer development and carcinogenesis (Shen and Abate-Shen 2003). The GST pull-down assays conducted by Guan et al. (2008) demonstrated that NKX3.1 directly interacts with Topors *in vivo*. *In vitro* studies revealed that NKX3.1 is both mono- and poly-ubiquitinated by Topors. The overexpression of Topors resulted in downregulation of NKX3.1 protein levels. The colocalization study revealed that both NKX3.1 and Topors localize as nuclear speckles with TopoI in a LNCaP cell line (androgen-sensitive human prostate adenocarcinoma cells). Furthermore, *in vivo* studies discussed earlier suggest that Topors may actively degrade a pool of NKX3.1 protein in the PML bodies (Guan, Pungaliya et al. 2008).

The ubiquitination activity is conserved in the Drosophila Topors homolog although it may have different targets. Dtopors regulates early embryonic patterning by ubiquitinating the transcription factor Hairy. The levels of hairy protein decrease as *dtopors* is overexpressed in the cell line and deletion of *dtopors* suppresses the embryonic

patterning defect seen in embryos with a hypomorphic *hairy* mutation. Thus Dtopors decreases the levels of Hairy in embryos (Secombe and Parkhurst 2004). Dtopors was not found to ubiquitinate p53 (Secombe and Parkhurst 2004), which indicates that there may be a different requirement of the p53-mediated pathway in flies (Rong, Titen et al. 2002; Xie and Golic 2004).

In flies, several novel roles of Dtopors in transcriptional regulation and nuclear organization have been discovered. Dtopors interacts with proteins of the Gypsy insulator complex and is required for Gypsy insulator function (Capelson and Corces 2005). Chromatin insulators are proposed gene regulatory elements involved in the establishment of independent chromatin domains, and are thought to play an important role in regulating the proper expression of independent gene units. This is hypothesized to be achieved by organizing the chromatin into structural domains that enable the autonomy of gene activity (Geyer and Corces 1992). Chromatin insulators have been shown to have enhancer-blocking activity, as they are able to oppose promoter-enhancer communication (Geyer and Corces 1992 ; Kellum and Schedl 1992) . They have also been proposed to have barrier activity, as they protect incorporated transgenes from the influence of the neighboring chromatin (Chung, Whiteley et al. 1993 ; Kellum and Schedl 1992). The gypsy insulator of *D. melanogaster* is a protein complex that consists of three known components, Suppressor of Hairy wing (Su(Hw)), Modifier of mdg4 2.2 (Mod(mdg4)2.2), (Ghosh, Gerasimova et al. 2001 ; Gause, Morcillo et al. 2001), and Centrosomal Protein 190 (CP190) (Pai, Lei et al. 2004). Analysis of Drosophila polytene

chromosomes shows that insulator complexes are found at multiple endogenous sites dispersed throughout the fly genome (Gerasimova and Corces 1998).

Capelson and Corces (2005) have proposed that the role of Dtopors in gypsy insulator activity is to direct the formation of chromatin domains by promoting the association between nuclear insulator complexes and the nuclear lamina. From a combination of co-immunolocalization assays, co-immunoprecipitation experiments and yeast two-hybrid assays, they have shown that dTopors interacts directly with the proteins of the gypsy insulator and with the major lamina protein Lamin Dm_0. Mutation of Mod(mdg4)2.2 leads to disruption of nuclear clustering of insulator complexes and perturbs insulator activity, but overexpression of Dtopors in the *mod(mdg4)2.2* null mutant restores insulator activity and the formation of nuclear insulator bodies. Capelson and Corces (2005) demonstrated that mutations in Lamin Dm_0 not only perturb Dtopors localization but also Gypsy insulator activity and nuclear organization

Based on knowledge of the Topors and Dtopors activity as E3 ubiquitin ligases (Rajendra, Malegaonkar et al. 2004 ; Secombe and Parkhurst 2004) and Topors as an E3 SUMO ligase (Weger, Hammer et al. 2005 Capelson and Corces (2006) explored the possibility that Dtopors ubiquitination or sumoylation activity may be involved in regulating gypsy insulator activity. They generated a *dtopors* transgenic construct carrying a point mutation, which changes a highly conserved cysteine of the RING domain to a serine (C118S). Mutation of this conserved residue has been demonstrated to disrupt the ubiquitin ligase activity of MDM2, a mammalian RING finger protein

(Honda and Yasuda 2000). This mutation disrupted insulator function. None of the known insulator complex proteins however, could be demonstrated to be ubiquitinated by Dtopors.

Further experiments, both *in vivo* and *in vitro*, showed that Dtopors may negatively regulate the sumoylation of CP190 and Mod(mdg4)2.2. In an *in vitro* experiment, Su(Hw), Mod(mdg4)2.2 and CP190 were used as substrates in a sumoylation reaction with or without dTopors. All three insulator proteins are potential targets for sumoylation as they possess lysines that are located in a SUMO modification consensus motif ψKxE (Capelson and Corces 2006). Each reaction consisted of incubating the E1, E2 enzymes, SUMO, with *in vitro*-transcribed and -translated ^{35}S-labeled substrate protein and *in vitro*-generated or -purified recombinant Dtopors. The results showed that CP190 and Mod(mdg4)2.2 were SUMO-modified as characterized by higher molecular weight bands in presence of sumoylation machinery. However, adding Dtopors decreased CP190 and Mod(mdg4)2.2 sumoylation instead of enhancing it (Capelson and Corces 2006). In an *in vivo* experiment, Capelson and Corces (2005) overexpressed Dtopors in larvae using a *UAS-dtopors* transgenic construct driven by an *actin-GAL4* (*ActGAL4*) promoter. Western blot analysis of protein extracts from larvae showed a decrease in sumoylated forms of Mod(mdg4)2.2 and CP190 when Dtopors was induced compared to uninduced. Furthermore, mutations in components of the SUMO conjugation pathway improved the enhancer-blocking function of a partially active insulator (Capelson and Corces 2006). Based on these findings, it was proposed that SUMO modification of insulator complex proteins negatively regulates the activity of the gypsy insulator. From

these studies, dTopors has been proposed to regulate the gypsy insulator activity by downregulating insulator sumolylation.

Insulator complexes may play a yet unrecognized role in male meiosis. A meiotic isoform of the insulator complex protein Mod(mdg4), MNM is required for maintaining pairing during male meiosis (Thomas, Soltani-Bejnood et al. 2005). Although MNM has not been demonstrated to bind to Dtopors, it shares a common BTB-POZ protein interacting domain with its somatic isoform Mod(mdg4)2.2 which interacts with Dtopors (Capelson and Corces 2005). Thus there can be a link between MNM and Dtopors in relation to interaction between chromatin and nuclear lamina that is important for maintaining pairing in male Drosophila. It can be further studied whether MNM is sumoylated by Dtopors and whether sumoylation of MNM affects pairing.

This study will report the alteration in sumoylation by *dtopors* and propose a model to understand the role of *dtopors* in sumoylation in male *D. melanogaster*.

CHAPTER II

MATERIALS AND METHODS

Competent cells

The Scott-Simanis transformation protocol (M. Montiero, personal communication) was used to prepare competent cells. On the first day, a frozen stock of *E.coli* DH5α cells was used to streak a Ψa plate (5g/L Bacto-yeast extract, 20g/L Bactotryptone, 5g/L $MgSO_4*7H_2O$, pH 7.6, 14g/L BactoAgar) and incubated overnight. On the second day, a single resulting colony was picked to inoculate 5 ml Ψ_β (5g/L Bacto-yeast extract; 20g/L Bactotryptone, 5g/L $MgSO_4*7H_2O$, pH 7.6) medium and incubated overnight at 37°C at 250 rpm in an orbital shaker. On the third day, the 5 ml overnight culture was used to inoculate 500ml of prewarmed Ψ_β medium and further grown at 37°C at 250 rpm in an orbital shaker until the $OD_{590}$was approximately 0.48. Cells were cooled on ice for 5 min then spun down at 6k for 5 min at 4°C. Pre-chilled pipettes were used to resuspend the cells gently in 100 ml of ice cold TfbI (30 mM potassium acetate, 100 mM $RbCl_2$, 10 mM $CaCl_2$ * 2 H_20, 50 mM $MnCl_2$ * 4 H_20, 15% glycerol (v/v), pH 5.8). The mix was left on ice for 5 min then spun down at 6K for 5 min at 4°C. The cell pellet was then resuspended in 12.5 ml of ice cold TfbII (10 mM MOPS, 75 mM CaCl2*2H_20, 10 mM $RbCl_2$, pH 6.5, 15% glycerol (v/v)) and

incubated on ice for 10 to 15 more min. 200 µl aliquots were snap frozen in liquid nitrogen and stored at -80°C.

Bacterial transformation

Competent DH5α cells were thawed on ice. 100 µl of cells were used per transformation. Different amounts (10 ng, 5 ng and 1 ng) of pCaSpeR-hs plasmid DNA (containing an ampicillin-resistance gene) were added to cells and incubated on ice for 20 min. The cells were then heat-shocked at 42°C for 2 min then returned on ice for 2 min before adding 1 ml LB (10g/L Bacto-Tryptone, 5g/L Bacto-yeast extract, 10g/L NaCl, pH 7.0) and incubating for 1 hr at 37°C. Cells were then plated on LB amp plates (10 g/L Bacto-tryptone, 5 g/L Bacto-yeast extract, 10 g/L NaCl, 15g/L Bacto-agar, 50 ug/ml Ampicillin, pH 7.0) and incubated at 37°C overnight.

Vector preparation

The fly vector used was pCaSpeR-hs which expresses the *white* gene and contains a multiple cloning site preceded by the *hsp70* basal promoter (Figure 1). The starting vector contained a portion of the *D. melanogaster teflon* cDNA cloned into the *Bam*HI-*Not*I sites, in-frame with the EGFP cDNA, which was cloned into the *Not*I-*Xba*I sites, (pCaSpeR-hs/tefEGFP331-end). A sample of the purified vector (5µl) was examined by electrophoresis on a 1% agarose gel to estimate the DNA concentration. DNA was quantified by absorbance at 260 nm.

PCR amplification of SUMO cDNA and generation of the fragment

The *smt3* cDNA (*D. melanogaster* SUMO homolog, LD07775, Stock # 2904) cloned in pBluescript_SK(-) vector was obtained from the Drosophila Genomics Resource Center (DGRC,https://dgrc.cgb.indiana.edu) as spots dried on FTA filter paper discs. The plasmid containing *smt3* cDNA was extracted from the filter paper by following the protocol supplied by DGRC (Kris Klueg Whatman Protocol). 1X TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA) was added into the microfuge tube containing the disc quickly two times and removed immediately. The microfuge tube was then kept on ice and 50µl of competent cells were added. The cells/disc mixture was allowed to incubate on ice for 30 min. Halfway into the ice incubation period the cells/disc mix was vortexed for one sec and immediately returned to the ice. Then the *smt3* cDNA-bearing plasmid was transformed in to *E.coli* DH5α cells. A single fresh colony from the transformation plate was used to inoculate 500 ml LB amp medium and incubated at 37°C overnight at 250 rpm in an orbital shaker. Cells were harvested and *smt3*cDNA plasmid was purified using a plasmid maxi kit (Qiagen, Valencia, CA). A sample of the purified plasmid (5 µl) was examined by electrophoresis on a 1% agarose gel to estimate the DNA concentration and verify its integrity. DNA was quantified by absorbance at 260 nm.

The full-length *smt3* cDNA was amplified from the plasmid DNA by PCR using the primers *Eco*RI_SUMO_Forward primer (5'-GCGAATTCATGTCTGACGAAAAGAAG - 3') and *Not*I_SUMO_Reverse primer (5'-TAGCGGCCGCAGTAATCTTATGGAGCGC - 3'). PCR amplification cycling

conditions were 30 cycles of denaturation at 95°C for 30 sec, annealing at 58°C for 30 sec and elongation at 72°C for 30 sec. All primers were purchased from MWG biotech (Huntsville, Alabama). PCR products were purified using Qia-quick kit (Qiagen, Valencia, CA) and each DNA product was verified by agarose gel electrophoresis before and after purification.

Cloning *smt3 cDNA* into *pCaSpeR-hs/tef(331-end)-EGFP* vector

The *pCaSpeR-hs/tef(331-end)-EGFP* vector and *smt3* cDNA were doubly digested with *Eco*RI and *Not*I restriction enzymes (Promega, Madison, WI). Digest products were purified using Qia-quick kit (Qiagen, Valencia, CA) and yield was estimated using agarose gel electrophoresis. The *pCaSpeR-hs/tef(331-end)-EGFP*vector digested product was run on a agarose gel to verify that the *tef* fragment was cleaved off the vector. The digested *smt3* was ligated into the resulting vector downstream from the *hsp70* promoter and upstream of *EGFP* (Figure 1) using 1x ligase buffer and T4 ligase (Promega, Madison, WI). Ligation products were transformed into DH5α competent cells and transformants grown overnight at 37°C on LB amp plates. Resulting colonies were used to inoculate 5 ml LB amp media and were grown overnight at 37°C in an orbital shaker at 250 rpm. The plasmid DNA from each clone was extracted and purified following the mini-prep protocol (Sambrook 1989) and the DNA yield was estimated using agarose gel electrophoresis.The mini-prep DNA was digested with *Eco*RI and *Not*I enzymes and the resulting products were separated by agarose gel electrophoresis to test for the presence of the *smt3* insert. One colony containing the insert was selected then

used to inoculate 500 ml LB amp medium, and was grown at 37°C overnight at 250 rpm in an orbital shaker. The plasmid DNA was extracted and purified using a maxi-prep DNA purification kit (Qiagen, Valencia, CA). After confirming the purification yield by gel electrophoresis and absorbance at 260 nm, the plasmid DNA was sent to MWG Biotech (Highpoint, NC) for DNA sequencing of the cDNA insert.

Drosophila culture and stocks

A w^{1118}fly line was used for establishing the transgenic lines for the *smt3* transgenic flies. For *smt3* transgene rescue assays the fly stocks used were *P{ry[+t7.2]=PZ}smt3[04493] cn[1]/CyO; ry[506] , Df/CyOS*^{2}*cnbw* and *w[1118]; PBac{w[+mC]=WH}Topors[f05115]/CyO*. The *smt3* transgenic lines were generated as described in Figure 2. All flies were grown on standard cornmeal, molasses, yeast, agar medium at room temperature (25°C).

Transgenic fly stocks

The *pCaSpeRhs smt3-EGFP*construct was sent to a commercial fly injection company to be injected in the fly embryos (Genetics Services, Inc., Salisbury, MA). We received 200 injected embryos per clone which gave our first generation (G_0) of transgenic flies. Only 95 of the injected embryos survived. All the G_0 males were individually crossed with 5 w^{1118} virgin females and all G_0 females were crossed with 3 w^{1118} males. From $G_{1,}$ or the second generation, only w^{+} flies were collected and used to establish independent transgenic fly lines. The flies were selected from G_1 flies were crossed with w^{1118} flies and the offspring produced in G_2 were intercrossed. Homozygous

transgenic flies were selected based on the eye color intensity and were used to establish stable transgenic fly stocks for each line.

Transgene mapping

Transgenes were mapped to individual chromosomes on the basis of the patterns of segregation of the [w^+] marker from second and third chromosome dominant markers. We crossed 2-3 males transgenic flies (*w1118/Y*) from each of the transgenic lines with 5 virgin *yw; tef* ^{k15914}*/Cy; Sb/TM3, Ser; spa*^{pol}females. F1 [w+], Cy and either Sb or Ser males were crossed to w^{1118}females and the offspring were scored to map the transgenes. If all males were white then the transgene was on X chromosome. If all Curly flies were white then the transgene was on 2nd chromosome. If all Serrate or Stubble flies were white then the transgene was on 3rd chromosome. If none of the above, the transgene was on 4thchromosome.

Transgene rescue assay

The *P{ry[+t7.2]=PZ}smt3[04493] cn[1]/CyO; ry[506]* fly line containing a null mutation in *smt3* gene (Schnorr, 2001) was obtained from the Bloomington Stock Center (www.flybase.org). The w^{1118}*; PBac{w[+mC]=WH}Topors[f05115]/CyO* and *Df/CyO Roi cn bw* flies were used in the rescue assay. The *smt3; PCaSpeR hs smt3-EGFP* flies were generated by the crossing scheme shown in Figure 2. The fly lines generated were kept at room temperature, or alternatively, were heat-shocked for 1 hr each 8 hrs of development. The flies were scored every 2 days starting from the 12th day till the 18th day of the cross.

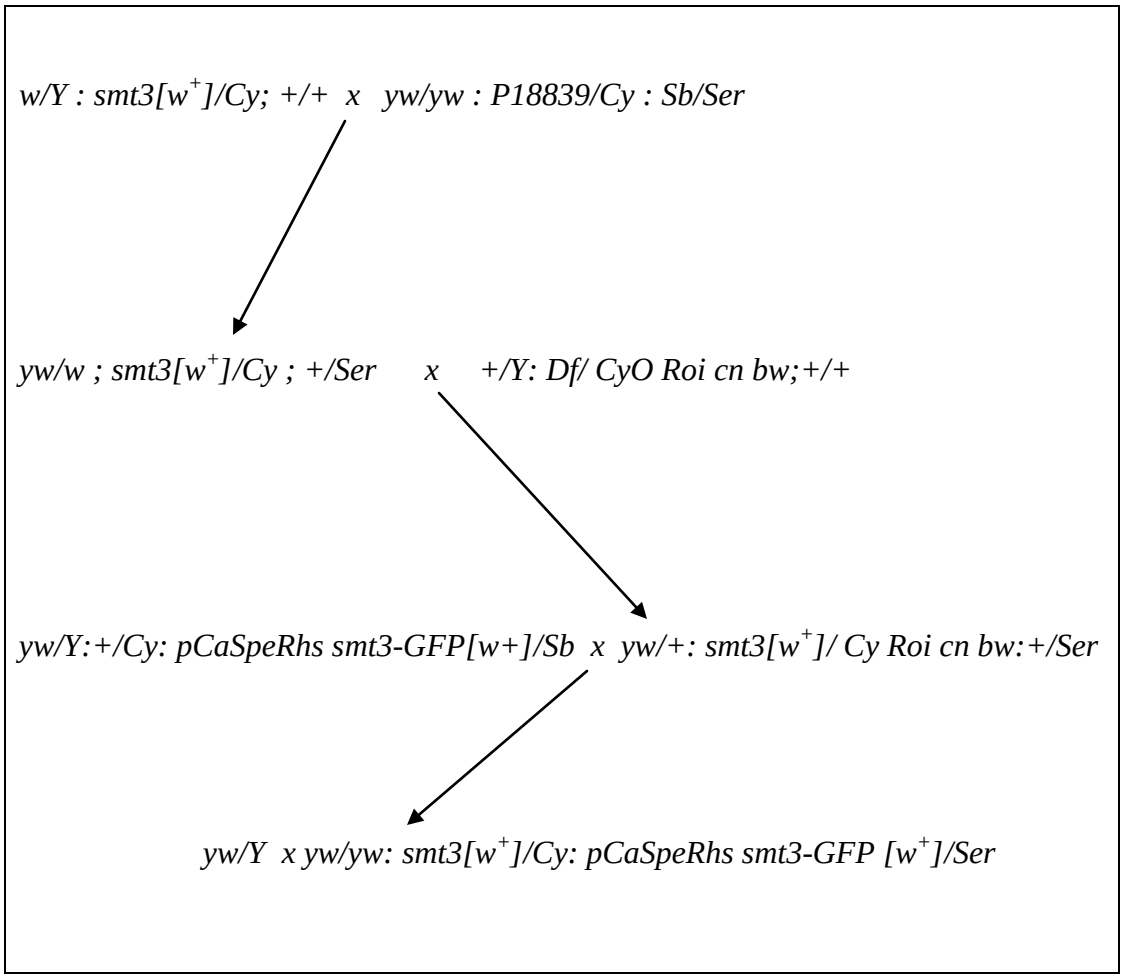


Figure 1: The scheme for *smt3* transgene rescue experiment.

Expression of the Smt3-EGFP fusion proteins

Homozygous *smt3-EGFP* flies and larvae were heat-shocked by incubation at 37°C for 1 hr each 8 hrs throughout development. Male flies were dissected to collect testis. Salivary glands were dissected from third instar larvae. All dissections were performed in Schneider's insect tissue culture media (GIBCO BRL, Gaithersburg, MD). Tissues were transferred to a fresh drop of Phosphate Buffered Saline (PBS; 137 mM

NaCl, 10 mM Phosphate, 2.7 mM KCl, pH 7.4) on a microscope slide and covered with a coverslip. The tissues were then viewed immediately using an Olympus Fluoview FV500 confocal laser scanning microscope to detect and record EGFP signal in living tissue.

Preparation of protein extracts and Western Blot analysis

Df(2R)Topors^{AA} and Canton-S strains were used for collecting testes for Western blot analysis. Testes were dissected from 1-day-old adult flies in Schneider's Medium (GIBCO BRL, Gaithersburg, MD) and extracts were sonicated for 1 min in Sample Buffer (50 mM Tris:HCl,pH 6.8,15% Sucrose, 2 mM EDTA, 3% SDS and 0.01% Bromophenol Blue) with or without Inhibitor Cocktail (0.1 mM MG132, 0.1 M P2714, 80 mM NEM and 0.4 mM IAA) (Sigma-Aldrich, St Louis, MO). An equal number of testes of each sample group were loaded into separate wells of a 10% polyacrylamide gel. Molecular weight marker (Amersham Biosciences, Piscataway, NJ) was loaded into one lane of the gel. Proteins were resolved by 10% SDS-PAGE for 12-18 hrs at 50-60 V and then transferred to Immobilon-P PVDF membranes (Millipore, Billerica, MA) in transfer buffer (25 mM Tris, 192 mM Glycine, 10% Methanol).

Western blots were initially stained with Ponceau S to visualize the proteins and confirm equal loading and transfer. Then the blots were probed with rabbit anti-C-terminal Smt3 at a 1: 100 dilution (Abgent, San Diego, CA**)** and mouse anti-tubulin (Developmental Studies *Hybridoma Bank*, Iowa City, IA) at a 1:1000 dilution. Antibodies were diluted in either PBS containing 3% fraction V bovine serum albumin (Fisher Scientific, Fair Lawn, NJ) or in Tween-Tris Buffered Saline (TTBS) (0.15 M

NaCl, 0.01 M Tris pH 8.0, 500 µl Tween) containing 1% Non-fat dried milk (NFDM). Goat anti-rabbit or goat anti-mouse horseradish peroxidase- conjugated secondary antibodies (Sigma-Aldrich, St Louis, MO) were used at a 1:5000 dilution in 1% NFDM TTBS. Hybridization was detected by Enhanced Chemiluminescence (ECL) using the Supersignal West Pico Chemiluminescent detection kit (Thermo Scientific, Rockford, IL). Imaging was performed on a Biorad Chemi Doc XRS imager using the Quantity One software. All signals were normalized using the anti-tubulin signals to adjust for minor variations in protein loads. The blots were done in triplicate, and the means and standard deviations for band intensities were measured using Quantity One software and a Bio Rad image aquisition apparatus.

Statistics

For Western Blots three biological replicas were used to determine the sumoylation of testis protein. *P*-values were determined using a two-tailed student T-test. For the transgene rescue assay the percentage rescue is calculated as (Cy+ flies observed)/(Cy+ expected if 100% survived), e.g. (Cy+ flies observed)/(1/3). The *P*-values were determined based on chi-squared values determined from a contingency test.

CHAPTER III

RESULTS

Alteration of sumoylation by *dtopors*

While it is known that human topors acts as both a ubiquitin and SUMO ligase *in vitro* and *in vivo* (Rajendra R, 2004; Weger, 2005; Pungaliya P, 2007) the ability of the fly homolog Dtopors to directly sumoylate other proteins has not been demonstrated. There is, however, evidence that Dtopors can at least indirectly affect the status of sumoylation of proteins in the fly soma. It has been suggested that the function of the gypsy chromatin insulator complex is negatively regulated by sumoylation. The components of gypsy insulator complex CP190 and Mod (mdg4)2.2 are hyper-sumoylated when the dose of *dtopors* is halved (Capelson and Corces, 2006). To determine if *dtopors* might similarly affect sumoylation in the testis, we looked for changes in the patterns of sumoylated testis proteins in *dtopors/+* and *dtopors* flies. On western blots probed with anti-SUMO antibodies, we observed variation of sumoylation in both genotypes compared to that of a wild type Canton S strain. These blots were done in triplicate, and the means and standard deviations for band intensities were measured. All intensities were normalized for a signal generated by probing the same blots with an anti-beta tubulin antibody. Tubulin appears to be a suitable control for this tissue, as estimation of protein loads from Coomassie-stained gels and Ponceau-stained blots were comparable to estimations made using anti-tubulin signal intensities. An example of the blots is shown in Figure 2. Surprisingly, for a number of protein bands, we found greater differences between wildtype and *dtopors/+* flies than *dtopors* homozygous flies. First,

the pool of free SUMO was lower in *dtopors*/+ versus *dtopors/ dtopors* testis (P< 0.014). The pool of free SUMO was also significantly less in *dtopors*/+ than in wild type Canton S (P <0.04) (Figure 3). These results suggest an effect of *dtopors* in the germline on SUMO production, stability and/or amount of sumoylation or cleavage of SUMO from target proteins. When we looked at sumoylated proteins we also observed alterations in the abundance of sumoylated forms. The approximate molecular weights of these proteins were calculated by comparison to the migration of molecular weight standards. Based on these calculations we assigned the approximate mass of each band in the Western blot. We observed significant increases in sumoylated proteins of approximate molecular masses of 150 and 105 kd in *dtopors* homozygous mutants when compared with both Canton S and *dtopors*/+ testis (P <0.045, Figure 2 and 4). Although there is an apparent difference in protein band intensity at about 190 kd between *dtopors* homozygous mutants and the wild type Canton S, it is not statistically significant (P >0.035, Figure 4). The protein bands at ~62 kd show significant differences in intensity between homozygous and heterozygous *dtopors* mutants (P <0.03, Figure 4), but there was no significant difference observed between wild type and mutants. For the lower molecular weight bands of ~33 kd and 29 kd the signal decreased significantly when the dose of *dtopors* was halved when compared to the wild type Canton S (P <0.02, Figure 4). In case of the *dtopors* null mutant, however, the signal was increased in comparison to heterozygous mutant (P < 0.043), while it was less than observed in wild type (P <0.05, Figure 4).

As the nuclear lamins are found to be disrupted by *dtopors* mutation (Matsui, Sharma, Tomkiel, unpublished data), we examined the sumoylation status of Lamin C to see if it was one of these proteins differentially modified in mutant testis. Western blot analysis was performed in the presence of both sumo isopeptidase inhibitors and proteosome inhibitors, allowing visualization of higher molecular weight bands corresponding to either ubiquitinated or sumoylated forms. Our results indicated that Lamin C is not detectably ubiquitinated or sumoylated. This is shown in the lower half of Figure 2.

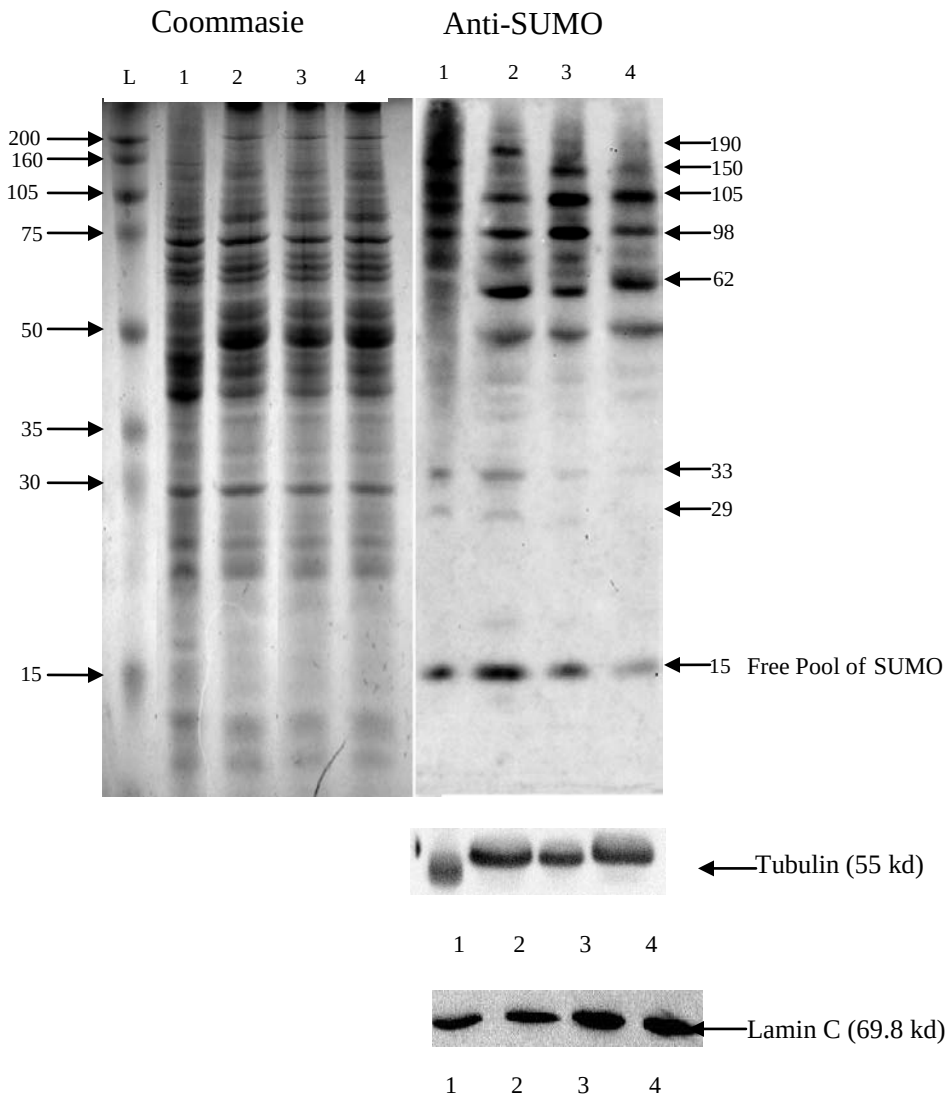


Figure 2: Alteration of sumoylation by *dtopors*. The left half of the picture is a Coommasie stained gel, where L is the molecular weight marker and lane 1 is Canton S testis sample without the Inhibitor Cocktail (IC), lane 2 is Canton S testis with IC, lane 3 is homozygous *dtopors* testis with IC and lane 4 is heterozygous *dtopors*/+ testis with IC. The right half of the picture is a Western blot probed with anti-SUMO antibodies, where lanes 1-4 are the same as the Coommassie stained gel lanes.

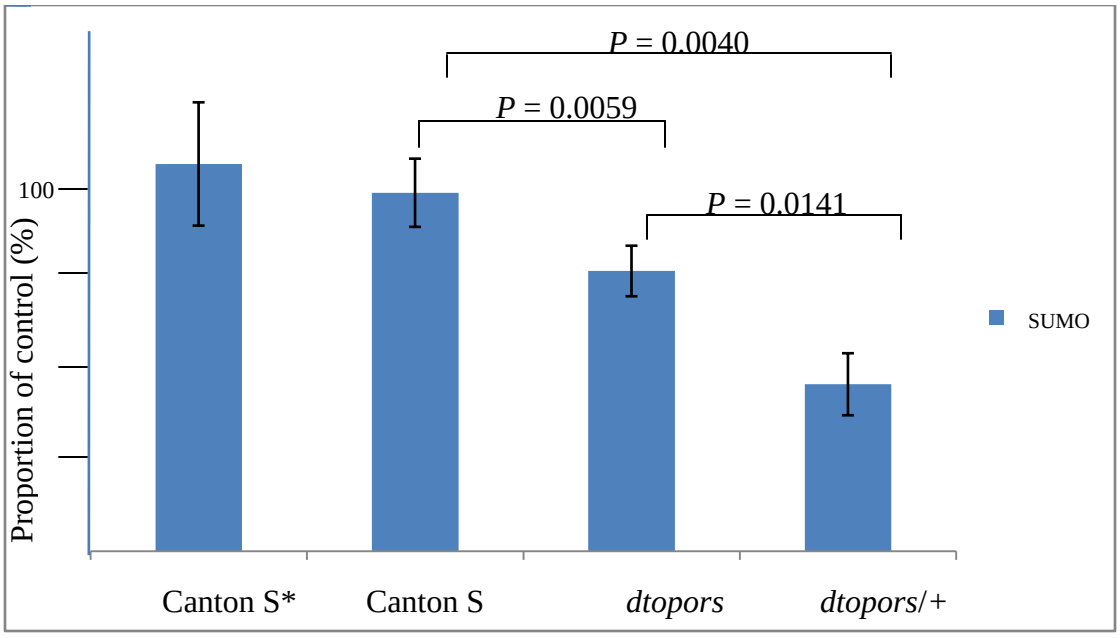
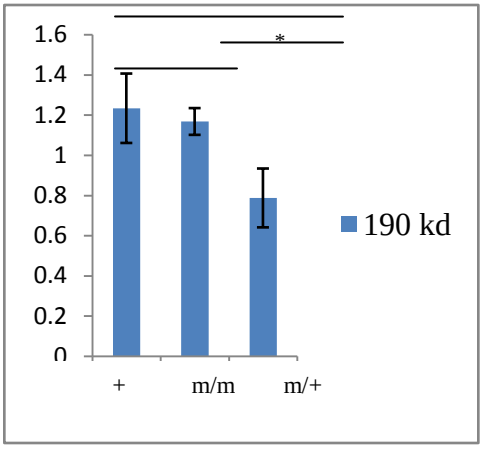


Figure 3: The pool of free SUMO is altered by *dtopors*. Quantitation of anti-SUMO signal on Western blots of total testis protein from flies of the indicated genotypes. The asterisk indicates the absence of Inhibitor cocktail (See Materials and Methods for details). All signals were normalized using an anti-beta tubulin signal as a loading control, and then adjusted by setting the Canton S value to 1.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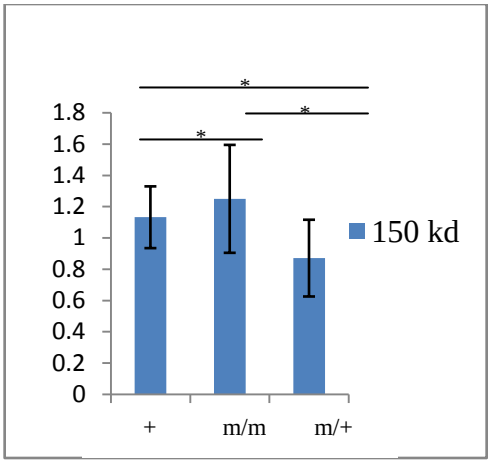
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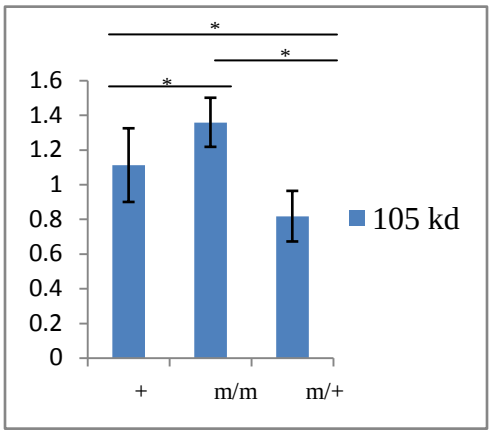
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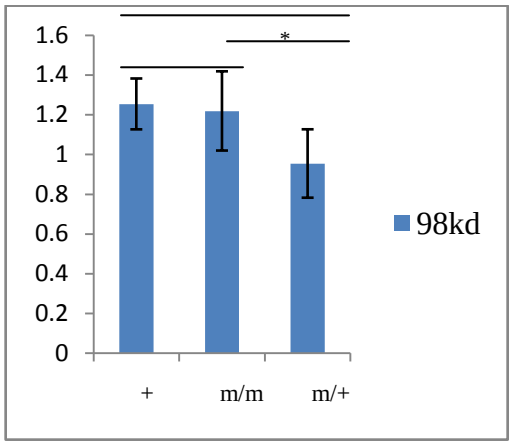
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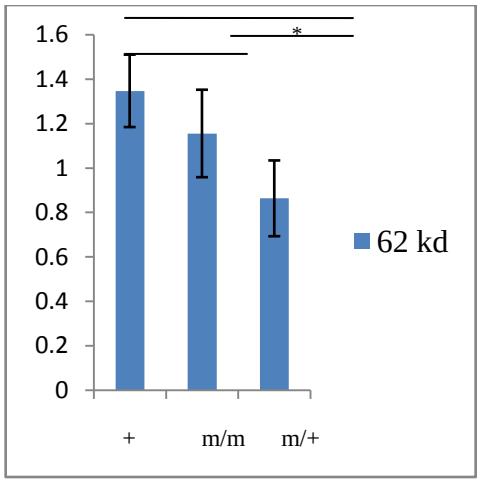
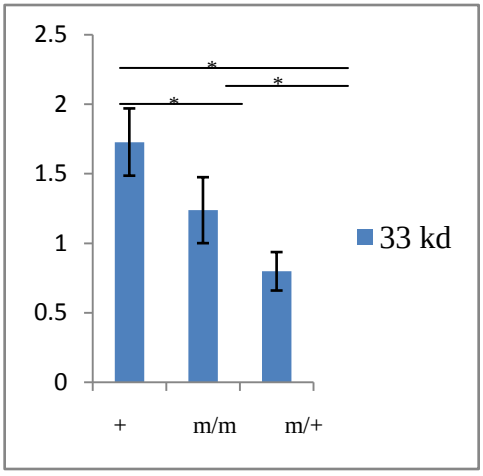
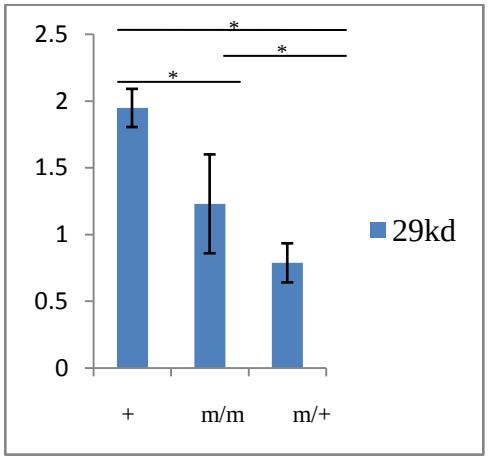






Key for P value

* indicates P value <0.05

Figure 4: Analysis of alteration of sumoylation by *dtopors*. +, m/m and m/+, indicate Canton S, *dtopors* and *dtopors*/+ genotypes, respectively. All protein samples were prepared in IC. All signals were normalized using an anti-beta tubulin signal as a loading control, and then adjusted by setting the Canton S value to 1.

Generation of vector for expressing SUMO-EGFP fusion protein

We have shown that Dtopors alters the profile of sumoylated proteins and the pool of free SUMO in the germline of the male. These changes could result from altering the dynamics of SUMO, i.e. changing the activity of isopeptidases, or the rate of turnover or recycling of SUMO. Alternatively, the pool of free SUMO may change because more or less SUMO is conjugated or because more or less SUMO is synthesized. To examine these possibilities we developed a tool to investigate the pool of unused SUMO versus recycled or processed SUMO.

We created a construct that would produce a SUMO protein that was tagged at its C-terminus with EGFP. In the process of sumoylation, the SUMO molecule undergoes cleavage by SUMO isopeptidases at the C-terminus (Li SJ, 2000; Takahashi Y, 2000). Our construct will allow us to compare the amount of intact fusion protein versus processed protein to estimate the relative changes in processed versus unprocessed via isopeptidase cleavage.

We used PCR to generate a cDNA fragment that encodes full-length SUMO and verified the correct size of the resulting PCR product by agarose gel electrophoresis (Figure 5). A fly transformation construct expressing a SUMO-EGFP fusion protein was generated by subcloning the SUMO cDNA into the pCaSpeRhs expression vector, in which the EGFP cDNA had already been inserted. This vector contains the hsp70 promoter that drives expression of the fusion protein upon heat shock. Putative clones were transformed into DH5α cells and plasmid DNA purified (see Materials and

Methods). The purified DNA was digested with *EcoR*I and *Not*I restriction enzymes and separated by agarose gel electrophoresis to verify the presence and size of the insert. The expected fragment size of 398 kb was confirmed (Figure 6). To ensure that no mutations had been introduced by PCR the insert of each clone was verified by DNA sequencing.

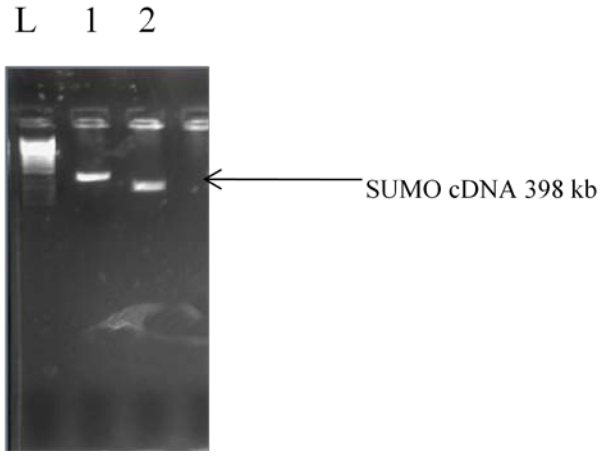


Figure 5: PCR amplification of SUMO cDNA. The arrow indicated the 398 kb PCR amplified SUMO cDNA in lane 2 of an agarose gel. L represents exACTGene 24kb Max DNA ladder Lane 1 shows PCR product of EGFP molecule.

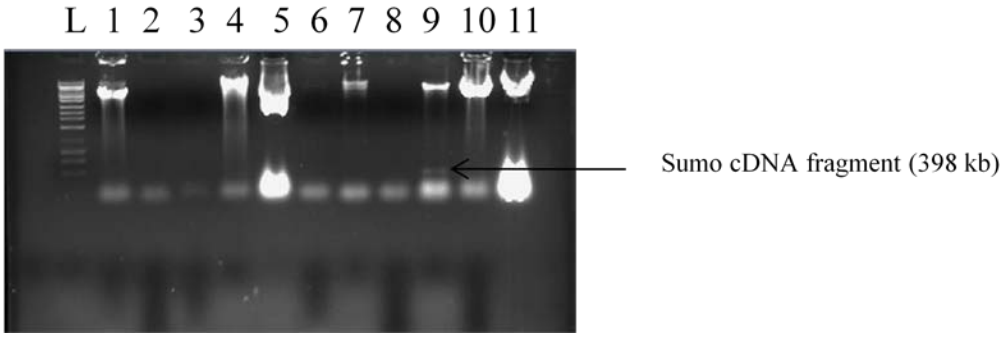


Figure 6: pCasperhs/SUMO-EGFP clone. Fragments of all prospective SUMO cDNA clones were separated by agarose gel electrophoresis after restriction enzyme digestion with *EcoR*I and *Not*I. Only the clone at the lane number 9 contains the SUMO fragment of the correct size. L represents exACTGene 24kb Max DNA ladder.

Generation of transgenic flies

Two hundred injected embryos were received for the clone, and 95 flies survived to give our first generation (G_0) of transgenic flies. Each G_0 fly was crossed with 5 w^{1118} flies (see Materials and Methods) to produce the second generation (G_1). From each G_0 cross, a maximum of two transgenic w^+ G1 flies were collected, and these flies were used to generate 22 (8♀, 12♂) independent transgenic fly lines. Ultimately, only a single line was kept from each G_0 parent to insure that each line established was independent. Resulting w+ G2 offspring were collected and intercrossed. Homozygous G3 transgenic flies were selected based on the eye color intensity and were intercrossed to establish homozygous transgenic flies stocks for each line.

Transgene mapping

As transgenes could have inserted into any of the four chromosomes, we had to identify which chromosome was carrying the transgene for each transgenic line. This was accomplished by setting up crosses that enabled us to score the patterns of segregation of the [*w+*] transgene from second and third chromosome dominant markers (see Materials and Methods). The mapping results are shown in Table 1.

Table 1: Chromosomal location of transgene insertions sites

Transgene	Line #	Chromosome
SUMO full length	1,2,3,4,5,7,10,12,18 ,20	3
	6,8,9,11,13,14,15,16,17,19,21,22	2

Transgene rescue assay

Because we did not know if a C-terminal GFP moiety would interfere with SUMO processing and conjugation, it was first important to demonstrate that our transgene could substitute for endogenous SUMO. The *smt3* homozygous mutation is lethal (Schnorr, 2001), so we asked if the *smt3-GFP* transgene was able to rescue this lethality. From a cross between *smt3/SM1. Cy; pCaSpeRhs smt3-GFP* males and females, it was expected that one-third of the F1 should be homozygous for *smt3* (as homozygous *Cy/Cy* flies do not survive). We found that 27% of the surviving flies were of this genotype when the progeny were raised under heat-shock conditions (see Materials and Methods). Our findings show that there is 82.1% rescue of the *smt3* mutation by the SUMO transgene under cyclical heat shock at 37˚C. The flies kept in room temperature did not rescue to the same extent, as only 35.2% of the expected *smt3* offspring survived (Table 2). The difference is statistically significant ($P < 0.01$, Figure 7). This result suggests that there is some "leaky" transcription of the construct under non-heat-shock conditions and supports the conclusion that it is the expression of the transgene, rather than some genetic background effect that results in rescue. From these results, we infer

that that the carboxyl-tagged EGFP SUMO molecule can be cleaved by the SUMO isopeptidases and used to sumoylate target proteins. This means that this construct may be useful to monitor the proportion of processed SUMO in the cell.

Table 2: Rescue of the *smt3* lethality by expression of a *smt3-GFP* transgene. F1 from crosses between *smt3/SM1, Cy ; pCaSpeRhs smt3-GFP* males and females. hs indicates that flies were heat-shocked throughout development as described in Materials and Methods. % rescue is calculated as (Cy+ flies observed)/(Cy+ expected if 100% survived) = (Cy+ flies observed)/(1/3).

Genotype	Cy males	Cy females	+ males	+ females	% Rescue in males	% Rescue In females	% Overall Rescue
smt3 (no hs)	134	117	0	0	0	0	0
smt3 (hs)	70	78	0	0	0	0	0
smt3 + SUMO(no hs)	170	168	23	22	35.7	36.6	35.2
smt3 + SUMO(hs)	132	136	49	52	81.2	82.9	82.1

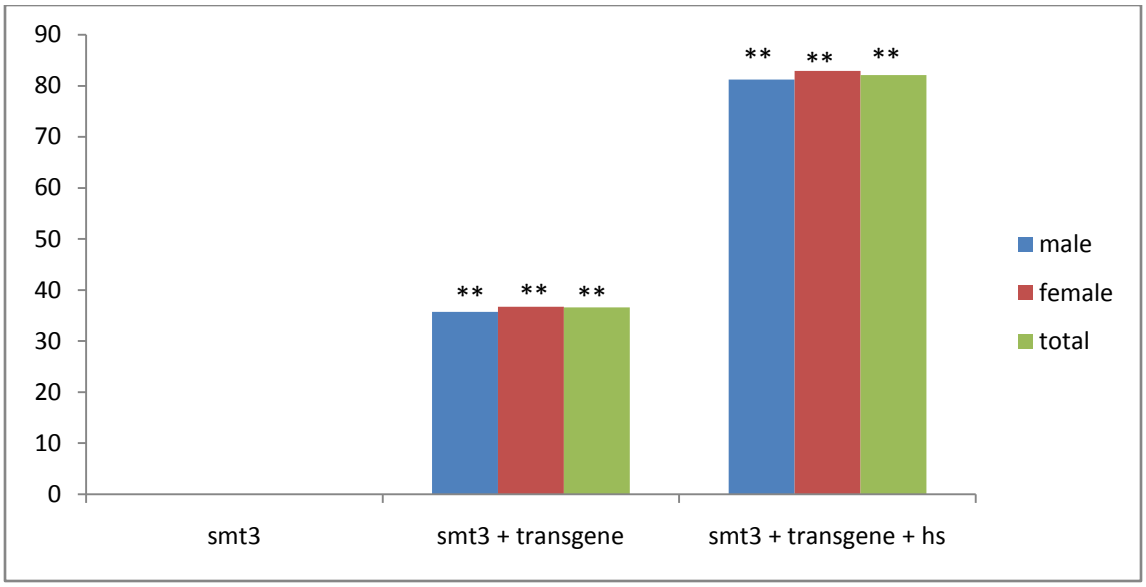
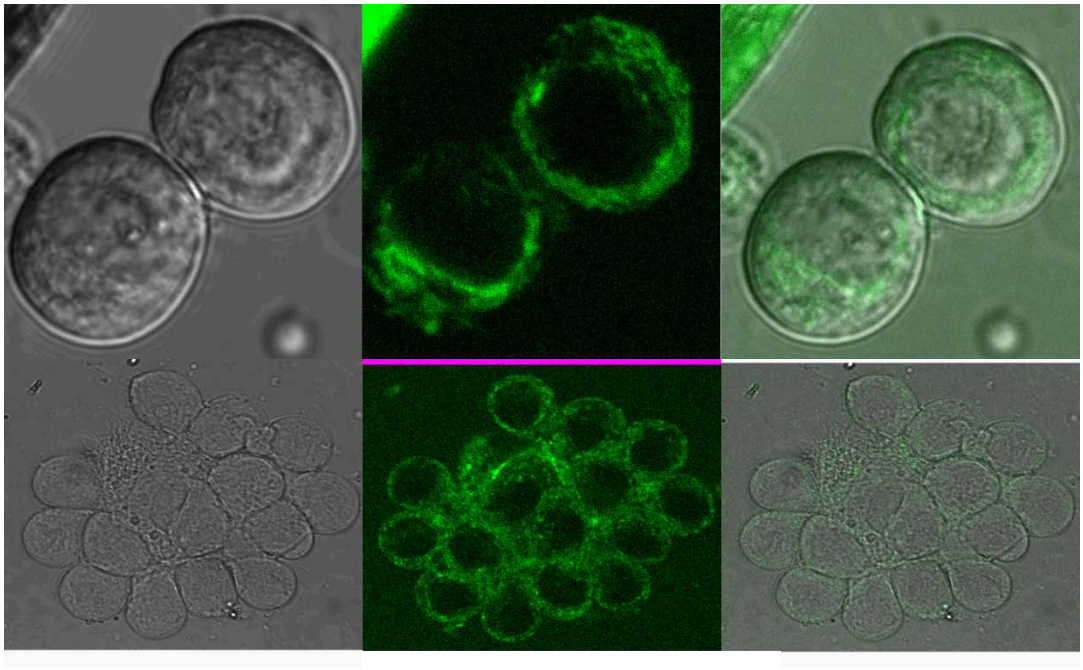


Figure 7: *smt3* transgene rescue assay. Comparison of survival of *smt3* homozygotes with or without the *smt3-GFP* transgene. Each class was compared with the like class in the control. ** P< 0.01

Expression of SUMO-GFP transgene

In order to investigate the expression of the SUMO-GFP transgene, we performed Western blots of testis protein using anti-GFP antibodies and examined the expression of the transgenic protein directly in living tissues by confocal microscopy. To induce the expression of the transgene, the transgenic flies were heat-shocked at 37°C for 1 hr every 8 hrs throughout their life cycle. Unfortunately, no signal could be detected by Western blotting. As the rescue assay indicated that the transgene was expressed in the soma, this may mean that the amount of protein expressed in testis was merely below the level of detection by this approach. We did, however, detect an EGFP signal by the more

sensitive method of examining live testis using confocal microscopy. Spermatocytes showed a subcellular expression in the cytoplasm (Figure 8). We do not know if this corresponds to the full-length fusion protein or to the cleaved EGFP moiety. Because the EGFP was expressed at the carboxyl terminus, however, this confirms that the fusion protein was being expressed.



A **B** **C**

Figure 8: Expression of Smt3-EGFP transgenic protein in testis. Panel A shows a DIC image, panel B shows the EGFP signal and panel C is a composite picture of A and B merged. All images are captured at 400X magnification.

These results from the expression and rescue assays indicate two things. First is the presence of EGFP moiety, which means that the transgene is expressed, and second, the SUMO molecule is properly processed by the isopeptidases in the cell. The rescue assay and expression assay indicated that the transgene with EGFP on its carboxy-terminus was expressed and processed to be tagged onto SUMO targets. This tool can be used to investigate and calculate the relative amounts of free unprocessed SUMO and processed SUMO (Figure 9).

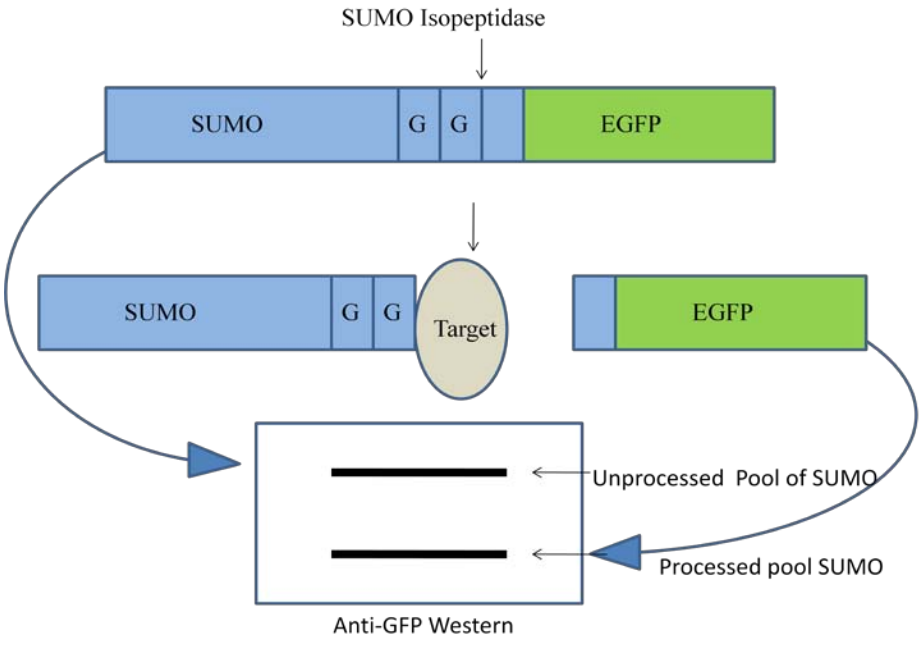


Figure 9: The tool used to investigate amount of unprocessed and processed SUMO in the cell.

CHAPTER IV

DISCUSSION

In our studies we examined sumoylation in flies both heterozygous (*dtopors*/+) and homozygous (*dtopors/dtopors*) for a null mutation. Prior observations indicated that Dtopors plays an important role in chromosome segregation in male meiosis. Mutations in *dtopors* disrupt chromosome segregation and cause chromosome non-disjunction, disruption of the nuclear lamina, nuclear blebs and male sterility (Matsui, Sharma and Tomkiel, unpublished data). Other studies have suggested that sumoylation negatively regulates insulator function (Capelson and Corces 2006). In a related study, it was proposed that *dtopors* is required for proper function of insulator complexes (Capelson and Corces 2005). In our study we investigated whether *dtopors* mutations alter the sumoylation of proteins in the testis.

The simplest explanation for these changes is that Dtopors is directly involved in conjugating SUMO to target proteins in the germ line, thereby affecting the remaining amount of unconjugated free SUMO. We cannot rule out other possibilities resulting indirectly from changes in *dtopors*. For example, *dtopors* may alter protein abundance by ubiquitinating or sumoylating transcription factors that determine levels of synthesis of specific proteins. Thus, changes in band intensities may reflect a change in abundance rather than the level of sumoylation *per se*. Dtopors may alternatively regulate the

proteosomal degradation of a particular protein. This may also result in a change in the abundance of a sumoylated band. The changes we observe may also reflect differences in mono-sumoylation and polysumoylation. The determination of whether a protein is mono- or polysumoylated may depend upon the number of free lysine molecules available for sumoylation. Finally, ubiquitination and sumoylation may compete for the same lysine molecule. Decreasing ubiquitination by decreasing Dtopors may free lysines for sumoylation. All these possibilities should be taken in consideration while interpreting these data.

We found that several high molecular weight protein bands (150 and 105 kd) show a difference in intensity when wildtype testis proteins are compared with those of *dtopors* mutants (Figure 2). When we reduced the dose of *dtopors* gene to half we observed a decrease in sumoylation compared to wildtype. Surprisingly when we observed the same band in the dtopors homozygous mutant, the intensity of the anti-SUMO signal looked more like wildtype. This phenomenon somewhat mimics the function of *dtopors* with respect to the gypsy insulator. In a genetic assay using the *cut6* mutation in which a gypsy insulator is inserted into the gene regulatory region, activity of the insulator disrupts *cut* transcription resulting in a severe wing phenotype. When the dose of *dtopors* is halved insulator function is disrupted, suppressing the *cut6* mutant phenotype (Capelson and Corces, 2005). In homozygous *dtopors* null mutants, however, gypsy insulator function is restored and appears to be similar to wild type (Matsui and Tomkiel, unpublished data). One model to explain these paradoxical results is that there is a balance between Dtopors ubiquitination and sumoylation activities. For example, if

Dtopors ubiquitination function alters the levels of sumoylation components then a small decrease in Dtopors may favor one activity over the other. An example of how this may work is shown in Figure 10. This model could be tested by making separation-of-function mutations in *dtopors* that differentially affect either ubiquitination or sumoylation.

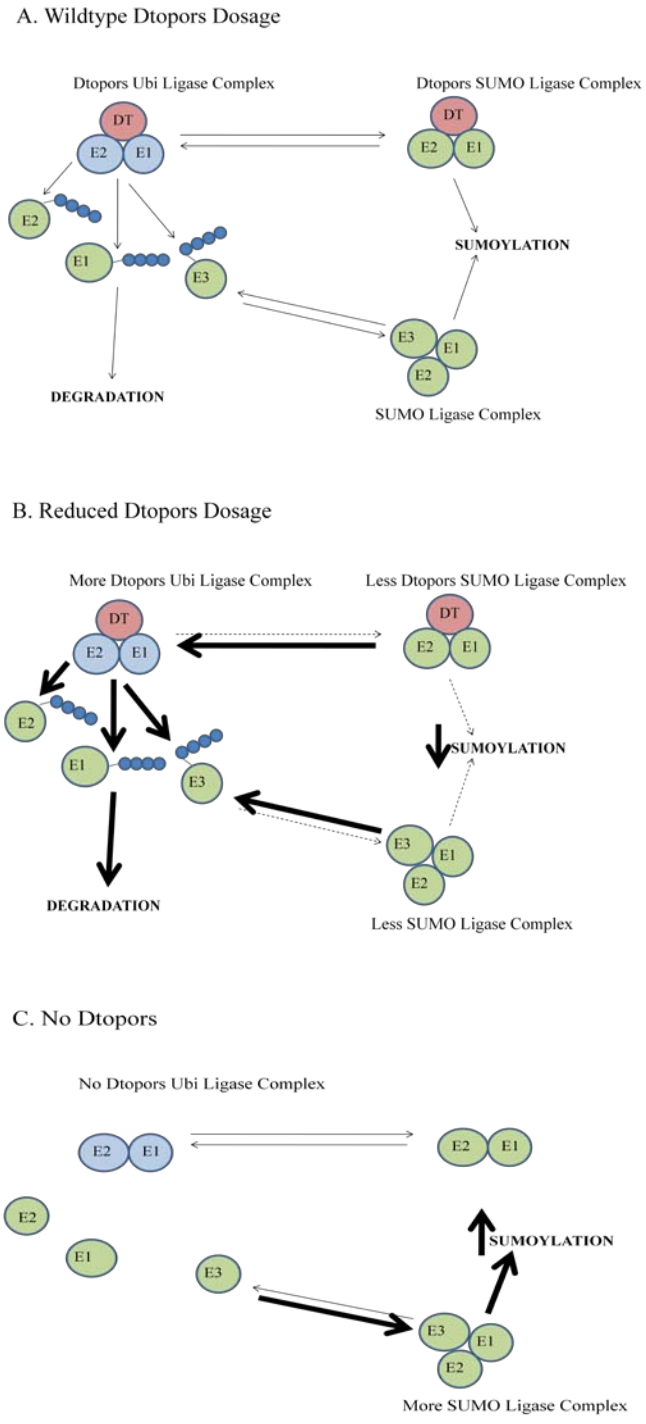


Figure 10: Proposed model of dose dependent Dtopors function in sumoylation and ubiquitination. A. Ubiquitination and Sumoylation activities are balanced in wildtype flies. B. Decrease in Dtopors favors assembly of ubiquitin ligases and decreases sumoylation by increasing degradation of SUMO ligases. C. Absence of Dtopors

enhances sumoylation by other E3 ligases that are stabilized by lack of Dtopors ubiquitination. Dark arrows indicate altered activity.

We also observed that the effects of Dtopors are not the same for all proteins. All the bands of sumoylated proteins were least intense in the *dtopors* heterozygotes when compared to homozygous mutants and the Canton S wild type. Bands of ~150 and 105 kDa sizes were least intense in the wild type and heterozygous mutants when compared to the homozygous mutants. These observations indicate that the *dtopors* mutation may alter the sumoylation patterns of proteins in a differential way specific for the protein in question. This data set also points out that the effect of *dtopors* on the sumoylation signal is dependent on the dose of *dtopors* gene. Overall, the pattern of sumoylation was more severely altered in heterozygous *dtopor* flies versus homozygous *dtopors* mutants. Thus while studying the effects of *dtopors* mutation on sumoylation, the level of dtopors expression must be considered. From previous studies it has been shown that human Topors is downregulated in seminomas and ovarian cancers, while in bladder, myeloma, endometrial, prostate and salivary gland tumors it is found to be overexpressed (Rhodes, Kalyana-Sundaram et al. 2007). This may occur due to stoichiometric differences in the balance of ubiquitination and sumoylation functions of *dtopors* in different tissues.

In the future it will be very interesting to identify the proteins that correspond to the bands altered in the *dtopors* mutants. Our initial attempts at pull-down studies using anti-SUMO antibodies were unsuccessful. Using this approach, however, it should be possible to isolate these proteins on two dimensional gel electrophoresis and determine their identity via MALDI mass spectrophotometric analysis. Another method to identify

these proteins would be to use a candidate gene approach. Western blot analysis could be performed using anti-SUMO antibodies on proteins derived from strains of flies mutant for a specific protein of interest (such as MNM, the meiotic isoform of Mod(mdg4)2.2)). Alternatively, antibodies specific for a candidate proteins of interest could be used to probe Western blots of proteins derived from wildtype and *dtopors* mutants. We used this latter method to examine whether lamin C was altered by *dtopors*, as the nuclear lamina shows defects in *dtopors* mutants. But our results reveal that Lamin C is not modified by ubiquitination or sumoylation by *dtopors* because there is no shift or change in band migration of Lamin C in *dtopors* mutant flies (Figure 2). This indicates that the disruption of the nuclear lamina integrity observed in *dtopors* spermatocytes is not caused by changes in ubiquitination or sumoylation of Lamin C. Rather, Dtopors may play a structural role in spermatocyte lamina assembly, or may regulate some as-yet-unidentified protein that is critical to lamina assembly and/or stability.

If MNM and Mod(mdg4)2.2 are similarly altered, it may suggest similarities between *dtopors* function in soma and germ line, and perhaps a role of insulators in meiotic chromosome organization. New sumoylation targets in the male germline will provide an idea how this particular modification plays its role in cell cycle progression.

The second half of the study was dedicated to making a tool to study SUMO dynamics with respect to *dtopors* to further investigate in detail how the regulation might occur. Our Western blot analyses showed that the pool of free SUMO gets altered by the dosage of *dtopors* mutation in testis tissue lysates. When the dose of *dtopors* is halved,

the free SUMO pool decreases and when *dtopors* is eliminated it increases, relative to wildtype. This may indicate a role of *dtopors* in SUMO processing and functioning in cell. One difficulty in interpreting these observations is that we do not know if the pool of free SUMO has changed because of changes in SUMO synthesis, or because of changes in conjugation to or cleavage from targets. To help distinguish between these alternatives, we made a transgenic construct with EGFP protein tagged onto the carboxyl terminus of SUMO. There are SUMO-specific proteases that both process SUMO precursors to the mature form with C-terminal di-glycine and deconjugate SUMO from modified proteins (Li SJ, 2000; Takahashi Y, 2000).

To determine if this tool would be useful, it was first important to demonstrate that the fusion protein would be recognized and processed by SUMO isopeptidases. To do this, we asked if our SUMO-EGFP construct was able to rescue a lethal *smt3* null mutation in flies, making the assumption that viability would require that the transgene protein be properly processed. We report that it rescues 82.1% of the *smt3* mutant flies. This result indicates that sufficient SUMO-EGFP is expressed and properly processed in the organism to allow for complementation of the essential function. Furthermore, our confocal microscopy observations of living spermatocytes indicate that this construct is expressed in spermatocytes, as we observed EGFP signal in the cytoplasm in these cells. These data together confirm that the carboxy-terminal tag allows for the normal processing of the SUMO molecule in cell, and suggest that this will be a useful tool to examine germline events.

This tool might be made even more useful by the addition of an amino-terminal GFP tag as well for use in a FRET assay (Fluorescence Resonance Energy Transfer). By expressing a FRET acceptor blue fluorescent protein tag on the amino-terminus, one could visualize the intact molecule by FRET, and also both the processed amino and carboxyl termini. GFP and BFP are FRET compatible pairs (reviewed by Brian AP, 1999), as the emission spectrum from BFP will excite GFP. When the BFP portion of the fusion protein is excited at 380nm it will emit at 420nm, which is in the excitation spectrum for GFP. The different emissions can be measured and calculated to determine how much SUMO is conjugated to proteins, how much is processed and how much is recycled during cell cycle progression. Western blot analysis of *smt3* null mutants would allow us to quantitate the different processed forms of SUMO, as there will be a size difference in SUMO bands with only BFP and both BFP and GFP. Together, these data may be useful to examine SUMO dynamics in the cell. Use of this tool to compare a *dtopors+* and *dtopors* mutant background might tell us specifically how SUMO processing and conjugation is effected by mutations in *dtopors*.

This tool may open up numerous options to study sumoylation and male meiosis in *D. melanogaster*. Sumoylation pathways of proteins involved in meiosis can be monitored. This tool will allow differentiating between tagged and untagged SUMO in the cell, when emission is measured at a specific wavelength. Cellular localization of proteins involved in male meiosis can be more conveniently studied whether they get modified via sumoylation. The two different tags give this benefit of tracking a single molecule for differential signal. This will be a novel tool to use and study SUMO

dynamics which has not been studied before in this approach in male meiosis of *D. melanogaster*.

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