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Ubiquitination and sumoylation are post-translational modifications that control a variety of cellular processes. Topors, a protein that can act as both an E3 ubiquitin and SUMO-1 ligase, is critical for regulation of gene expression, DNA repair and cell division. The Drosophila homolog, dTopors, is required for establishing proper nuclear structure and chromosome segregation in male meiosis. The relationship between ubiquitination and/or sumoylation in these functions, however, is unknown.

Using CRISPR-Cas9, I created separation-of-function mutants to specifically alter the ubiquitin ligase activity and used these to investigate the role of ubiquitination in these processes. In combination with mass spectrometry, I also utilized these mutants to identify potential targets of dTopors' ubiquitination and sumoylation in testis. Furthermore, to determine which E2 ligase acts with dTopors in the germline, I performed an RNAi knockdown screen of each E2 ligase and assayed for nondisjunction and nuclear defects. Positives were then tested for enhancement of a dtopors hypomorphic allele.

I found that the nuclear structure was more sensitive to lack of dTopors' ubiquitination than was chromosome segregation, suggesting that there may be different targets of dTopors ubiquitination important for each phenotype. Mass spectrometry identified over 800 peptides that were differentially ubiquitinated in the presence or absence of dTopors' ubiquitination. Notably these included chromatin proteins, Lamin Dm0 and numerous components of the ubiquitination pathway. Similarly, differentially sumoylated targets were identified including proteins involved in chromatin structure and transcription. Effete (UbcD1) and Bruce (BIR repeat containing ubiquitin-conjugating enzyme) were identified as potential E2 ligase partners of dTopors.

Expanding our knowledge of targets and E2 ligase partners are critical steps towards understanding the relationship between ubiquitination/sumoylation and germline nuclear structure and chromosome segregation.

THE ROLE OF DTOPORS IN NUCLEAR STRUCTURE AND MEIOTIC CHROMOSOME SEGREGATION IN *DROSOPHILA MELANOGASTER*

by

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DEDICATION

I am dedicating my dissertation to my wife Penelope, who always believed in me, supported me, and comforted me throughout my graduate journey, and to my children Sean and Amelie, for being the source of my perseverance.

I Am Because We Are!

APPROVAL PAGE

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CHAPTER I: INTRODUCTION

In eukaryotic cells, a protein's fate is regulated by post-translational modifications like ubiquitination and sumoylation. These modifications can affect protein turnover and may even alter the protein's function or its localization pattern. In both processes, a small peptide is covalently conjugated to lysine residues of a target protein via a glycine-lysine isopeptide bond. Although ubiquitination and sumoylation are very similar, involving an E1 activating enzyme, E2 conjugating enzyme and E3 ligases, the outcomes for the target proteins are quite different. In contrast to ubiquitination, which usually marks proteins for degradation, the addition of a SUMO (Small Ubiquitin-like Modifier) group does not lead to protein degradation. Moreover, it may stabilize proteins by competing for lysine residues with ubiquitin (Desterro et al., 1999). To initiate the sumoylation pathway, SUMO proteins need to be activated by means of a C-terminal cleavage of amino acids via SENP (Sentrin/SUMO-specific protease) resulting in an exposed di-glycine motif, ready to be conjugate to the lysine residue of a target protein (S.-J. Li & Hochstrasser, 1999). Afterwards sumoylation commences in three steps: first, SUMO is attached to the heterodimeric SUMO E1 activating enzyme (SAE 1/2), then it is passed to an E2 conjugating enzyme (Ubc9), which catalyzes the SUMO transfer to the target protein mediated by an E3 SUMO ligase (Hochstrasser, 2000). Sumoylation is important for multiple cellular processes, including changes in protein localization (RanGAP, Matunis et al., 1996), cellular signaling (HSF1, regulation of heat shock factors, (Hietakangas et al., 2003), chromatin modification of Histones (Shiio & Eisenman, 2003), HATs (Histone Acetyl Transferase) and histone modifiers such as HDACs (Histone Deacetylase) (Girdwood et al., 2003; Kirsh et al., 2002; David et al., 2002), regulation of transcription (for review Gill, 2004) and DNA damage repair (Hardeland et al. 2002). Similar to sumoylation, ubiquitination is also three-step process,

which starts with the ATP-mediated activation of Ub via E1 ubiquitin ligase, followed by conjugation of Ub to an E2 conjugating enzyme, which then complexes with an E3 ligase. The E3 ligase recognizes the substrate and catalyzes the Ub transfer to a lysine residue on the target protein leading to its degradation by the 26S proteosome (Wilkinson et al., 1995). The ubiquitination process may modify ubiquitin itself, resulting in the formation of polyubiquitin chains. Such polyubiquitin modifications usually result in proteolytic degradation of the targeted protein via the 20S proteosome. Other proteins, however, such as p53, may be monoubiquitinated, resulting in alterations to their function. (Grossman et al. 1998). Like sumoylation, ubiquitination alters a variety of targets and regulates multiple cellular processes. some targets may become polyubiquitinated like HDAC1 by IFIXalpha (Yamaguchi et al., 2008) and p53 by the histone acetyltransferase p300 and CBP (CREB-binding protein) (Grossman et al., 1998, 2003; Lill et al., 1997). The processes of ubiquitination and sumoylation are highly conserved from single cell eukaryotes to multicellular plants and animals.

The Role of Ubiquitination and Sumoylation in Signaling and Cancer

Several E3 ligases have been implicated in various cancers due to their ability to selectively ubiquitinate various targets in cell cycle regulation, signaling and cellular proliferation. The NFkB pathway plays an essential role in for a series of physiological responses including cell cycle regulation through the CDK/CDKi system. The pathway is initiated by phosphorylation (serine 32 and 36) induced activation of IkB (Inhibitors of kappa B) proteins by IKK and their subsequent modification with Ub. Ubiquitination of IkBs leads to their degradation by the proteasome thus allowing NF-kB to enter the nucleus and turn on transcription of oncogenes. Fukushima et al. showed that ubiquitination of the NFkB precursor NFkB2/p100 by SCF(Fbw7), an E3 ubiquitin ligase and tumor suppressor (Welcker et al., 2008),

leads to NFkB degradation by the proteasome, thus regulating oncogene expression (Fukushima et al., 2012). A different study also showed NFkB is also regulation regulated via ubiquitination of another component of a different NFkB activation pathway. IRAK-2 (Interleukin-1 receptor associated kinase-2), an essential component of the TLR (Toll-like receptors) mediated NFkB activation pathway, is involved in TRAF6 (TNF (Tumor Necrosis Factor) Receptor Associated Factor 6)) ubiquitination (Keating et al., 2007). TRAF6, has an amino terminal RING domain and interacts with two E2 conjugating enzymes (UBE2V1/UEV1A and UBE2N/UBC13) to form polyubiquitin chains required for activation of IKK (Keating et al., 2007). Once activated, IKK phosphorylates IkB which triggers IkB ubiquitination and subsequent degradation by the proteosome. This allows NFkB to enter the nucleus and initiate transcription of oncogenes. Studies showed that TRAF6 is overexpressed in a variety of cancers including colon, gastric, breast carcinomas and melanoma (Sun et al., 2014; Han et al., 2016; Shen et al., 2017) and affects cell proliferation, survival, and tumor invasion (Zhang et al., 2018).

SUMO modifications have also been shown to play a role in cancer as a number of cellular proteins implicated in cancer are sumoylated. In HeLa cells, p53, a tumor suppressor and c-Jun, an oncogenic transcription factor are sumoylated *in vitro* and *in vivo* (Müller et al., 2000) and Weger et al., also observed increased levels of Topors-mediated sumoylation of p53 *in vitro* and *in vivo* (Weger et al., 2005). Both c-Jun and p53 are well known targets of ubiquitination, however, they are also regulated by sumoylation at different residues (Müller et al., 2000). Sumoylation of c-Jun negatively regulates its activity, while sumoylation of p53 only impairs its apoptotic activity (Müller et al., 2000). Availability of p53 is also regulated by its degradation via Mdm2, an E3 ligase with RING ubiquitin ligase activity (Haupt et al., 1997). Mdm2 has the capability of self-ubiquitination, and sumoylation interferes with this activity, thus increasing

Mdm2 ubiquitination activity towards p53 (Buschmann et al., 2000, 2001). Sumoylation and ubiquitination may occur on the same residue and may influence protein function and availability as is the case with of IκBα (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor), where sumoylation antagonizes ubiquitination and protects IκBα from degradation (Alarcon-Vargas & Ronai, 2002). IκBα functions as an inhibitor of NF-κB, thus preventing NF-kB from turning on transcription of oncogenes.

Regulation of the Nuclear Lamina by Ubiquitination and Sumoylation

Ubiquitination plays an essential role at the nuclear lamina, a meshwork of filamentous proteins providing structural integrity to the nucleus and is essential for chromatin organization, DNA replication and gene expression (Bridger et al., 2007; Dechat et al., 2007; Dorner et al., 2007). Interactions between chromatin and the nuclear lamina are also thought to be important for chromosome behavior in many organisms (Bähler et al., 1993; Chikashige et al., 1997; Cooper et al., 1998; Nimmo et al., 1998; MacQueen et al., 2005; Sato et al., 2009; Wynne et al., 2012; Ding et al., 2007; Horn et al., 2013; Link et al., 2013; Morimoto et al., 2012; Schmitt et al., 2007) including flies (Cenci et al., 1994). In humans, several components of the lamina (pRb, LAP2a, lamin B1,lamin A, and Emerin) are ubiquitinated by the E3 RING domain ligase RNF 123 (Khanna et al., 2018). Sumoylation also plays an important role at the nuclear lamina by regulating the assembly of nuclear pore complex, a major transporter conduit between the nucleus and the cytoplasma (Folz et al., 2019). The nucleoporin RanBP2/Nup358 is a SUMO1 E3 Ligase and sumoylates SP100, a major component of the PML (promyelocytic leukemia) bodies (Sternsdorf et al., 1999).

Lamins (A, B and C) are intermediate filament proteins that provide structural stability to the nucleus. Lamin A function and localization is also regulated by SUMO modification (Y.

Li et al., 2019; Y.-Q. Zhang & Sarge, 2008). Lamin A interacts with several other nuclear proteins including DNA binding protein complexes like the highly conserved essential protein BAF (barrier-to-autointegration factor) and nucleoporins. When sumoylated, BAF binds to Lamin A filaments, which hold nuclear pore complexes in place, and to LEM-domain proteins, thus supporting nuclear integrity (Q. Lin et al., 2020).

Numerous mutations in genes encoding lamina proteins have been identified and cause a class of diseases known as laminopathies. Hutchinson-Gilford Progeria Syndrome (HGPS) is perhaps the most profound example. HGPS is a premature aging disorder caused by a rare *de novo* single base substitution in the *lamin A* gene, which codes for both Lamin A and C proteins (B. Liu et al., 2005). In HGPS, the nuclear lamina is destabilized leading to nuclear dysmorphology in the form of blebs (Eriksson et al., 2003), and is also associated with mitotic chromosome segregation defects (Eisch et al., 2016). It is possible that a distorted nuclear morphology and chromosomal damage may impact chromosome segregation, however, the exact mechanism how this may occur is still unclear.

Interestingly, mutations in two different genes encoding lamina proteins in the fruit fly cause somewhat similar phenotypes. Mutants in the nucleoporin-encoding gene *rae 1 (ribonucleic acid export 1*) and in *dtopors (Drosophila Topoisomerase I-interacting Arginine Serine rich protein*), display both chromosome segregation errors and nuclear blebbing in the male germline (Matsui et al., 2011; Volpi et al., 2013). In male flies associations between the nuclear lamina and chromatin appear to be particularly important for meiotic chromosome behavior (Cenci et al., 1994), but the precise relationship between lamina disruption and chromosome segregation errors is not known. To investigate a potential relationship between

nuclear structure and chromosome segregation, I will focus on the role of one of these genes, *dtopors*.

TOPORS is a Dual E3 Ubiquitin SUMO-1 Ligase

TOPORS was first identified in human as an *in vitro* interaction partner of Topoisomerase I (Haluska, 1999a) and *in vivo* of p53 (p53BP3, (Zhou et al., 1999)) and as LUN, expressed in lung cells (Chu et al., 2001). Topors was the first protein identified as a dual E3 ubiquitin and SUMO-1 ligase (Rajendra et al., 2004; Weger et al., 2005) and is expressed in both soma and germline (Chu et al., 2001). Topors is a conserved protein, containing an amino-terminal RING (Really Interesting New Gene) domain, bipartite nuclear localization sequences (NLS), an arginine/serine rich (RS) region, and numerous PEST domains (P (proline), E (glutamate), S (serine), and T (threonine)) for degradation (Chu et al., 2001; Secombe & Parkhurst, 2004; Zhou et al., 1999). The RING domain has been shown to be required for DNA-binding and ubiquitin ligase activity (Rajendra et al., 2004) but is not necessary for sumoylating activity (Desterro et al., 1999).

It has been shown that Topors can modify itself, and such modification can influence its own activities. Human Topors can sumoylate targets and can also be a sumoylation target itself at specific lysine residues (Weger et al., 2003). Furthermore, phosphorylation of Topors' serine 98 residue eliminates ubiquitin ligase activity but does not affect sumoylating activity (Park et al., 2008), while phosphorylation of serine 718 residue on Topors by PLK-1 (Polo-like Kinase 1) has been linked to p53 degradation by downregulating p53 sumoylation and inducing p53 ubiquitination (Yang et al., 2009). These observations suggest that the dual enzymatic activity of Topors may be a binary switch function regulated by post-translational modification.

TOPORS is Involved in DNA Damage Response Pathways

In HeLa cells, Topors has been shown to localize to PML-NBs (Promyelocytic Leukemia Nuclear Bodies), multiprotein complexes linked to transcription regulation (Zhong et al., 2000), cell growth (Fagioli et al., 1998), DNA damage (Carbone et al., 2002) and apoptosis (Wang et al., 1998) via PML protein (tumor suppressor, required for PML-NB assembly) binding (Rasheed et al., 2002). The size and number of PML-NBs have been observed to increase in response to double strand breaks (Dellaire et al., 2006). Topors involvement in different aspects of the DNA damage response is evidenced by its dual ligase activity of sumoylating or ubiquitinating unrelated targets in different DNA damage-sensing and repair pathways. Topors was shown to colocalize and sumoylate IKKe (Inhibitor of Kapp-B Kinase epsilon, regulates antiviral signaling pathways including NF-KappaB) in PML-NBs (Renner et al., 2010). A Topors sumoylated IKKe is linked to the phosphorylation of NF-KappaB and p65, thus, plays a role in the anti-apoptotic pathway initiated by DNA damage response (Renner et al., 2010). The first response to DNA damage in form of double strand breaks (DSBs) is hyperphosphorylation of the Histone 2 variant, H2AX at Serine 139 residue (γ-H2AX) (Pan et al., 2011; Rogakou et al., 1998). γ-H2AX is thought to anchor broken chromosomes to a large complex of proteins, which then recruit the DNA damage repair machinery (Stucki et al., 2005). Ubiquitination of H2AX is a prerequisite for its phosphorylation and thus for the start of DNA repair (Doil et al., 2009; Pan et al., 2011). H2Ax is ubiquitinated (mono-, di-, tri-ubiquitinated) *in vitro* by Topors and this ubiquitination leads to the phosphorylation-mediated activation of H2AK (Giannattasio et al., 2005; Hung, n.d.; G. Liu et al., 2021; Zheng & Petes, 2018).

TOPORS is Implicated in Various Cancers

The complexity of Topors enzymatic activity is not yet fully understood but multiple lines of evidence suggest involvement in cell cycle regulation and in cancers. Interestingly, the expression level of Topors is increased in some cancers and decreased in others, suggesting it may function in a tissue-specific manner. In colon adenocarcinomas, the CpG islands in *topors* promoter appear hypermethylated and *topors* expression is downregulated (Saleem et al., 2004). Alternatively, when upregulated, *topors* halts the cell cycle, trapping cells in G0/G1 phase, suggesting a possible role as a tumor suppressor (Saleem et al., 2004). In non-small cell lung cancer, *topors* mRNA levels were significantly decreased and showed correlation with less favorable cancer progression (Oyanagi et al., 2004). In prostate cancer, Topors is overexpressed and ubiquitinates the tumor suppressor NKX3-1 for proteasomal degradation (Guan et al., 2008), while in ovarian cancer elevated levels of TOPORS-AS1 (lncRNA TOPORS Antisense RNA1) in the early stages of the disease corresponded to decreased levels of β-catenin and disruption of Wnt/β-catenin signaling pathway (Fu et al., 2021). High TOPORS-AS1 levels correlated to more favorable disease outcome, as TOPORS-AS1 suppressed cell proliferation and metastasis, while TOPORS-AS1 expression levels were low in more progressed stages. Around half of human cancers harbor mutations in p53, a tumor suppressor gene (Levine, 1994). Expressed among many species, p53 is pivotal in regulating the cell cycle at the G1/S checkpoint, activating DNA repair genes, and initiating apoptosis in case of extensive DNA damage. In HeLa cells, Weger et al., observed increased levels of Topors-mediated sumoylation of p53 *in vitro* and *in vivo* (Weger et al., 2005), and in mice, mTopors was shown to be a coactivator and stabilizer of p53 and to enhance the p53-mediated expression of p21 (cyclin-dependent kinase inhibitor, regulates cell cycle at G1/S), of MDM2 (Mouse double minute 2, E3 ubiquitin ligase and negative regulator of

p53), and Bax (Bcl-2-associated X protein, p53 mediated apoptosis) (L. Lin et al., 2005). Topors expression is inducible by the anticancer drugs camptothecin (topoisomerase 1 inhibitor) and cisplastin and its overexpression causes cell cycle arrest and apoptosis (Lin et al. 2005). Embryonic fibroblasts of Topors knock-out mice have increased chromosomal segregation defects and genetic instability resulting in a 7-fold increase in tumor development and malignancies in different tissues (Marshall et al., 2010).

The Pleiotropic Effects of TOPORS

Another curious aspect of Topors is its localization pattern in distinct cells of different tissues. In humans Topors was observed in PML bodies (Weger et al., 2003; Zhong et al., 2000) but was not observed at the lamina (Rasheed et al., 2002; Weger et al., 2003), yet plays a role in chromosome segregation (Marshall et al., 2010). In the eye retinal photoreceptor cells, Topors localizes to the connecting cilium junction of the photoreceptor inner and outer segments, while in the retinal ganglia cells it is either aggregated into punctate spots in the nuclei or is associated with the centrosomes during cell division (Chakarova et al., 2011). Mutations in Topors have been associated with retinitis pigmentosa (adRP) perivascular retinal pigment epithelium atrophy, an autosomal dominant disorder resulting in night blindness and eventually vision loss (Bowne et al., 2008; Chakarova et al., 2011) and recently with syndromic ciliopathy, an oral-facial-digital syndrome causing abnormal oral cavity (Strong et al., 2021). In flies, dTopors localizes to the nuclear lamina in both somatic (Capelson & Corces, 2005) and male germ cells and *dtopors* mutants exhibit chromosome segregation defects and nuclear blebbing only in male germline (Matsui et al., 2011). These tissue-specific phenotypes, localization patterns and functions in Topors and homologs might all reflect a pleiotropic role of Topors in regulating numerous pathways, that may or may not be otherwise related. Therefore, it will be important to

identify the downstream targets of both ubiquitin and SUMO - modification by Topors as modification of different targets may be independently associated with the different phenotypes.

Conservation of dTopors' Role in Drosophila

Drosophila melanogaster is a great model organism to study the diverse functions of this conserved protein because of the many similarities that exist between hTopors and dTopors (the fly homolog of hTopors), including the enzymatically active RING domain (Haluska, 1999a; Rasheed et al., 2002; Secombe & Parkhurst, 2004). dTopors is an interaction partner of Hairy, a transcriptional repressor required for segmental patterning during early embryonic development (Secombe & Parkhurst, 2004). dTopors polyubiquitinates Hairy, and like hTopors also interacts with Topoisomerase I (Top I) and p53 (Dmp53) *in vitro*. Unlike mammalian Topors, however, it does not ubiquitinate Dmp53 (Secombe & Parkhurst, 2004). Despite the structural similarities between hTopors and dTopors, their localizations differ.

Not all aspects of Topors function may be conserved, however. In flies dTopors colocalizes with Lamin Dm0 and Lamin C in both mitotic and meiotic cells (Capelson & Corces, 2005; Matsui et al., 2011), while multiple studies of mammalian Topors localization in mitotic cells failed to detect it at the nuclear lamina (Haluska, 1999a; Rasheed et al., 2002; Weger et al., 2003).

In somatic cells, dTopors colocalizes with components of the *gypsy* insulator complex (gene regulatory elements involved in the establishment of independent chromatin domains) and is required for proper insulator function. None of the other known insulator components, however, are ubiquitinated by dTopors (Capelson & Corces, 2005). Mutations in LaminDm0 disrupt *gypsy* insulator function and interfere with dTopors localization to the nuclear lamina (Capelson & Corces, 2005). No direct role of dTopors in sumoylation has been demonstrated,

although there is evidence of an indirect one. Levels of sumolyation of several insulator complex proteins are decreased by dTopors in *in vitro* sumolyation assays (Capelson & Corces, 2006).

Mutations in *dtopors* that affect meiotic chromosome transmission were first identified as part of a large screen for male chromosome loss mutants by Wakimoto et al., 2004 and were further characterized by Matsui et al., 2011. In spermatocytes, dTopors localizes to the nuclear lamina and accumulates in punctate spots that may be analogous to PML bodies in mammals. (Matsui et al., 2011). In *dtopors* homozygous deletion, both Lamin Dm0 and Lamin C Lamin localization to the lamina is disrupted and the nuclei bleb (Matsui et al., 2011). Moreover, both Lamin Dm0 and Lamin C are distributed within the nucleus but in a somewhat different pattern. While Lamin C appears to accumulate on large intranuclear spots, Lamin Dm0 is distributed more evenly in the nucleus (Matsui et al., 2011). In addition to the nuclear blebbing phenotype, *dtopors* mutants also display a multiplicity of meiotic defects including failure of proper centriole separation, chromatin condensation defects, Anaphase I bridges/lagging chromosomes and chromosome nondisjunction (NDJ) resulting in aneuploid gametes (Matsui et al., 2011). It is unknown however, if the nuclear blebbing and meiotic defects are independent phenotypes. Since chromosomes occupy distinct domains at the nuclear lamina at prophase, would perturbance of the laminar structure influence chromosome segregation during meiosis or are these two distinct events? Does dTopors play a structural or an enzymatic role to preserve intact lamina structure and proper chromosome segregation in male meiosis? What type of enzymatic activity is needed for lamina structure and for chromosome segregation, is it ubiquitination or sumoylation, or are there different requirements and different targets for each?

CHAPTER II: NUCLEAR LAMINA STRUCTURE AND CHROMOSOME SEGREGATION HAVE DIFFERENT REQUIREMENTS FOR DTOPORS-MEDIATED UBIQUITINATION IN THE GERM LINE OF MALE *DROSOPHILA MELANOGASTER*

Introduction

The nuclear lamina is a meshwork of filamentous proteins providing structural integrity to the nucleus and is essential for chromatin organization, DNA replication and gene expression (Bridger et al., 2007; Dechat et al., 2007; Dorner et al., 2007). Alterations in lamina/chromosome interactions underlie defects in chromosome behavior in humans. Mutations in genes encoding lamina proteins cause a class of diseases known as laminopathies, with Hutchinson-Gilford Progeria Syndrome (HGPS), being the most profound case. HGPS is a premature aging disorder caused by a rare *de novo* single base substitution in the *lamin A* gene, which codes for both Lamin A and C proteins (Eriksson et al., 2003). In HGPS, the nuclear lamina is destabilized, leading to nuclear dysmorphology in the form of blebs (Eriksson et al., 2003) and is also associated with chromosome segregation defects (Eisch et al., 2016). The precise relationship between distorted nuclear morphology and chromosome segregation, however, is unclear.

Interactions between chromosomes and the nuclear lamina are also observed during meiosis in many species including in plants (Bähler et al., 1993), the nematode roundworm Caenorhabditis *elegans* (MacQueen et al., 2005; Sato et al., 2009; Wynne et al., 2012), mouse (Ding et al., 2007; Horn et al., 2013; Link et al., 2013; Morimoto et al., 2012; Schmitt et al., 2007), and in male *Drosophila melanogaster* (Cenci et al., 1994). In other organisms, such as the fission yeast *Schizosaccharomyces pombe*, which lacks Lamins, interactions between chromosomes and structures associated with the nuclear envelope (Chikashige, 1997.; Cooper et

al., 1998; Nimmo, 1998.) may be analogous to lamina interactions. Numerous observations underlie the importance of these interactions in respect to meiotic chromosome behavior. In many plant species, meiotic chromosomes exhibit bouquet formation (Thomas & Kaltsikes, 1976), having their telomeres anchored to an attachment plaque at the inner nuclear membrane (Zickler & Kleckner, 1998). Bouquet formation may facilitate homolog pairing by limiting the homology search to two rather than three dimensions, thus limiting homolog interactions to a small area of the nucleus (Bähler et al., 1993).

Caenorhabditis elegans a conserved LINC complex makes connections across the nuclear lamina and envelope between chromosomes to cytoskeletal actin, and is important for meiotic chromosome movement (Sato et al., 2009). Chromosomal sequences called pairing centers (Phillips et al., 2005) connect chromosomes via zinc finger proteins to a lamina-associated KASH/SUN-domain protein complex (Klarsicht/ANC-1/Syne/ homology; meiosis specific, Sad-1/UNC-84) the LINC complex) across the nuclear envelope to the cytoskeleton (Sato et al., 2009). Actin-dynein forces are transmitted through the lamina mediate movement of the chromosomes and are believed to help stabilize proper homolog pairing while shaking lose potential weaker unfitting heterologous associations.

This complex is highly conserved and is also observed in mouse germ cells, where the telomeric protein KASH5, connects across the nuclear membrane with the telomere-associated SUN1 protein and this complex interacts with the microtubule-associated dynein-dynactin complex resulting in meiotic chromosome movement (Morimoto et al., 2012). The nuclear lamina, a dense mesh-like structure made of Type-V intermediate filaments that provides stability to the nucleus, is located on the inner side of the nuclear membrane (Paouneskou & Jantsch, 2019). It provides regions for chromatin attachment (Gruenbaum & Foisner, 2015) and

interacts with components of the nuclear envelope (i.e., SUN1, nucleoporins, and LEM (LAP2, Emerin, MAN1) domain proteins) (Cohen-Fix & Askjaer, 2017).

Even though *Schizosaccharomyces pombe* does not have Lamins, the connection between chromosomes and nuclear structure is still important. Dramatic meiotic chromosomes movements are mediated by attachment across the nuclear membrane to the SPB (Spindle Pole Body, a microtubule organizing center in yeast connected to the nuclear envelope, like centrosomes). During interphase, the chromosomes are gathered next to the SPB, and their centromeres clumped into a cluster (Chikashige et al., 1997). During meiotic prophase, the telomeres attach to the SPB, and the centromeres subsequently separate from it (horsetail stage). Chromosomes remain attached through their telomeres while the entire nucleus is drawn between cell poles by the SPB (Ding et al., 1998).

A particularly interesting system to study the connection between meiotic chromosomes and nuclear structure is in the Drosophila male, where paired meiotic chromosomes appear to be intimately associated with the nuclear periphery (Cenci et al., 1994), suggesting a possible role for the lamina in regulating chromosome behavior in this organism as well. By mid-prophase in this organism, paired bivalents have moved into discreet nuclear territories at the nuclear periphery and remain in these separate domains until prometaphase. While associated with the lamina, they undergo an unusual "unpairing" of both homologs and sister chromatids. These observations were made in live spermatocytes using chromosomes tagged with a GFP-tagged Lac repressor (GFP-Lac I)/Lac-operator (LacO) system at 14 different euchromatic loci. Homologs pair and remain associated through euchromatic interactions in early prophase (Vazquez et al., 2002). However, in older Stage 3 (S3) spermatocytes, chromosomes associations between LacO sites on both homologs and sisters simultaneously separate (Vazquez et al., 2002).

The formation of these territories in prophase of S3 (S3 is mid-prophase) spermatocytes separates bivalents into physically distinct domains, and may provide a sorting mechanism to disrupt non-homologous chromosome associations (Vazquez et al., 2002). The fact that these territories are located at the nuclear lamina suggest that a) interactions between the chromosomes and the lamina are important for territory formation and b) chromosomes may be attached to sites on the nuclear lamina to facilitate bivalent associations (Vazquez et al., 2002), although the specific nature of this relationship is not well understood.

Although the formation of these lamina-adjacent domains suggests their importance to subsequent meiotic chromosome segregation, it is unknown if chromosome segregation defects would result if they were disrupted. The chromatin-binding protein Dany (distal antenna-young, involved in regulation of transcription in spermatogenesis) disrupts the formation of these domains, however, *dany* mutants also affect transcription, and completely disrupt meiosis prior to anaphase (Trost et al., 2016). Recently it was demonstrated that mutants in the condensin complex component CapH also disrupt domain formation, and these mutants result in chromosome segregation defects. However, these segregation errors may reflect a role of CapH mutants in resolving centromere associations early in prophase, rather than the result of domain disruption *per se*. Prior to domain formation in early prophase, clustering between all centromeres is observed, and these associations are normally resolved as domains form. In *capH* mutants, however, centromeres fail to separate and lead to anaphase I bridges (Vernizzi & Lehner, 2021).

Interestingly, mutations in two different genes encoding lamina proteins cause lamina disruptions, and result in meiotic chromosome segregation errors as well. Mutants in the nucleoporin *Rae 1 (Ribonucleic acid export 1*) and in *dtopors (Drosophila Topoisomerase I-*

interacting Arginine Serine rich protein), display both nuclear herniations and chromosome segregation errors in the male germ line (Matsui et al., 2011; Volpi et al., 2013). To investigate a potential relationship between nuclear structure and chromosome segregation, I will focus on the role of one of these genes, *dtopors*.

dTopors Role in Drosophila Soma and Male Germline

Mutations in *dtopors* that affect meiotic chromosome transmission were first identified from a screen for male chromosome loss mutants by Wakimoto et al., 2004 and were further characterized by Matsui et al., 2011. dTopors had been previously identified as a ubiquitin ligase that regulates proteosome-mediated degradation of Hairy, a transcriptional repressor required for segmental patterning during early embryonic development (Secombe & Parkhurst, 2004).

dTopors shares structural similarities with hTopors including the conserved RING (Really Interesting New gene) domain, an RS (arginine-serine rich) region, NLS (nuclear localization signal), several PEST ((proline (P), glutamic acid (E), serine (S), and threonine (T) rich sequences)) sequences and a consensus sequence unique to Topors homologs, but there is only limited sequence homology outside of the RING domain (Haluska, 1999a; Matsui et al., 2011; Secombe & Parkhurst, 2004; Zhou et al., 1999).

Despite these structural similarities between hTopors and dTopors, protein localization is very different. In humans, Topors is not observed at the lamina (Rasheed et al., 2002; Weger et al., 2003), yet it also plays a role in chromosome segregation (Marshall et al., 2010). A TOPORS mouse knockout shows chromosomes segregation defects, but no nuclear deformations (Marshall et al., 2010).

dTopors colocalizes with Lamin Dm0 and Lamin C in mitotic cells and meiotic spermatocytes (Capelson & Corces, 2005; Matsui et al., 2011) and *dtopors* mutants display

nuclear herniations in spermatocytes and meiotic chromosome segregation defects (Matsui et al., 2011). In somatic cells, dTopors also colocalizes with components of the *gypsy* insulator complex (gene regulatory elements involved in the establishment of independent chromatin domains) and is required for proper insulator function, however none of the insulator components are ubiquitinated by dTopors (Capelson & Corces, 2005).

In spermatocytes dTopors was observed to localize to the nuclear lamina and to accumulate in punctate intranuclear spots (Matsui et al., 2011), however in a *dtopors* deletion dTopors localization to the lamina is disrupted and the nuclei bleb (Matsui et al., 2011).

Lamin Dm0 and Lamin C still localize to the spermatocyte nuclear lamina in a *dtopors* mutant, however, there is also an abnormal accumulation of these Lamins in the nucleoplasm (Matsui et al., 2011). In addition to the nuclear blebbing phenotype, *dtopors* mutants also display a multiplicity of meiotic defects including failure of proper centriole separation, chromatin condensation defects, Anaphase I bridges/lagging chromosomes and chromosome NDJ resulting in aneuploid gametes (Matsui et al., 2011).

It is unclear if the effects on chromosome segregation are related to lamina disruption, or if they reflect Topors regulation of components of separable pathways. Since chromosomes occupy distinct domains at the nuclear lamina at Prometaphase, would perturbance of the laminar structure influence chromosome segregation during meiosis or are these two distinct events? In this context, there does not seem to be a gross change in the formation of nuclear domains. Does dTopors play a structural or an enzymatic role to preserve intact lamina structure and proper chromosome segregation in male meiosis? What type of enzymatic activity is needed for lamina structure and for chromosome segregation? Is it ubiquitination or sumoylation or are there different requirements and different targets for either case?

Aim of Study

To investigate the relationship between nuclear structure and chromosome segregation, and the role of dTopors-mediated ubiquitination in each, I created a separation-of-function ubiquitin null allele of *dtopors*. The RING domain of human Topors is required for Topors' ubiquitin ligase activity (Rajendra et al., 2004) but not for sumoylation activity (Weger et al., 2003). I used CRISPR-directed mutagenesis to alter conserved residues in the RING domain, and then assayed the effects of these mutations on nuclear structure and meiotic chromosome segregation. I then used these mutations in combination with tandem mass spectrometry to identify potential targets of Topors ubiquitination that may be associated with each phenotype.

To expand the understanding of the differential enzymatic activities of dTopors, direct targets of dTopors need to be identified and dTopors effects on the activities of these substrates characterized. Previous research revealed that many targets of hTopors sumoylation are involved in chromatin modification and transcriptional regulation ((i.e., Sin3A (switch independent 3) and SAF-B2 (Scafold Attachement Factor-B2)) (Pungaliya et al., 2007) and in cell cycle regulation (p53, Weger et al., 2005) and TopI, Hammer et al., 2007). While sumoylating activities of hTopors were described in more detail little is known about its ubiquitination activities and targets. Based on the dual ligase activity of dTopors, it is possible that dTopors regulates the same targets alternatively by ubiquitination and sumoylation, or perhaps the two functions have entirely separate targets. Here, I present evidence that the nuclear structure and chromosome segregation are two independent phenotypes, potentially requiring different thresholds of ubiquitination by dTopors. Furthermore, I also identified possible targets of dTopors ubiquitination in the male germline by proteomic analysis.

Materials and Methods

Drosophila Stocks and Crosses

Drosophila melanogaster were reared at room temperature on a standard diet of cornmeal, molasses, agar, and yeast with propionic acid and tegosept added to prevent mold. The *Df(dtopors^{AA})* allele was provided by Susan Parkhurst. All other stocks were obtained from Bloomington Drosophila Stock Center at Indiana University (NIH P40OD018537) and are described on Flybase (http://flybase.bio.indiana.edu).

CRISPR-Directed Editing of dTopors RING Domain

Plasmid Preparation and gRNA Cloning. In two separate experiments I used CRISPR directed mutagenesis to introduce point mutations at different sites on exon 1 encoding the dTopors RING domain. I aimed to replace a cysteine with a serine at position 118 (C118S) and a cysteine with an alanine at position 102 (C102A). To accomplish this, I designed guide RNAs (gRNA) using primer sets to synthesize the gRNA (5'TGCACTGCAGGAGGAAGTGCTTCA 3' and 5'AAACTGAAGCACTTCCTCCTGCAG 3' for C118S; 5'TGCACCTGCAGCGGGACAAGCAGA 3' and 5' AAACTCTGCTTGTCCCGCTGCAGG 3' for C102A). The gRNAs were cloned into a commercially available multiplex gRNA plasmid pCFD5 (Addgene) which expressed the gRNA under the control of the strong, ubiquitous RNA polymerase III promoter, U6:3 (crisprflydesign.org). Recombinant plasmids were verified by sequencing (MWG Eurofins).

Single Stranded Template Design for Homology Directed Repair (HDR). Template strands were designed for HDR for each clone. For the C118S substitution the template strand 5'TCCGCCGCGGAGGAGAACGGAACAGTAGAGCGCAACTCGCCGCCGCCCAATT GCGCCATCTGCTTGTCCCGCTGCAGGAGGAAGTGCTTCACGGACAGC**AGT**ATGCAC

CAGTTCTGCTTCAAGTGCCTGTGCGAGTGGAGCAAGATAAAGCCCGAGTGTCCACTG TGTAAGCAGCCCTTCAGAACCATCATACACAATGT 3’ was designed to replace the nucleotides TGC (exon 1 at bp 352-354) with AGT (bold underlined). For the C102A substitution TGC (exon 1 at bp 313-315) was replaced with GCC (bold underlined) in the template 5’TCCGCCGCGGAGGAGAACGGAACAGTAGAGCGCAACTCGCCGCCGCCCAATTGCG CGATC**GCC**TTGTCCCGCTGCAGGAGGAAGTGCTTCACGGACTCGTGCATGCACCAG TTCTGCTTCAAG 3’. In addition, I made a GC**C** to GC**G** substitution at position 309 which created a restriction enzyme recognition site for PVU I (CGAT*CG) for initial screening of induced mutations.

Embryonic Injections and Verification of RING Mutants. The recombinant plasmid and the template were commercially injected (GenetiVision) into y[1] M{w[+mC]=nos-Cas9.P}ZH-2A w[*] (BDSC54591) embryos for C118S and y1 M{Act5C-Cas9.P.RFP-}ZH-2A w1118 DNAlig4169 (BDSC58492) embryos for C102A. The latter strain contains a mutation in DNA ligase to increase the frequency of insertions at the repair junction during homology directed repair (HDR) (Yu & McVey, 2010) and to increase ratio of HDR to non-homologous end joining (Beumer et al., 2008) after Cas9-induced DNA break. Germline transformants were identified and homozygous stocks were established for each G1 offspring due to the possibility of parental germline mosaicism. Genomic DNA was extracted from males in each line using a single fly DNA extraction protocol (Gloor et al. 1993). The presence of the desired CRISPR-induced change was first verified by PCR with primers 5’GGATAATGCCAACGCCATCG 3’and 5’CTGGTACCACGCCTCACAATGTGGTAACG 3’ and then by restriction enzyme (RE) digest with NspI (New England Biolabs), as the mutant template did not contain the restriction

site (R-CATG-Y) for NspI. The presence of the C102A alteration was verified by PCR with primers 5'GAGGAGAACGGAACAGTA 3' and 5'CTTGAAGCAGAACTGGTG 3' and by RE digest with PVU I (CGAT*CG, a site not found in the wildtype). Positive lines identified by the RE digests were subsequently confirmed by sequencing (Eurofins Genomics).

Genetic Assays of Meiotic Chromosome Segregation

Crosses were set up between *y w sn; C(4)ciey* females and *yw/y+Y; spa* males either homozygous or heterozygous for the following *dtopors* alleles recovered for the CRISPR- based mutagenesis: *Df(dtopors$^{114\text{-}118}$) (*from here on *Df(114-118)), dtopors^{C102A}* (*C102A*), and *dtopors^{fsF114SX19}* (*FS*) to monitor sex and fourth chromosome NDJ. Normal progeny consisted of *y+ w* males and *y w* females, while progeny arising from sex chromosome NDJ were detected as either *y w sn* males (*nullo-XY,* which received no paternal sex chromosome) or as *y+ w* females (*diplo-XY,* which received both paternal X and Y chromosomes). Fourth chromosome NDJ was detected as *spa* progeny produced from *diplo-4* sperm or *ci ey* progeny from *nullo-4* sperm. To test for NDJ of the major autosomes, crosses were set up between males homozygous or heterozygous for *dtopors* and females with *Compound 2* (*C(2)EN cu sp*) chromosomes, where chromosome 2 homologs are attached to the same centromere resulting in the production of either *nullo-2* or *diplo-2* eggs. Since major autosome aneuploidy is lethal, the only viable offspring of these females result from aneuploid sperm produced from NDJ events. I counted embryo hatch rates first and then the number of progenies per male for homozygous *dtopors* males versus their heterozygous siblings to assess frequencies of chromosome 2 NDJ.

Quantitative Assessment of the Spermatocyte Nuclear Phenotype

Testes of 1-day old homozygous *C102A, Df(C114-118), Df(dtopors^{AA})* and *wildtype(+)* adults were dissected and one testis pair per slide was mounted under a coverslip in 3 µl of Schneider's tissue culture medium. The tissue was immediately observed Nikon Eclipse E600 phase microscope and images were taken with a Moticam X^3 WiFi color camera (Motic Scientific). For each image, the number of S5-S6 spermatocytes with and without nuclear herniation (blebbing) was recorded. Images were collected from at least 12 individual males and at least 850 cells were assessed per genotype.

Fluorescent In Situ Hybridization

Testes were dissected in Schneider's Drosophila medium (GIBCO BRL, Gaithersburg, MD) and were placed in a small drop of Schneider's medium on a silanized coverslip, were opened with a thin needle and carefully squashed onto a Poly-L-Lysine coated slide (Electron Microscopy Sciences, Hatfield, PA). The slides were briefly submerged in liquid nitrogen and the coverslips were subsequently removed. The testes were fixed in a 55% methanol/25% acetic acid solution for 10 min, then dehydrated for 10 minutes in 95% ethanol.

FISH Preparation. A modified protocol of Beliveau et al. (2014) was used. The slides were rehydrated for 10 min at room temperature in 2X SSCT (saline sodium citrate/Tween 20) and then incubated in 50% formamide/2X SSCT for 2.5 min at 92°C followed by 60°C for 20 min. To remove Formamide residue, slides were rinsed in 2X SSCT for 5 min and then air dried. 5 µl of a probe master mix containing 12.5 µl hybrid cocktail (50% dextran sulfate, 20X SSCT), 12.5 µl formamide, 1 µl of 10 mg/ml RNase, 2 µl of probe 1 (5 pmol/µl), and 2 µl of probe 2 (5 pmol/µl) was added onto a silanized 18x18 mm coverslip which was carefully mounted onto the tissue slide and sealed with Elmer's rubber cement. The slides were placed on a heat block for

2.5 min at 92°C and were transferred to a moist chamber for overnight (>18 hours) incubation at 42°C. At the end of the incubation period, unbound probes were washed off first with 2X SSCT for 20 min at 60°C, then with 2X SSCT for 10 minutes at room temperature, and lastly with 0.2X SSC (saline sodium citrate) for 10 min at room temperature. Chromosomes were stained for 2 min with 1X DAPI (1 µg/µl 4',6-diamidino-2-phenylindole) (Sigma, St. Louis, MO), excess stain was rinsed off in 0.2X SSC. Slide was mounted in 11µl ProLong Gold antifade (Invitrogen, Carlsbad, CA) and the signals were visualized with a Keyence BZ-X700 Fluorescence Microscope. Meiocytes were selected in early – late anaphase of meiosis I, and signals in were evaluated based on their presence or absence in chromosome bridges and/or trailing chromosomes in Anaphase 1.

Probe Design. The probes were designed as described by Hylton et al., (2020). Probe Design pools were generated to selected sequences at a density of 10 probes/Kb and a complexity of ~10,000 probes per pool (Arbor Biosciences, Ann Arbor, MI). For chromosome 2, a triple-labeled Atto-488 probe was generated to bp 20368577-21368577 (56F-57F) and for the Y chromosome, an Atto-488 probe (Eurofins MWG Operon, Louisville, KY) was synthesized to the Y-specific AATAC heterochromatic repeat (Lohe and Brutlag 1987). For the X chromosome, a triple-labeled Atto-594 oligonucleotide probes were made to X salivary gland chromosome bands 2E1-3E4, bp 2606837-3606837; 12A4-12F4, bp 13824004-14826069; and 16F7-18A7, bp 18193946-19193592.

Assessment of Anaphase Defects

Testes squashes of heterozygous control and of homozygous *C102A* and *FS* adults were dissected in Schneider's tissue culture medium and gently squashed under silanized coverslips. The slides were frozen in liquid nitrogen, the coverslips were instantly removed, and the tissue

was fixed in cold methanol for 10 minutes. Chromosomes were stained with 1X DAPI and the slides were mounted in 50% glycerol. The signals were visualized with a Keyence BZ-X700 Fluorescence Microscope. For each line a minimum of 100 meiosis I cells from at least 5 males were assessed at anaphase-telophase transition for chromosome bridges and lagging chromosomes.

Indirect Immunofluorescence

Testes of homozygous *C102, Df114-118, and dtopors*^{f05115} mutants were dissected in Schneider's tissue culture medium and fixed as described above. After fixation, slides were rehydrated in 1X PBS for 5 minutes 3 times. Primary antibodies were applied in the following dilutions in 1X PBST (PBS with 0.1% TritonX): rabbit anti-dTopors 1:20 (Capelson & Corces, 2005) and mouse anti-Lamin Dm0 (ADL67.10) 1:100 (Iowa Hybridoma Bank). The slides were incubated in a humid chamber at 4^0C overnight. Primary antibodies were removed with 3 X 5 min washes in 1X PBS. Secondary antibodies of Alexa Fluor 488 goat anti-mouse (Molecular Probes) and Alexa Fluor 546 goat anti-rabbit (Molecular Probes) were applied in a dilution of 1:1000 in 1X PBST + 3% BSA (bovine serum albumin, Fisher Scientific). Secondary incubation was 2 hours at room temperature (rt), followed by a 3X 5 min washes in 1X PBS. Slides were stained for 2 min with 1X DAPI, briefly rinsed in 1X PBS and mounted in 50% glycerol. The signals were visualized with a Keyence BZ-X700 Fluorescence Microscope.

Mass Spectrometry

Tissue Preparation. For each mass spectrometry analysis two thousand pairs of testes of 1-3 Day old males of either yw*/y*$^+$*Y; spa*^{pol} and *yw/y*$^+$*Y; dtopors*^{C102A}*; spa*^{pol} *genotypes* were collected in Schneider's Drosophila medium (GIBCO BRL, Gaithersburg, MD). Testes were centrifuged at 1700 rpm for 2 minutes, Schneiders was removed, and testes were gently washed

in 200µl 1X PBS (Phosphate Buffer Saline pH 7.4). The centrifugation was repeated, and the PBS was removed. An Inhibitor Cocktail (IC, 0.1 mM MG132, 80 mM NEM and 0.4 mM IAA (Sigma-Aldrich, St Louis, MO), and 0.1 M Halt 100X to final concentration of 1X (Thermo Scientific, Rockford, IL) was added in amounts of 1 µl per 2 pair of testes. The tissues were sonicated in short 20 second intervals for a total of 10 repeats and a standard Bradford assay was done to quantitate protein concentrations (Thermo Scientific, Rockford, IL).

Mass spectrometry analysis was performed by Creative Proteomics, Shirley, NY. Briefly, the samples were digested by Lys-C followed by trypsin. The resulted diglycine remnant containing peptides were enriched through Anti-Ubiquitin Remnant Motif (K-ε-GG) Antibody conjugated agarose beads and then identified by applying a nano-LC-MS/MS platform.

Protein Preparation. Disulfide bridges were reduced by 10 mM Tris(2-carboxyethyl) phosphine (TCEP) at 56℃ for 1 hour. Reduced cysteine residues were alkylated by 20 mM iodoacetamide (IAA) in the dark at room temperature for 30 min. The lysates were then diluted to < 1M urea by addition of 50 mM Tris-HCl, pH 8.2 before digestion. For the analysis, 4.16 mg protein was digested by Lys-C using an enzyme to substrate ratio of 1:400 (w/w) followed by trypsin using an enzyme to substrate ratio of 1:200 (w/w). After overnight digestion at 37℃, TFA was added to 1% final concentration. Any precipitate was removed by centrifugation at 1,780g for 15 min.

Peptide Purification. Purification of peptides was performed at room temperature on C18 reversed-phase columns. The columns were first conditioned by 100% ACN followed by 0.1% TFA, 50% ACN and then equalized by 0.1% TFA. The acidified and cleared digest were then loaded onto column. After wash by 0.1% TFA, the peptides were eluted from the column by 0.1%TFA, 50% CAN and dried using a SpeedVac.

Anti- K-ε-GG Peptide Enrichment and Fractionation. Before incubation, the beads were washed in pre-chilled PBS 4 times. The peptides were resuspended in IAP buffer (50 mM MOPS, pH 7.2, 10 mM sodium phosphate, 50 mM NaCl). The peptide solution was then added into the vial containing motif antibody beads and incubated on a rotator for 2 hr at 4°C. After incubation, the beads were washed with pre-chilled IAP buffer 2 times and HPLC grade water 3 times. The enriched peptides were eluted from the beads by 0.15% TFA and were separated into 3 fractions using an Ultimate 3000 nano UHPLC system (ThermoFisher Scientific, USA) with a trapping column (PepMap C18, 100Å, 100 μm × 2 cm, 5 μm) and an analytical column (PepMap C18, 100Å, 75 μm × 50 cm, 2μm). A sample volume of 1 μg was loaded along a mobile phase made of A: 0.1% formic acid in water; B: 0.1% formic acid in 80% acetonitrile. The total flow rate for 250 nL/min LC linear gradient was from 2 to 8% buffer B in 3 min, from 8% to 20% buffer B in 50 min, from 20% to 40% buffer B in 26 min, then from 40% to 90% buffer B in 4 min.

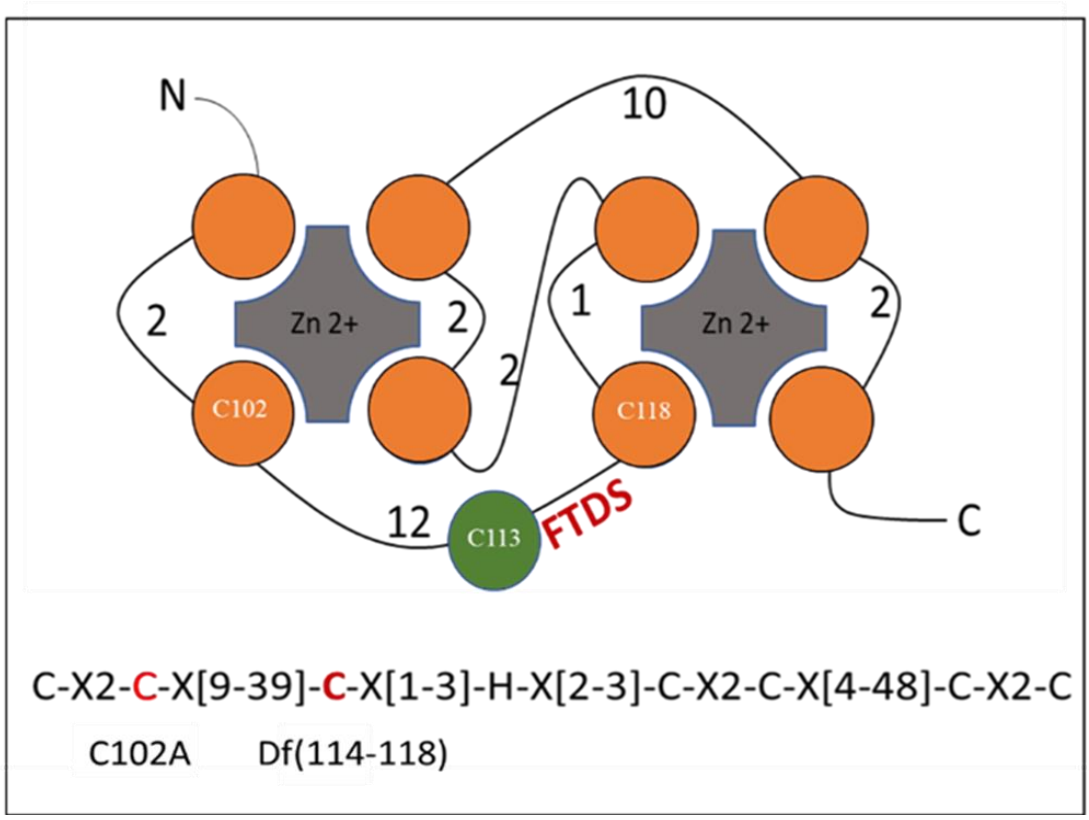
Analysis of Mass Spec Data. The full scan was performed between 300-1,650 m/z at the resolution 60,000 at 200 m/z, the automatic gain control target for the full scan was set to 3e6. The MS/MS scan was operated in Top 20 mode using the following settings: resolution 15,000 at 200 m/z; automatic gain control target 1e5; maximum injection time 19ms; normalized collision energy at 28%; isolation window of 1.4 Th; charge sate exclusion: unassigned, 1, > 6; dynamic exclusion 30 s. Raw MS files were analyzed and searched against *Drosophila melanogaster* protein database based on the species of the samples using Maxquant (1.6.2.14). The parameters were set as follows: the protein modifications were Carbamidomethylation (C), oxidation (M) (variables), GlyGly(K); Lys-C and Trypsin were set as the enzymes for ubiquitinated-proteomic analysis. The maximum missed cleavage was set to 4, the precursor ion mass tolerance was set to

10 ppm, and MS/MS tolerance was 0.6 Da. The data was sorted by gene ontology enrichment analysis and categorized for molecular function and cellular components (geneontology.org).

Results

To ask if dTopors ubiquitin ligase activity was required for proper nuclear structure and/or for chromosome segregation in spermatocytes, I used CRISPR-directed mutagenesis to induce mutations (*C102A* and *C118S*) in the conserved RING finger required for ubiquitin ligase activity (Secombe & Parkhurst, 2004). The C102A mutation in dTopors was previously shown to eliminate ubiquitin ligase activity *in vitro* (Secombe and Parkhurst, 2004)and the C118S mutation was shown to abolish the activity of a homologous RING domain in mammalian MDM2 ligase (Honda & Yasuda, 2000). Three alleles were recovered: the desired *C102A* point mutation, a 15 bp deletion that removed the codons for 5 amino acids including the conserved cysteine at position 118 (*Df(114-118),* and a frameshift within the RING-encoding domain *dtopors*^{fsF114SX19}*)*. In this latter mutation, a cytosine was deleted at bp 339 in the open reading frame causing a *frameshift* at F114 resulting in a 113 amino acid peptide from dTopors with a 19 amino acid fusion (Figure 1).

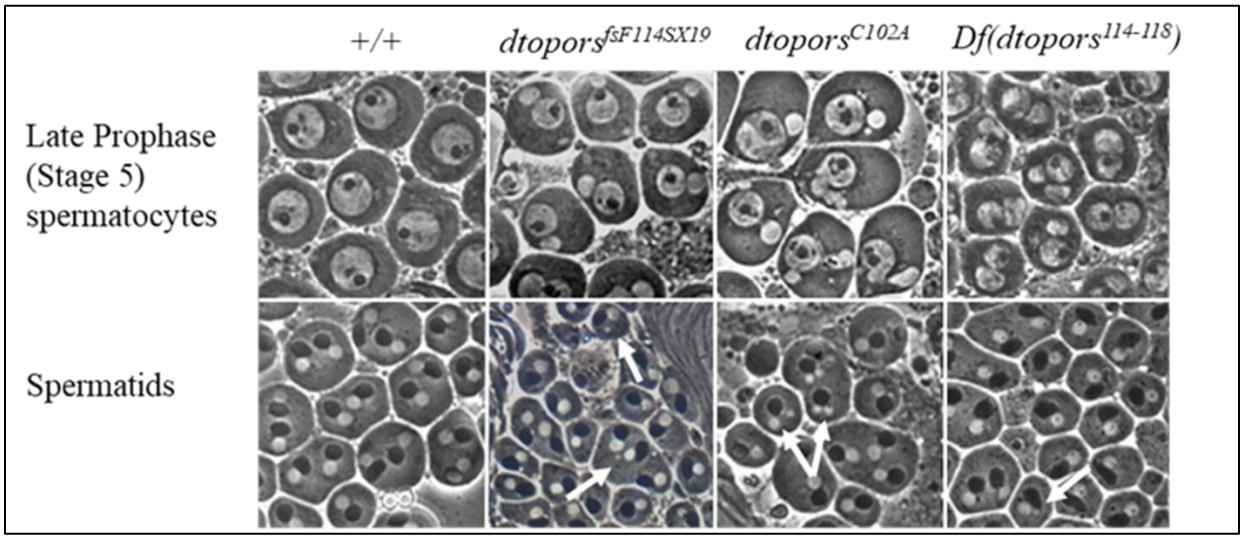
Figure 1. Diagram of dTopors C3HC4 RING Domain Showing Residues Altered in CRISPR/Cas9-Induced Mutations. Conserved residues are displayed in orange, and the number of amino acids between conserved residues is indicated. In *Df(dtopors$^{114\text{-}118}$),* the FTDS residues (red) were deleted in addition to C118 potentially allowing C113 (green) to substitute for C118 in the RING domain. (Figure modified from https://www.mcb.ucdavis.edu/faculty-labs/Callis/).



dTopors Ubiquitin Ligase Activity is Required for Spermatocyte Nuclear Structure

Testes of homozygous mutant males were dissected, and late prophase (Stage 5-6, S5-S6) spermatocytes were observed by phase microscopy for nuclear herniations (blebs). In addition, post-meiotic onion stage cells (early spermatids) were examined for the presence of micronuclei and/or nuclear size differences, indicative of chromosome missegregation. Both *RING* mutants and the frameshift (*FS*) exhibited blebbing (Figure 2, top panels) and micronuclei, indicative of NDJ (Figure 2 bottom panels). Thus, dTopors ubiquitin ligase activity is required for both nuclear structure and germline chromosome segregation.

Figure 2. Phase Contrast Images of S5-S6 Spermatocytes and Onion Stage Spermatids Nuclei. Genotypes are indicated above each column. Unequal sized nuclei and micronuclei (arrows) resulting from nondisjunction.



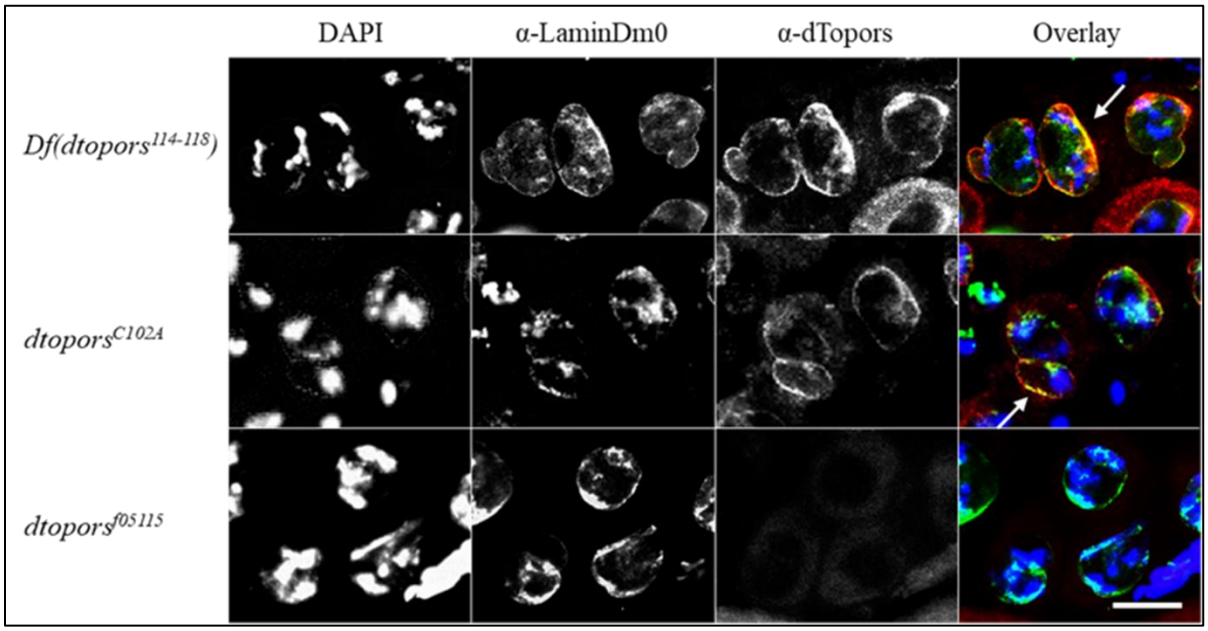
Although the cytological observations revealed blebbing and NDJ in both *RING* mutants, I noticed some differences in the frequency of the blebs and in the number of observed micronuclei. To quantify these differences, I scored the frequency of blebs in double blind cytological assays on testes of at least 12 individual 1-day old homozygous *C102A, Df(C114-118)* and controls (*Df(dtopors^{AA})* and *wildtype*) adult males. To minimize variation induced by different degrees of squashing of dissected cells, I mounted single pairs of testes under a coverslip in a defined amount of tissue culture medium and examined the tissue without additional squashing. The number of S5-S6 spermatocyte nuclei with and without blebs were counted immediately. Chi square statistical analysis of at least 850 cells per genotype was conducted. I found that both *Df(114-118)* and *C102A* mutants displayed blebbing at least as frequently as complete deletion of the gene, *Df(dtoporsAA)* (31%, 50.3% and 34.4%, respectively). This indicates that the ubiquitin ligase activity is required for proper nuclear

structure, and suggests other possible functions of dTopors (i.e., a structural role at the lamina, or sumoylation) may be of secondary importance to nuclear structure.

Ubiquitin Ligase Activity of dTopros is Not Required for Its Localization to the Lamina

It was possible that the nuclear defects observed in the *RING* mutants were an indirect consequence of dTopors mislocalization rather than a loss of ubiquitin ligase activity *per se*, as it had not been demonstrated that lamina localization is independent of this activity. To determine if the blebbing was a consequence of dTopors mislocalization to the nuclear lamina, I examined dTopors localization at the nuclear lamina by indirect immunofluorescence using rabbit anti-dTopors (Capelson & Corces, 2005) and mouse anti-Lamin Dm0 antibodies (ADL67.10) (Iowa Hybridoma Bank). I found that dTopors colocalized with LaminDm0 at the nuclear lamina in both *RING* mutants suggesting that ubiquitin ligase activity is not required for localization (Figure 3).

Figure 3. dTopors and Lamin Dm0 Localization in Primary Spermatocytes. S5-S6 stage spermatocytes stained with DAPI (blue), α-LaminDm0 antibodies (green) and α-dTopors antibodies (red). Arrows indicate co-localization of dTopors and LaminDm0. Scale bar 20 μm.



In the *frameshift (FS)* mutant, which is expected to produce a truncated protein containing only the N-terminal 118 amino acids, lamina colocalization was not observed. This suggests that this fragment is not sufficient for lamina localization or is unstable, or perhaps the antibody does not recognize any of the epitopes of the truncated protein. It is also of note that Lamin Dm0 localization appeared grossly normal in these mutants, although I cannot rule out the possibility that this technique failed to detect a subtle change in LaminDm0 localization. Overall, I conclude that dTopors ubiquitin ligase activity is not required for its assembly at the nuclear lamina. This suggests that dTopors' ubiquitin ligase activity, possibly acting on some component of the lamina, plays a critical role in nuclear structure.

dTopors Ubiquitin Ligase Activity is Required for Meiotic Chromosome Segregation

While cytological observations indicated that the two RING mutations and a *dtopors* null allele had a similar effect on nuclear morphology, my initial observations on chromosome

segregation suggested differences between the mutants. Micronuclei and nuclear size differences were much more apparent in the *FS* mutant than in either *C102A* and *Df(114-118)* mutants (Figure 2). To quantify the effects on chromosome segregation, I performed genetic assays of NDJ. In these assays, I simultaneously monitored the frequencies of chromosome loss and NDJ of the sex chromosomes and the fourth chromosomes (see Materials and Methods) (Table 1). I found that there were significant differences in both sex and fourth chromosome transmission among the mutants. When compared to *Df(dtopors^{AA})*, sex chromosome NDJ was significantly less frequent for both *C102A* and *Df(114-118)*, and *Df(114-118)* frequencies were significantly lower than the *C102A* levels (Table 1). The difference was even greater for the fourth chromosome NDJ. Both *RING* mutants had significantly less fourth chromosome NDJ than *Df(dtopors^{AA})* mutants. The frequency of fourth NDJ in *Df(114-118)* mutant was not significantly elevated over wildtype (Table 1).

Table 1. Sex and Fourth Chromosome Nondisjunction Frequency from Crosses of yw/y^{+}Y; *dtopors*; spa^{pol} Males to y w sn; C(4)RM ci ey/0 Females

Recovered male gametes:	Y; 4	X; 4	0; 4	X/Y; 4	X; 0	Y; 0	X; 4/4	Y; 4/4	0; 0	0; 4/4	X/Y; 0	X/Y; 4/4	#totals
Paternal genotype													
dtopors^{fsF114SX19}/Df (dtopors^{AA})	294	401	381	184	87	67	89	86	207	66	26	55	1943
dtopors^{C102A}/Df (dtopors^{AA})	263	242	222	53	25	29	17	31	39	7	5	7	940
Df(dtopors$^{114-118}$)/Df (dtopors^{AA})	316	317	357	77	22	28	18	16	31	18	5	3	1208
dtopors^{fsF114SX19}	88	95	153	32	23	14	14	29	51	21	5	19	544
dtopors^{C102A}	481	492	233	66	11	7	7	3	9	4	1	2	1316
Df (dtopors$^{114-118}$)	852	1247	78	4	6	5	0	0	2	0	0	0	2194
dtopors^{fsF114SX19}/Cy	1018	1504	9	4	2	0	1	1	0	1	0	0	2540
dtopors^{C102A}/Cy	499	560	0	1	0	1	0	0	9	0	0	0	1070
Df(dtopors$^{114-118}$)/Cy	710	982	2	2	1	0	0	2	3	0	0	0	1702

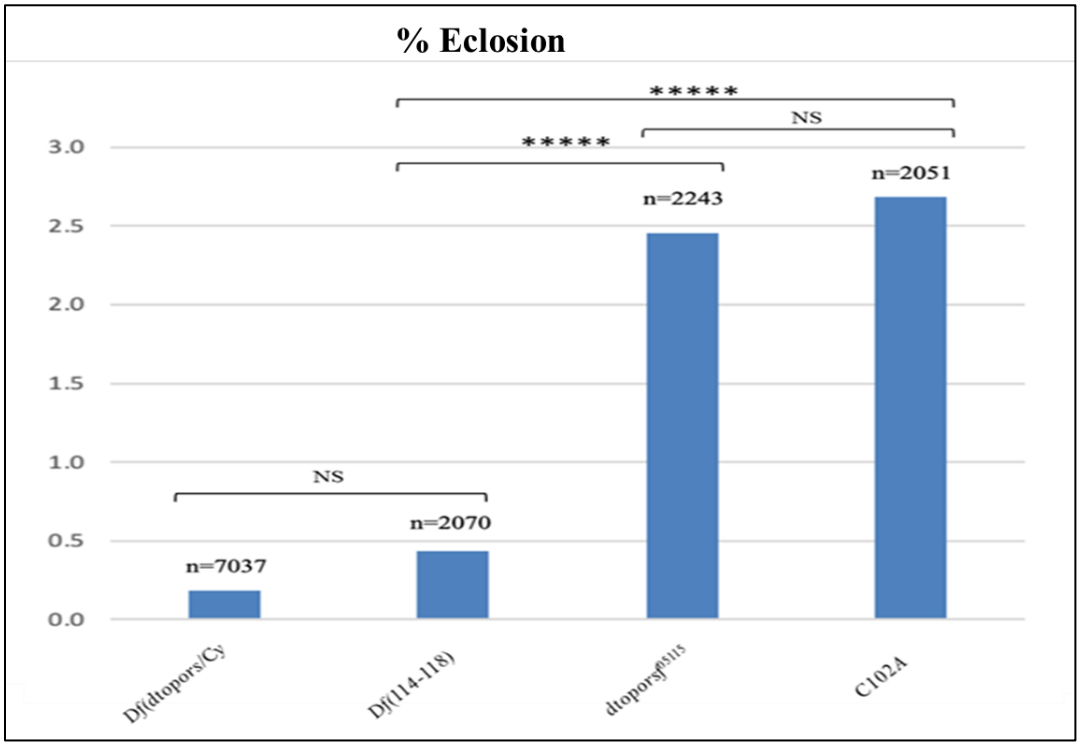
% Nondisjunction	Nullo XY	Diplo XY	Nullo 4	Diplo 4	XY	4
dtopors^{fsF114SX19}/Df(dtopors^{AA})	33.7	13.6	19.9	15.2	47.3	35.2
dtopors^{C102A}/Df(dtopors^{AA})	28.5	6.9	10.4	6.6	35.4	17.0
Df(dtopors$^{114-118}$)/Df(dtopors^{AA})	33.6	7.0	7.1	4.6	40.6	11.7
dtopors^{fsF114SX19}	41.4	10.3	17.1	15.3	51.7	32.4
dtopors^{C102A}	18.7	5.2	2.1	1.2	23.9	3.3
Df(dtopors$^{114-118}$)	3.6	0.2	0.6	0.0	3.8	0.6
dtopors^{fsF114SX19}/Cy	0.4	0.2	0.1	0.1	0.6	0.2
dtopors^{C102A}/Cy	0.8	0.1	0.9	0.0	0.9	0.9
Df(dtopors$^{114-118}$)/Cy	0.3	0.1	0.2	0.1	0.4	0.4

The significantly lower NDJ frequency in the ubiquitin null *C102A* mutants compared to the *null Df(dtopors^{AA})* mutants indicates that loss of ubiquitin ligase activity does not disrupt chromosome segregation to the same degree as does loss of all dTopors functions. This suggests that another function of dTopors, perhaps sumoylation, contributes to chromosome segregation. I was surprised to find a difference in NDJ between the *Df(114-118)* and *C102A* mutants, as mutation of either C102 or C118 was previously reported to abolish ubiquitin ligase activity. My data suggest instead that *Df(114-118)* may be behave as a hypomorph. The deletion of the C118 target residue and four additional residues (FTDS) downstream, potentially allows C113 to substitute for C118 in the RING domain (Figure 1). This would result in a RING domain with an altered variable region between conserved cysteines, which might only partially disrupt the ability of the domain to properly fold.

Another interesting observation was that for each of the *RING* mutants, there was a greater effect on sex than on fourth chromosome segregation, whereas in null alleles sex and fourth chromosome NDJ are both nearly completely randomized (Table 1). It is possible that this difference might be related to chromosome size (sex chromosomes are much larger than the 4th) or chromosome specificity (sex chromosome versus an autosome). Overall, in all NDJ assays, chromosome 4 *nullo-* exceptions were slightly elevated over the diplo-exceptions amongst all *dtopors* alleles suggesting that suggesting that there may be some chromosome 4 loss as well. For the sex chromosome exceptions, the *nullo*-exceptions were significantly higher than the diplo-exceptions. This difference is unlikely to reflect chromosome loss, rather it is likely due to meiotic drive, where sperm with lower overall chromatin content (i.e., *nullo-XY*) are preferentially recovered among progenies. This phenomenon occurs when sex chromosome segregation is disrupted in male meiosis (McKee, 1991).

To discern between sex chromosome specificity versus size specificity, I examined the transmission of chromosome 2, a major autosome. In this assay, I crossed mutant males homozygous or heterozygous for *dtopors* to *Compound 2* (*C(2)*) females. These females produce eggs with either both chromosome 2 homologs attached at a single centromere, or a chromosome 2-deficient egg, and when mated to chromosomally normal males, the only surviving progeny are produced from aneuploid sperm. Therefore, the percent of embryos which gave rise to eclosed adults is a measure of autosomal NDJ. I collected embryos and counted the frequency that survived to adults from *C102A*, *Df(114-118) and Topors*^{f05115} (a P-element induced *null* allele previously described by Matsui et al., 2011) fathers and their heterozygous brothers to estimate NDJ frequency. The chromosome 2 NDJ frequencies of *C102A* and *f05115* were nearly identical, whereas the frequency was significantly lower for the *Df(114-118)* allele (Figure 4).

Figure 4. The Effects of *dtopors RING* Mutants on Autosomal Nondisjunction. Frequencies indicated as percent of eclosed progeny per male produced from males bearing the indicated *dtopors* alleles. (n = number of progeny). NS = Not significant. ***** $p < 0.001$.

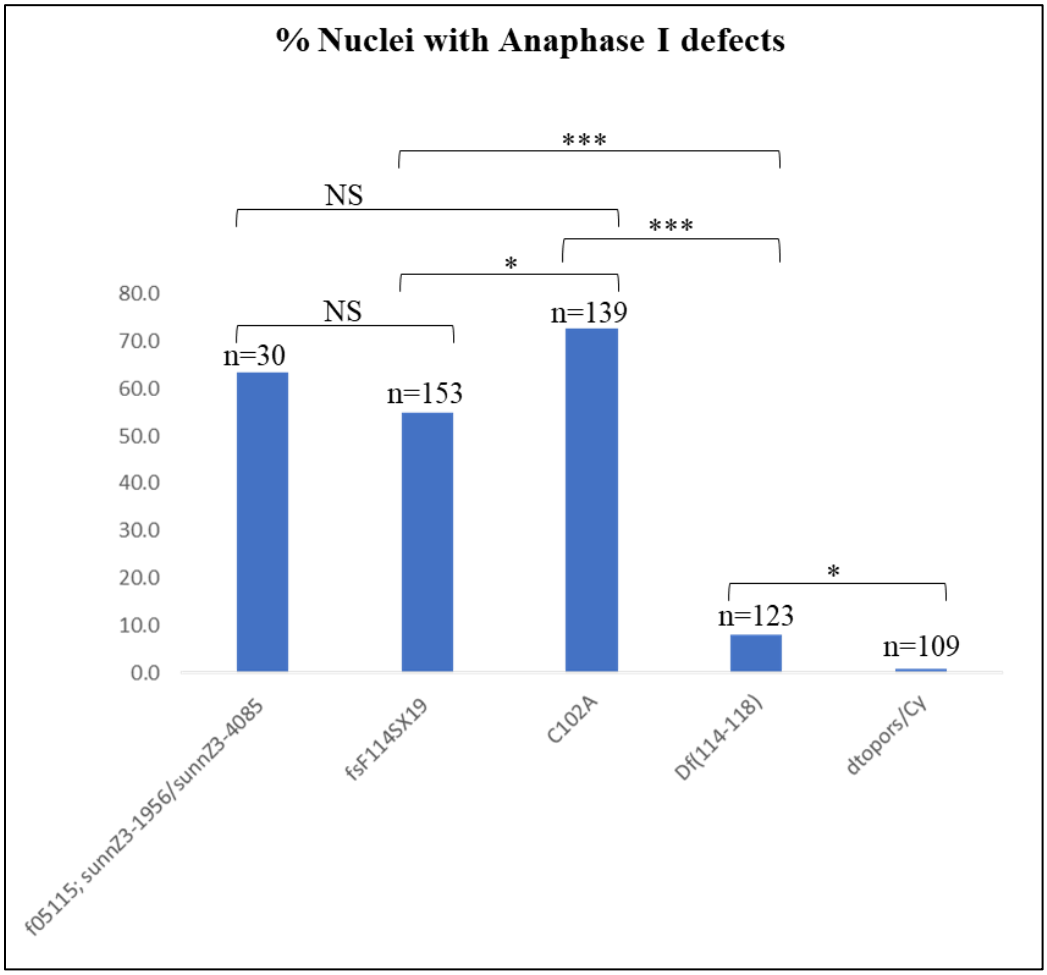


These data show that chromosome 2 segregation is also affected by the *RING* mutations, indicating that both types of chromosomes (autosomes and sex chromosomes) are affected by the mutation, regardless of size. However, it is interesting to note that although chromosome 2 is larger than chromosomes X and Y, yet it shows much lower frequency of NDJ. This suggests that chromosome specificity exist, however, the significance of this is elusive and should be further investigated. The difference in the NDJ frequencies among progeny of *C102A* and *f05115* males showing that segregation of chromosome 2 is affected equally by the *null* and the ubiquitin *null*. As observed for the sex chromosomes, the frequency of chromosome 2 NDJ was significantly lower among progeny of males bearing the for the *Df(114-118)* allele (when compared to the ubiquitin null), consistent with the hypothesis that *Df(114-118)* is hypomorphic.

The Absence of dTopors Ubiquitination Results in Anaphase I Bridge Formations

Prior examination of *dtopors* chromosome behavior suggests that meiotic NDJ is caused by the formation of Anaphase I bridges (Matsui et al 2011). To investigate whether dTopors ubiquitin ligase activity was required to prevent the formation of such bridges, I examined Anaphase I meiotic chromosomes in *RING* mutant males as well as in *FS null* males. I assessed at least one hundred meiosis I cells per mutant. The frequency of Anaphase I bridges and lagging chromosomes in *C102A* was similar to that of the null mutant, but was significantly lower in the *Df(114-118)* mutant (Figure 5). This indicates that Anaphase I bridges result from lack of dTopors ubiquitination.

Figure 5. Anaphase I Defects in Various *dtopors* Mutants. Frequencies indicated as percent of cells with bridges and lagging chromosomes at anaphase I. (n = number of progeny). NS = Not significant. *p<0.05 *** p < 0.00001.

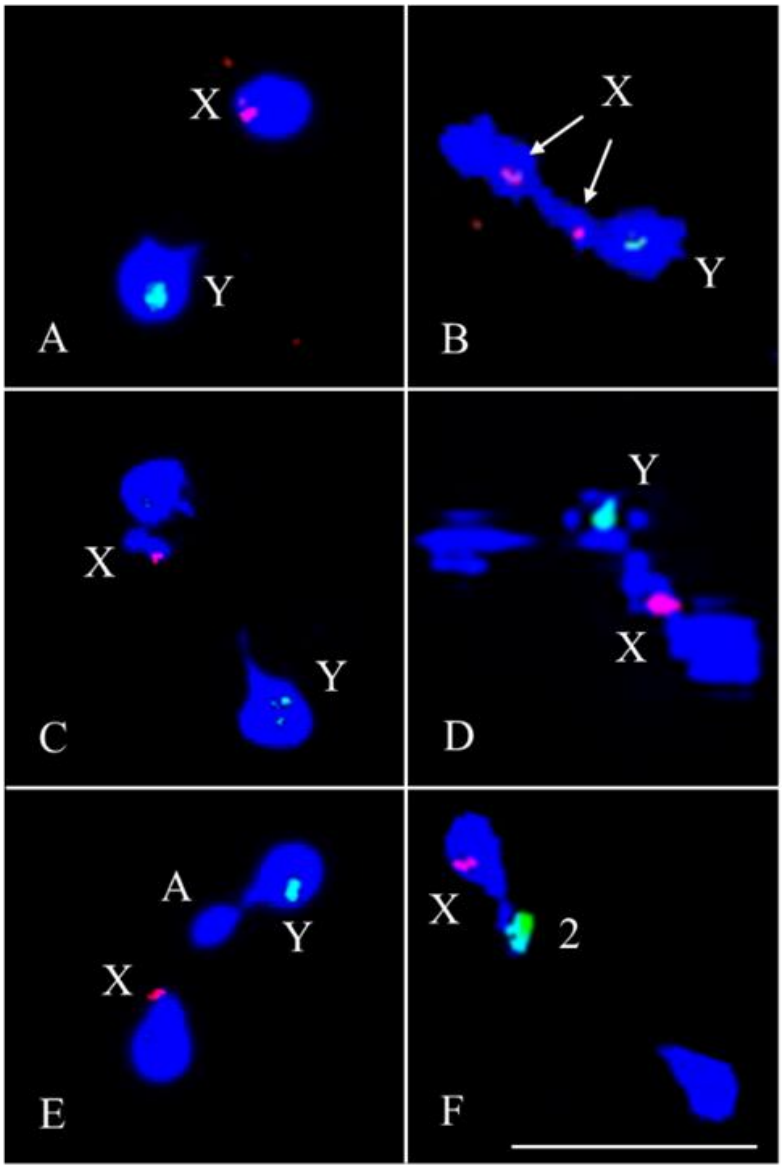


There are several possible causes of Anaphase I bridges. Matsui et al., (2011) ruled out that these bridges result from improper resolution of the conjunction complex that joins homologs together in male meiosis NDJ. In a double mutant homozygous for *dtopors* and *mnm*, the bridges were not resolved. A second possibility is that the bridges may result from improper orientation of sister chromatid centromeres to opposite poles at meiosis I. In yeast and mammals, the monopolin complex ensures that sister centromere co-orient to a single spindle

pole at Anaphase I (Corbett & Harrison, 2016; Tóth et al., 2000). Although this complex has not been identified in Drosophila, an analogous function must exist.

To investigate whether these bridges might result from the misregulation of a monopolin-like complex, I examined meiosis I in male flies double mutant for *f05115* and *sisters unbound* (*sunn)*, a gene require for sister chromatid cohesion in meiosis (Krishnan et al., 2014). Anaphase I bridges resulting from sister centromere misorientation would be expected to be dependent on sister chromatid cohesion. This, relieving connections between sister centromeres by *sunn* would be expected to resolve such bridges. I used flies transheterozygous for two alleles of *sunn* (*sunn*$^{Z3\text{-}1956}$ and *sunn*$^{Z3\text{-}4085}$) to avoid effects of background mutations on the *sunn* chromosomes. I found that the anaphase I bridges persisted despite the removal of cohesion between sister chromatids, suggesting that bridges do not results from inappropriate regulation of a monopolin-like function. (Figure 5). This posed the question, what chromosomes are together in bridges? To find an answer to this question, I performed fluorescent *in situ* hybridization (FISH) of meiosis I cells from homozygous *C102A* males, using probes to chromosomes X, Y and 2 and observed lagging chromosomes between X-Y homologs and X-2 and X-A heterologs (Figure 6). Based on this I conclude that both sex chromosomes and autosomes are present in the bridges. Although there is one example in panel B that suggests that some of these bridges may occur between sister chromatids, the mechanism of how these occur is unclear (it may be a case where each X sister was entangled with a different chromosome that segregated to opposite poles and dragged the X with it).

Figure 6. FISH Examination of *dtopors*^{C102A} Nondisjunction in DAPI-Stained Spermatocytes. Panels A-E hybridized with X probe (red) and Y probe (green). Panel F hybridized with X probe (red) and chromosome 2 probe (green). A. Telophase I with normal XY segregation. B. Anaphase I with X chromosome bridge. C. Lagging X chromosome at Telophase I. D. Lagging X and Y at Anaphase I. E and F. Lagging autosomes. Bar, 10 μm.



Ubiquitin Ligase Activity of dTopors is Not Required for Insulator Function

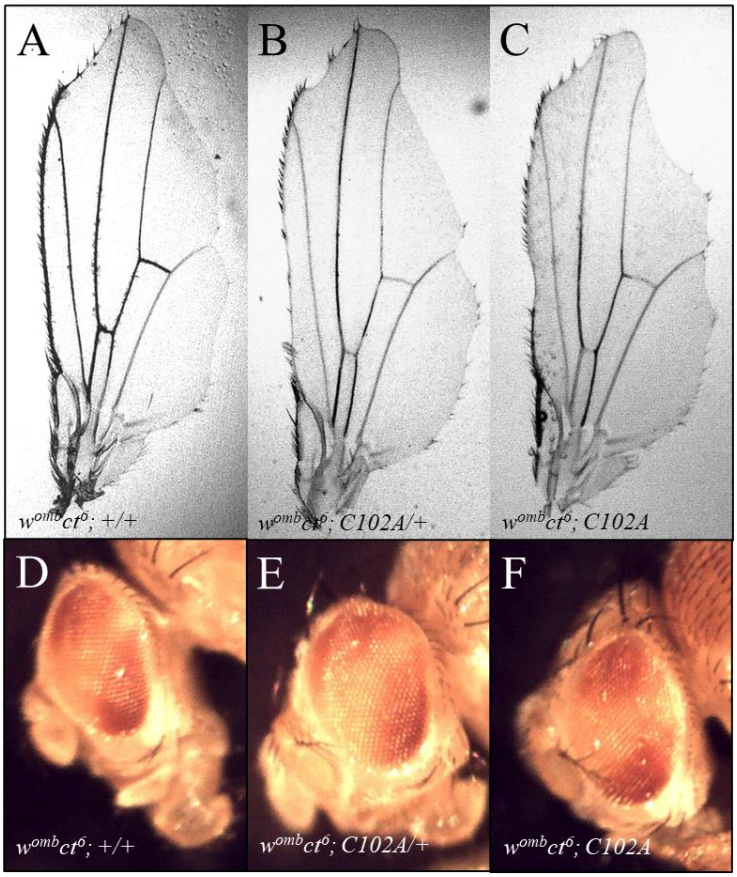
Insulator complexes are regulatory elements found in eukaryotic genome that influence gene transcription by either blocking enhancers from accessing the promoter of a gene (Geyer &

Corces, 1992; Kellum & Schedl, 1991), and/or preventing silencers from spreading of adjacent repressive chromatin (Chung et al., 1993; Kuhn & Geyer, 2003). The Drosophila *gypsy* insulator complex is a *cis*-regulatory element with enhancer blocking activity (Geyer & Corces, 1992) found at the borders between heterochromatin and euchromatic regions (Labrador & Corces, 2002). In somatic cells, dTopors colocalizes with components of the gypsy insulator complex and associates with the nuclear lamina (Capelson & Corces, 2005). To ask whether dTopors disrupts insulator function, Corces et al., used mutations generated by gypsy insertions in $y^2 w^{omb}$ ct^6 (*yellow-2* (y^2), *white optomotor blind-P1-D11* (*ombP1-D11*), *cut-6* (*ct6*)) genes as phenotypic markers for insulator activity. Each of these mutations is caused by the insertion of gypsy-transposon insulator into the regulatory regions of the gene, causing an insulator-dependent misexpression. Insertion of the gypsy insulator in the y^2 gene regulatory region causes drastic reduction of the black pigment production in the body cuticle (Geyer & Corces, 1992; Golovnin et al., 2008; Parnell et al., 2003). In the ommatidia, insertion of the *gypsy* transposon between the silencer element in the *omb* gene and the promoter of the *white* gene causes the appearance of red patches in the eye, dependent on the activity level of *gypsy*, with a less gypsy activity resulting in smaller patcher (Tsai et al., 1997).

In the wing, the insertion of gypsy between the enhancer of *wing margin* and the promoter of the *cut* gene results in the phenotype of jagged wing tips (Gause et al., 2001). Reduction of dTopors levels disrupts the *gypsy* insulator function resulting in phenotypes consistent with decreased insulator activity in these genetic assays (Capelson & Corces, 2005). To test whether dTopors ubiquitinates components of the insulators *in vivo*, Capelson et al., made an inducible *dtopors* construct carrying a C118S point mutation (Honda & Yasuda, 2000). The transgene was expressed in males hemizygous on the X chromosome for $y^2 w^{omb}$ ct^6.

Whereas overexpression of a *wildtype* dtopors transgene rescued disruption of insulator activity by an *mnm* mutation, expression of the C118S construct did not (Capelson & Corces, 2005). My genetic evidence suggests that *Df(114-118),* which targets the same C118 residue, behaves as a hypomorph and may still retain some ubiquitin ligase activity, therefore it was important to test the C102A in the same context, as this has been biochemically shown to be a ubiquitin ligase null. I tested male flies homozygous (or heterozygous) for the ubiquitin null *C102A* and hemizygous for two *gypsy* phenotype markers (w^{omb} and ct^{6}) on the X chromosome and found no changes in any of the phenotypes between test males and controls (Figure 7). This confirms Capelson and Corces' previous finding that dTopors ubiquitination is not required for insulator function (Capelson & Corces, 2005).

Figure 7. The Effect of *dtopors*^{C102A} on the *gypsy*-Induced *w*^{omb} *ct*^{6} Mutations. Phenotypes of flies either wildtype (A and D), heterozygous (B and E) or homozygous (C and F) for the indicated *topors*^{C102A} and hemizygous for *w*^{omb} *ct*^{6}.

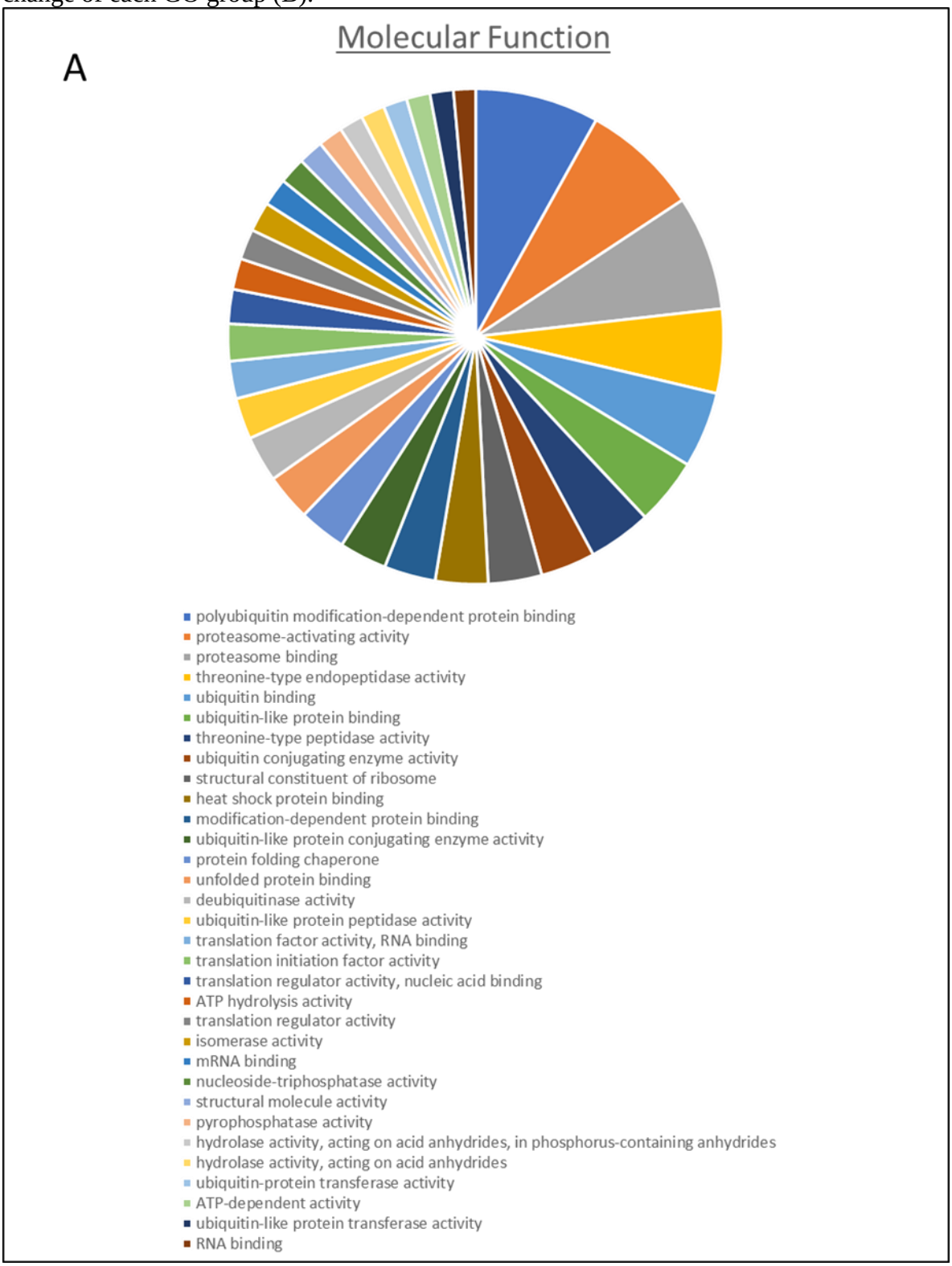


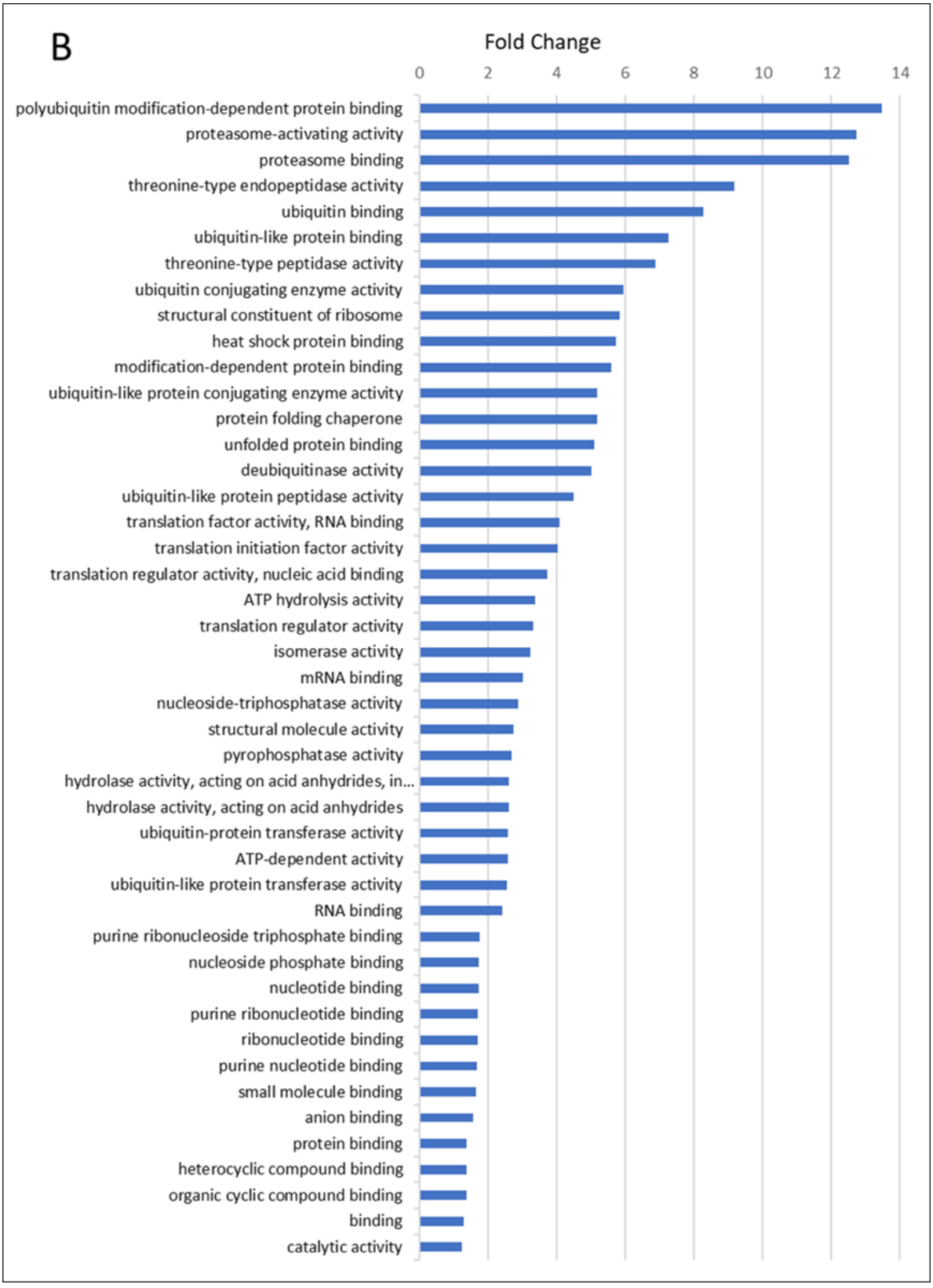
Targets of dTopors Ubiquitination

The mutants I created gave me a unique tool to identify *in vivo* targets of ubiquitination in the presence of functional *wildtype* dTopors versus *dtopors* (*C102A*) incapable of ubiquitination activity. To identify potential downstream targets of dTopors ubiquitination in the male germline, I made lysates of whole testis dissected from *wildtype* and *C102A* adults and immunoprecipitated ubiquitinated peptides using antibodies directed against the diglycine-lysine remnant (K-ε-GG) derived from preceding digestion with trypsin. A Ubiquitin molecule is conjugated to lysine residues of its target peptides by the -RGG (arginine-glycine-glycine)

residue sequence, resulting in a Ub-RGG-K bond. Trypsin cleaves the bond between arginine and glycine, thus exposing an -GGK epitope of the ubiquitinated target peptide. Immunoprecipitants were analyzed by tandem mass spectrometry (see Methods). I identified 924 differentially recovered ubiquitinated peptides. Of these, 69 were less than 0.67-fold frequent in the mutant and 855 were more than 1.5-fold frequent in the mutant when compared to the *wildtype* (supplementary data). When sorted for molecular function (describes activities that occur at the molecular level), more than half of the gene ontology groups were ubiquitination and proteasome related (Figure 8). Fold changes indicate representation of these gene ontology group members versus overall representation in the genome. The greatest number of targets were involved in polyubiquitin modifications, at nearly 14-fold higher than the overall representation in the genome (Figure 8), suggesting that dTopors is somehow involved in the regulation of other components of the ubiquitination pathway.

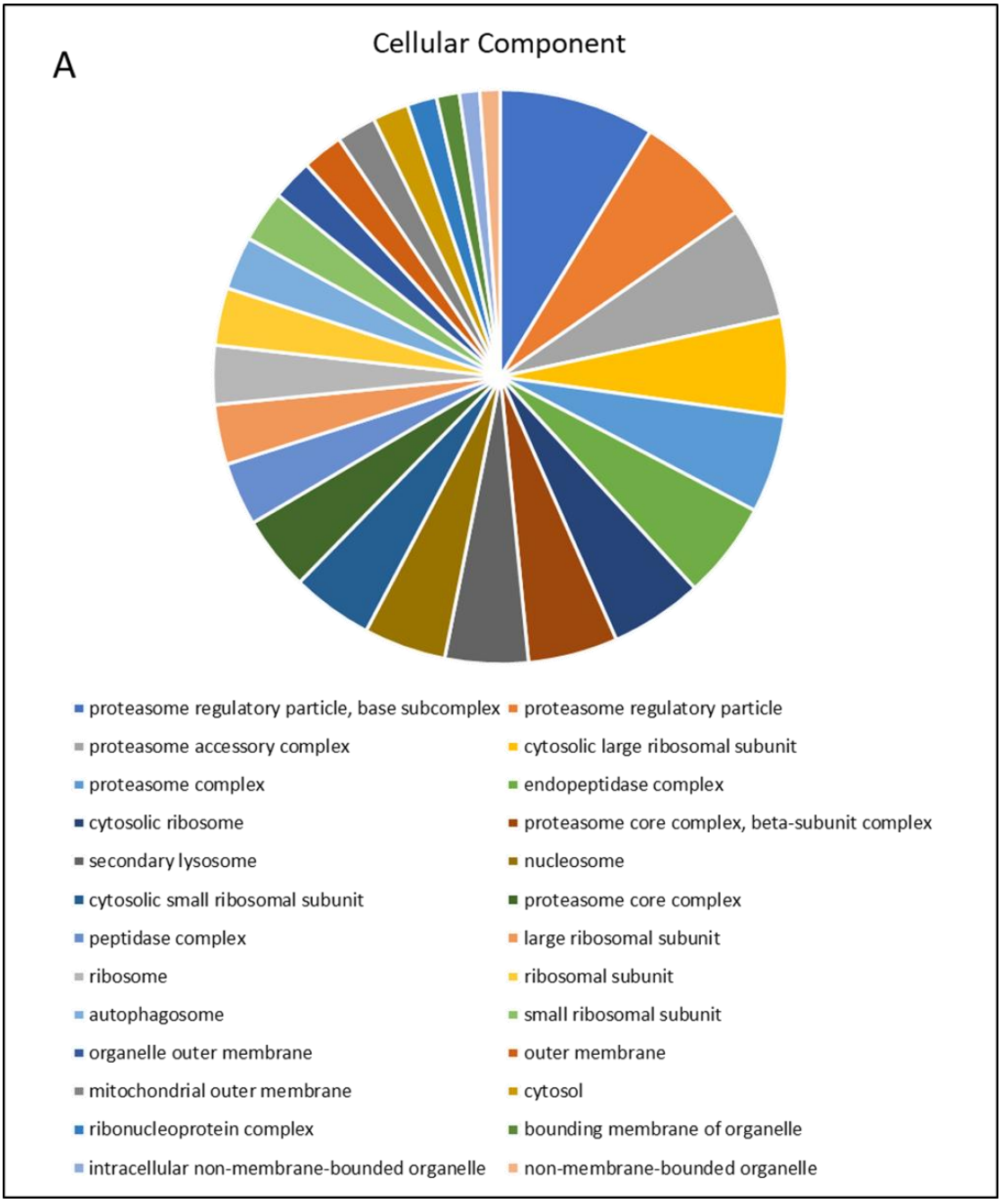
Figure 8. Gene Ontology Groups for Molecular Function of Differentially Ubiquitinated Targets. In the presence or in the absence of dTopors ubiquitin ligase activity (A) and fold change of each GO group (B).

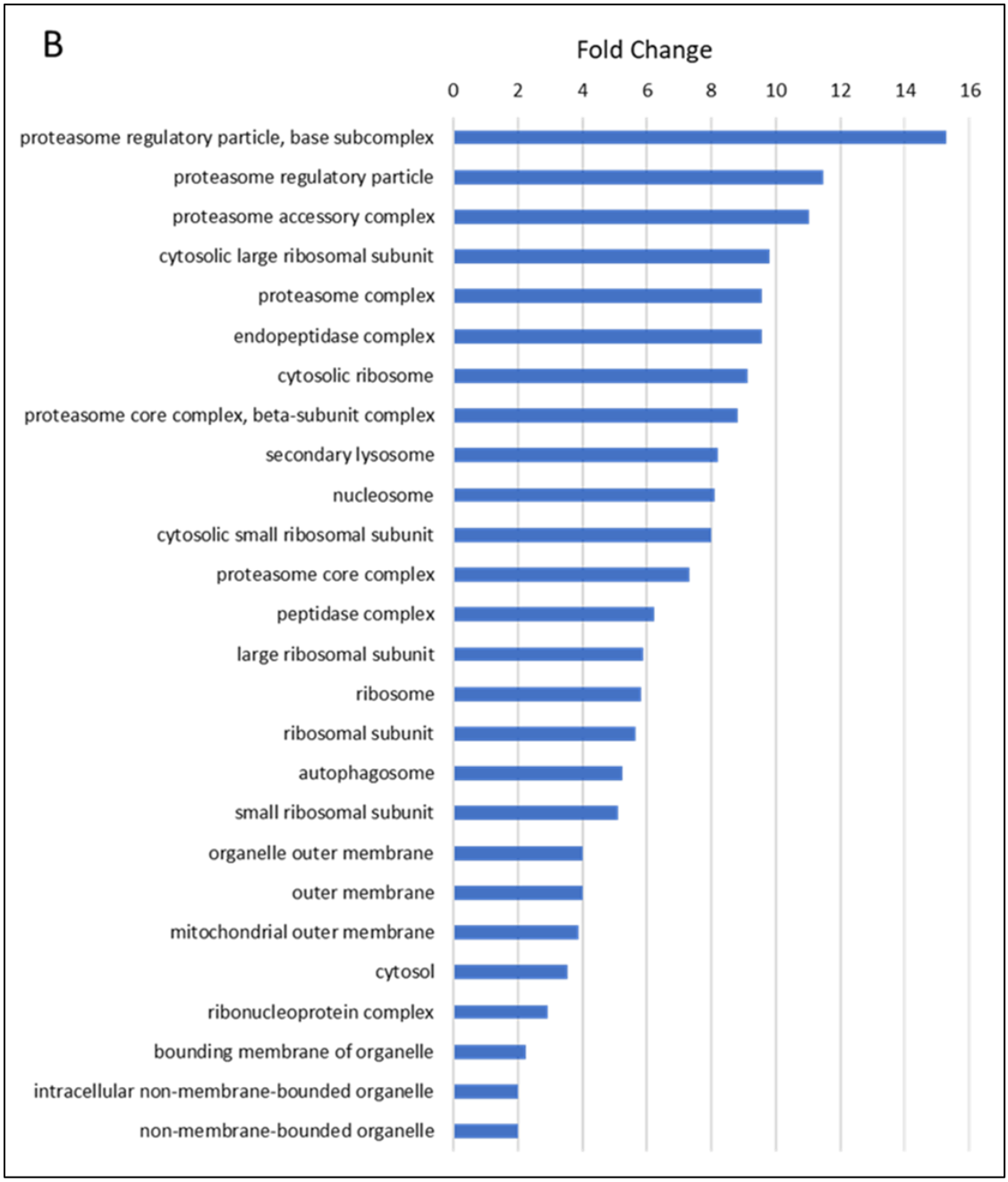



B
Fold Change
0
2
4
6
8
10
12
14
polyubiquitin modification-dependent protein binding
proteasome-activating activity
proteasome binding
threonine-type endopeptidase activity
ubiquitin binding
ubiquitin-like protein binding
threonine-type peptidase activity
ubiquitin conjugating enzyme activity
structural constituent of ribosome
heat shock protein binding
modification-dependent protein binding
ubiquitin-like protein conjugating enzyme activity
protein folding chaperone
unfolded protein binding
deubiquitinase activity
ubiquitin-like protein peptidase activity
translation factor activity, RNA binding
translation initiation factor activity
translation regulator activity, nucleic acid binding
ATP hydrolysis activity
translation regulator activity
isomerase activity
mRNA binding
nucleoside-triphosphatase activity
structural molecule activity
pyrophosphatase activity
hydrolase activity, acting on acid anhydrides, in...
hydrolase activity, acting on acid anhydrides
ubiquitin-protein transferase activity
ATP-dependent activity
ubiquitin-like protein transferase activity
RNA binding
purine ribonucleoside triphosphate binding
nucleoside phosphate binding
nucleotide binding
purine ribonucleotide binding
ribonucleotide binding
purine nucleotide binding
small molecule binding
anion binding
protein binding
heterocyclic compound binding
organic cyclic compound binding
binding
catalytic activity

When sorted for cellular components (locations relative to cellular structures in which a gene product performs a function), nearly half of the gene ontology groups were proteasome and ribosome related (Figure 9), suggesting that dTopors may be regulating components of the proteasome.

Figure 9. **Gene Ontology Groups for Cellular Components of Differentially Ubiquitinated Targets.** In the presence or in the absence of dTopors ubiquitin ligase activity (A) and fold change of each GO group (B).





The 924 peptides were also analyzed based on whether they were either present in wildtype but undetected in the mutant (dTopors-dependent) or undetected in the *wildtype* but present in the mutant (dTopors suppressed). Peptides classified as dTopors dependent are the most likely direct targets of dTopors ubiquitination. Peptides classified as dTopors suppressed

are peptides that may be ubiquitinated by other Ubiquitin ligases which in turn are regulated by dTopors. Among the dTopors-suppressed ubiquitination ontology groups we found that several Histones (His2B, His3, and His4) (Table 2).

Table 2. Gene Products Differentially Ubiquitinated in *dtopors*. (*ubiquitinated in the presence and absence of dTopors ubiquitin ligase activity).

Gene Ontology	dTopors-dependent	dTopors-suppressed
Chromatin/Transcription	*His3*; dikar; CG10887**	*ash1, CG12674, His2B, His3*, His4, Sirt2, CG10887**
Chromosome segregation	*Tcp-1zeta, CG6971, alphaTub84D*, alphaTub84B*, alphaTub85E **	*14-3-3zeta, Moe, Klp59C, alphaTub84D*, alphaTub84B*, alphaTub85E**
ER association	*Srp68**	*Rtnl2, Cnx99A, Rtnl1, Srp68*, Surf4*
Membrane association	*CG32512*	*FK506-bp, AnxB10, spartin, CG2930, CG7627, CG8153, l(2)k05819, MFS14, Pkd2, sut4, out, CG34126*
Metabolism	*Akr1B*, CG4546, CG8526*	*Argk, Pect, ACC, AdipoR, Akr1B*, AMPdeam, AOX1, Bacc, FucTD, CG32445*
Actine related	*cpb, tsr, Rtnl2*	*Act42A, Act5C, spn-F, cib, Act87E, Act88F, Act79B, Mhc, bent*
Ribosome/Translation	*Rpl11, Rps29*, bol, Ef1beta*	*RlP24, RpS29*, RpS3, RpS27, RpS17, CG6907, Ef1alpha48D, Ef1alpha100E, EF2, elF-4a*
RNA association	*Dhx15, Cpsf160, Saf-B*	*blanks, lost, pAbp, Ref1, spoon, exu, Dcr-2, qkr58E-3, Hrb98DE, Hrb87F, CG7907, bel, Rm62, SF2*
Signaling	*Gp150*, Hop**	*Inos, Gp150*, Hop*, CG34125, Sh3beta*
Sperm	*heph, CG15144, CG3330*	*Npc1a, Jyalpha*

Stress response	*Hsp27*	*CG16817, Hsc70-3, Hsc70-4, Hsp67Ba, Hsp8, UGP, Gss1, DnaJ-H*
Ubiquitin-mediated proteolysis	*Prosbeta4, Uba1, CG7220, CG3473, CG40045, Ubc10, hyd*	*CG10254, Np14, poe, CG4968, Rpn6, Rpt1, CG8786, CG8858, Rpn13R, Rpn3, Rpt6, Rpt6R, Atg8a, Atg8b, Prosalpha3, Fur1, klhl10, Uch, Usp7, Asx, Cul3, CG8042*
Vesicle	*comt, Rab2, Csp*	*Rab39, Syx7, Syb*
Miscellaneous (apoptosis, development, laminar, mitochondria, protein folding, sumoylation, oogenesis, dephosphorylation, courtship)	*Tom70*, Aos1, sds22, Pp1-Y2, qtc*	*Prx2540-1, CG12896, CG13516, Unc-13-4B, fax, Ntf-2r, Lam, Tom70*, Taz, COX6B, Sgt, Cctgamma, CG10635*
Unknown	*CG8136, CG2898, CG3760, CG3760, CG43675*	*CG10734, CG43121, CG42394, CG2861*

Histone 3 is an important target for different epigenetic modifications on its lysine residues, and here we report differential ubiquitination in a dTopors -dependent at lysine 23 (23K, KQLATK(gl)AAR) and in dTopors -suppressed manner at lysine 36 (K36, SAPSTGGVK(gl)KPHR). H3K23 is acetylated in flies and is implicated in courtship learning, memory impairment and cancer (Kai-Le et al., 2018; Klein et al., 2019). Here we show that the same H3K23 residue is ubiquitinated in the presence of dTopors, suggesting a competition between acetylation and ubiquitination for the same residue. H3K36 is a methylation site in flies for mono-, di-, and trimethylation associated with chromatin compaction and transcriptional regulation (Bell et al., 2007; Filion et al., 2010; Mukai et al., 2015). I found H3 peptides ubiquitinated at this site were enriched in the absence of dTopors, suggesting that dTopors

somehow regulates components of the ubiquitination pathway targeting this residue. However, dTopors' role is in this chromatin modification needs to be further investigated.

His2B is known to be part of the DNA repair pathway in many organisms. Here we show the first evidence that in Drosophila, His2B is ubiquitinated in the absence of dTopors, and furthermore, our screen revealed that the His2B lysine that is differentially ubiquitinated in the absence of dTopors, K118 (AVTK(gl)YTSSK), corresponds to the lysine mono-ubiquitinated in association with the DNA damage response K123 in yeast (Hung et al., 2017; G. Liu et al., 2021; Zheng & Petes, 2018), K120 in calf (Thorne et al., 1987), and in human cells (Chernikova et al., 2010, 2012). These results suggest that dTopors may be regulating components of the ubiquitination pathway for His2B. It is interesting to note that in the ubiquitin-mediated proteolysis group many of the targets ubiquitinated in the presence of dTopors are E2 ligases (*CG7220, CG3473, CG40045, Ubc10*), E1 (*Uba1*) and E3 ligase (*hyd*), suggesting that dTopors may be regulating the abundance of these ligases. Ubiquitination of E2 ligases may cause their degradation, and this may affect other targets indirectly. It also appears that dTopors regulates Uba, the only E1 ubiquitin ligase shared by all E2 ligases. This is very important since degradation of E1 would have a major impact on multiple ubiquitination pathways and thus on a myriad of different targets.

dTopors Regulates LaminDm0 Ubiquitination

dTopors physically interacts with LaminDm0 in somatic cells (Capelson & Corces, 2005) and also in spermatocytes, where these interactions have structural consequences for the lamina assembly (Matsui et al., 2011). In spermatocytes, there is no difference in the overall LaminDm0 levels in a *dtopors* deletion (null) mutant when compared to wildtype, however, there is variation in LaminDm0 distribution (Matsui et al., 2011). The mass spec analysis revealed an

accumulation of ubiquitinated LaminDm0 (K244) in absence of dTopors suggesting that dTopors somehow prevents LaminDm0 ubiquitination. dTopors colocalizes with both Lamins at the spermatocyte nuclear lamina in the wildtype (Matsui et al., 2011), and was shown to physically interact with Lamin Dm0 in somatic cells (Capelson & Corces, 2005).

Discussion

I set out to investigate the role of dTopors-mediated ubiquitination in nuclear structure and chromosome segregation, and to understand the relationship between nuclear lamina and chromosome segregation in meiosis. To accomplish this, I created a separation-of-function ubiquitin null allele of *dtopors* targeting different conserved residues in dTopors' RING domain. Fortuitously, the gene editing also yielded a *dtopors* frameshift and a genetic hypomorphic allele, a unique tool to uncover different requirements for ligase activity at the nuclear structure and in meiotic chromosome segregation.

It had previously been suggested that the meiotic chromosome segregation defects observed in *dtopors* mutants might be a consequence of perturbation of the nuclear structure (Matsui et al., 2011). Although this was a compelling argument, given the known examples of chromosome interactions with the lamina, our data argue that this may not the case. Rather, we suggest that the chromosome segregation defects and nuclear blebbing may be independent consequences of alterations in ubiquitination of different target proteins. Genetic and cytology assays of these different *RING* mutants revealed nuclear blebbing in all three mutants, but surprisingly the NDJ levels were very different. When compared to the *dtopors* deletion, both *Df(114-118)* and *C102A* mutants displayed similar frequencies of nuclear blebbing, but there were striking differences in NDJ frequencies and anaphase I defects. I suggest that this differential effect of the *Df(114-118)* on the two phenotypes might be explained by different

threshold requirements for ubiquitination of chromosome-associated versus lamina-associated dTopors' targets.

Examination of the primary amino acid sequence of the dTopors RING domain offers an explanation for the hypomorphic behavior of the *Df(114-118)* allele. In the consensus RING sequence, this deletion occurs in a variable region that is between 9 and 39 residue. (Borden et al., 1995). The deletion results in a variable region of 7 residues, just below the lower limit of the length observed in some wildtype RING domains. This may allow the domain to fold properly in some fraction of the dTopors proteins, thus allowing for some activity. Alternatively, as the RING domain is the region of the E3 that physically interacts with the E2 ligase (Joazeiro et al., 1999), alterations to this domain may have weakened or diminished assembly of the active ligase complex and reduced but not eliminated Ub ligase activity. While the deletion may affect protein folding causing some loss of function, the potential C113 to C118 substitution may partially restore ubiquitin ligase activity. As increase of temperature affects protein structures, I hypothesize that the deletion may exacerbate the folding defect. However, since blebbing occurs in all mutants while NDJ varies greatly, I assume that there may be different threshold requirements for dTopors-mediated ubiquitination in chromosome segregation and in nuclear structure. It also suggests that chromosome misbehavior may not be a consequence of the disturbed nuclear structure.

Interestingly, I found that different chromosomes showed different sensitivities to disruption by the Ub-null mutation. Sex chromosome segregation was disrupted to a greater degree than that of the tiny fourth chromosome. This difference was exaggerated in the *Df(114-118)* mutants, in which fourth chromosome segregation was nearly normal while sex chromosome NDJ was significantly elevated. This suggests that different chromosomes may

have different requirements for levels of dTopors ubiquitination activity. Surprisingly, I found that the larger autosome, chromosome 2, responded to the different alleles in a manner more similar to the 4th instead of the larger sex chromosomes. This may suggest that for dTopors ubiquitin ligase activity chromosome size does not play a significant role and that chromosome specificity exist. Generally, larger chromosomes tend to be more prone to entanglement, telomer fusion and breakage. In most cases DNA breakage is resolved by homology directed repair, but sometimes it may be repaired through NHEJ. All these cases may lead to chromosome segregation defects which are reflected in my findings. The significance of chromosome 2 having less NDJ than a sex chromosome of similar size is elusive and should be further investigated. Although both *RING* mutants showed NDJ, the differences in the frequencies were significant, *C102A* NDJ was comparable to the *f05115* null, while *Df(114-118)* was at wildtype levels, adding support for *Df(114-118)* being a genetic hypomorph. The significant lower NDJ frequency in the *C102A* ubiquitin null mutants compared to the *dtopors* deletional null *Df(dtopors^{AA})* mutants indicates that dTopors ubiquitin ligase activity is necessary but not sufficient for chromosome segregation. Therefore, chromosome segregation may be aided by some other function of dTopors, perhaps sumoylation.

NDJ may result from homolog, heterology and/or sister chromatid entanglements. FISH was used to verify involvement of both sex chromosome and autosomes in bridges and to investigate whether dTopors ubiquitin ligase activity plays a role in creating these bridges. Moreover, the frequency of anaphase I bridges and lagging chromosomes in *C102A* was similar to that of the null mutant, but was significantly lower in the *Df(114-118)* mutant, indicating that the bridges results from lack of or improper ubiquitination of some targets at chromosomes.

The anaphase I bridges persisted despite the removal of cohesion between sister chromatids, suggesting that bridges do not result from inappropriate regulation of a putative Monopolin complex.

In addition to NDJ, nuclear structure was also affected in both *RING* mutants as we observed nuclear blebs in both. We found that both *RING* mutants displayed blebbing at least as frequently as complete deletion of the gene, indicating that the ubiquitin ligase activity is required for intact nuclear morphology, but other possible functions of dTopors (i.e., a structural role at the lamina, or sumoylation) may be of secondary importance to nuclear structure. It remained possible that the nuclear defects observed in the *RING* mutants were an indirect consequence of dTopors mislocalization rather than a loss of ubiquitin ligase activity *per se*, as it had not been demonstrated that lamina localization is independent of this activity. I found that in the absence of its ability to ubiquitinate, dTopors protein still localized to the nuclear lamina, therefore enzymatic activity or interaction dependent on RING domain is not important for its localization. If the NDJ were a direct consequence of nuclear disruption, then I would have expected that more frequent nuclear disruption would lead to more frequent NDJ. Since this is not the case, the hypothesis is supported that the two phenotypes may be due to separate activities of dTopors – i.e., ubiquitination of different targets.

To test whether ubiquitination by dTopors was necessary for the components of the insulator complex *in vivo*, Capelson et al. made a transgene with an inducible *dtopors* construct carrying a C118S point mutation. The transgene was expressed in males hemizygous on the X chromosome for y^2w^{omb} ct^6 phenotypic markers for insulator activity. Each of these mutations is caused by the insertion of gypsy-transposon insulator into the regulatory regions of the gene, causing an insulator-dependent misexpression. Overexpression of the *C118S* construct did not

rescue disruption of insulator activity by an *mnm* mutation (Capelson & Corces, 2005), suggesting that a) ubiquitin ligase activity is not important for the insulator complex assembly or function, or b) the transgene was a hypomorph and retained some ubiquitin ligase activity due to a potential substitution of C113 for C118 in the RING domain. We identified *Df(114-118)* as a genetic hypomorph, and therefore it was important to test the *C102A* in the same context, since this was shown biochemically to be a definite ubiquitin ligase null in flies (Secombe & Parkhurst, 2004). My results confirmed Capelson and Corces previous results of dTopors ubiquitination not being required for insulator function. The RING mutants still localize to the lamina, and thus is consistent with Corces' model that dTopors role in insulator function may be limited to its ability to anchor insulators in the lamina. This could mean that the defects seen are not likely to be the indirect consequences of alterations of the transcriptional profile of spermatocytes, but rather directly result from lack of ubiquitination on some target(s).

The *C102A* mutant is a unique tool to identify in vivo targets of ubiquitination because it is a ubiquitin null, therefore it allows us to identify differences in ubiquitination when compared to dTopors wildtype. The great variety of targets confirm a broad and significant role of dTopors' ubiquitin ligase activity in the male germline. A prime candidate for involvement in the nuclear defects revealed by our comparative proteomic analysis was the Lamin Dm0 protein. In humans, post-translational modifications regulate Lamin function and integrity (Wagner et al., 2011), and many of these modifications (ubiquitination, acetylation, and sumoylation) target the same lysine at multiple locations on the protein (Murray-Nerger & Cristea, 2021). However, how these modifications interfere with each other and how such competition influences Lamin function and behavior is not yet fully understood. Ubiquitination by several E3 ligases are known to mediate proteasomal degradation of Lamins (Khanna et al., 2018; Krishnamoorthy et al.,

2018). Ubiquitination mediated degradation of Lamins is an important process to eliminate excess or unincorporated Lamins from the cell, and several E3 ligases (RNF123, HECW2, Smurf2, and Siah)1 are known to interact with Lamins in humans (Khanna et al., 2018). Phosphorylation at specific residues (S395 and S405 in Lamin B, S392 for LaminsA/C) leads to Lamin disassembly and solubilization, followed by ubiquitin-mediated degradation of the Lamins by E3 ligases (Shimizu et al., 1998; Y. Zhang et al., 2018), resulting in DNA breakage and apoptosis (Murray-Nerger & Cristea, 2021).

In the mass spec analysis, I found a 15-fold increase in ubiquitinated LaminDm0 in the absence of dTopors (see supplementary table), suggesting dTopors may prevent the ubiquitination and thus possibly the degradation of LaminDm0. This would fall in line with the blebbing phenotype present in all *dtopors* mutants, including the *RING* mutants. A possible explanation could be that in the presence of dTopors, LaminDm0 is not ubiquitinated and thus can be assembled into the nuclear lamina, while in the absence of dTopors, LaminDm0 is ubiquitinated and may not be effectively assembled into the lamina, thus weakening laminar structure resulting in blebbing. There is some evidence to support this assumption. Matsui et al., found no difference in the amount of LaminDm0 present in a *dtopors* null and in the wildtype, but they observed differences in intranuclear localization of LaminDm0. While in wildtype males virtually all LaminDm0 seemed to be found at the nuclear lamina, in the mutant, laminDm0 localizes at the lamina but is also dispersed throughout the nucleus (Matsui et al., 2011).

We also found that several Histones were ubiquitinated in the presence and in the absence of dTopors, suggesting a broader role for dTopors in chromatin modifications and transcriptional control. Histone 3 is an important target for different epigenetic modifications on its lysine

residues and we found two different histone sites of ubiquitination regulated by dTopors. These sites are known for epigenetic regulations and are also targeted by other post-translational modifications, such as acetylation and methylation. Ubiquitination of these residues may compete with other post-translational modifications at these sites, thus affecting chromatin structure and possibly regulating transcription of genes. Since H3K23 is ubiquitinated in the presence of dTopors, it is possible that this mark is a direct target of dTopors ubiquitin ligase activity. In flies, *enoki mushroom* (Enok), an acetyltransferase responsible for germline cell formation and abdominal segmentation in fly embryos acetylates H3K23 and regulates expression of genes involved in neuronal development and oogenesis (Huang et al., 2014). It is possible that in the male germline H3K23 is ubiquitinated to block acetylation and keep the chromatin closed, thus repressing expressions of genes not needed in the germline. H3K36 is mono-, di-, and trimethylated in wildtype flies, and here it is ubiquitinated in the absence of dTopors ubiquitin ligase activity. The methylation of this mark in the wildtype is associated with chromatin compaction and transcriptional gene silencing (Bell et al., 2007; Filion et al., 2010; Mukai et al., 2015), and in mammals with tumor suppression (Berthelet et al., 2018, p. 36; Zhu et al., 2017) and recombination in germ cells (Powers et al., 2016). These sites are important for chromatin modification events, and the fact that they are differentially regulated by dTopors, suggests that dTopors may play a role in their regulation. The exact role of dTopors at these epigenetic marks should be further investigated.

The other Histone ubiquitinated in the absence of dTopors is Histone 2B (His2B). His2B is of particular interest given what is known about dTopors and its own role in DNA damage repair. As part of the DNA damage response, H2Ax is first ubiquitinated by Topors and then activated by phosphorylation (γ-H2AX) (Doil et al., 2009; Pan et al., 2011; Rogakou et al., 1998;

Seong et al., 2012). An activated γ-H2AX recruits the DNA damage repair machinery (Stucki et al., 2005; Jackson et al., 2009). His2B is mono-ubiquitinated in yeast, mouse, calf, and human cells in response to DNA damage (Hung et al., 2017; G. Liu et al., 2021; Zheng & Petes, 2018; Thorne et al., 1987; Chernikova et al., 2010, 2012). dTopors does not exist in yeast and there is no evidence of Topors ubiquitinating His2B in the other organisms. I found that in Drosophila male germline, His2B is ubiquitinated in the absence of dTopors at K118 and that residue corresponds to the lysine mono-ubiquitinated in association with the DNA damage response K123 in yeast (Hung et al., 2017; G. Liu et al., 2021; Zheng & Petes, 2018), K120 in calf (Thorne et al., 1987), and in human cells (Chernikova et al., 2010, 2012). This suggests the possibility that dTopors somehow modifies this residue in response to DNA damage. The role of His2B ubiquitination in response to DNA damage has not yet been established in Drosophila and further experiments are needed to elucidate the dTopors role in this pathway. I observed that both LaminDm0 and His2B are ubiquitinated in the absence of dTopors ubiquitin ligase activity, and although these might be independent occurrences, it is interesting to note that His2B interacts with LaminDm0 by binding to the carboxy terminal end of the protein (Goldberg et al., 1999). It is possible that chromatin is linked to the lamina through His2B and LaminDm0 binding, and dTopors ubiquitin ligase activity plays a role in this interaction.

Interestingly, it seems that dTopors also regulates some of the ligases in the ubiquitination pathway as we found Uba1 (the sole Ub E1in Drosophila) and several E2 ligases ubiquitinated in the presence of dTopors. Since Uba1 is the only E1 ubiquitin ligase shared by all E2 ligases, its ubiquitination may have a major impact on multiple ubiquitination pathways and thus on a myriad of different targets. It is possible that dTopors is some sort of feedback regulator for Uba1 and functions to ubiquitinate excess Uba1 for degradation. Furthermore,

ubiquitination of E2 ligases may cause their degradation, and this may also affect many other targets indirectly. Based on this we suggest that dTopors may function as a "master regulator" of the ubiquitination pathway by interacting with several E2 ligases and by regulating the abundance of these pathway components through ubiquitination. Generally, E3-E2 interactions are determined by specificity of an E2 to an E3, however, a single E2 can interact with different E3 ligases, as seen with Ube2D/UbcH5 and the ubiquitination of a target depends on which E3 is chosen by an E2 (Ye & Rape, 2009). E2s utilize different residues to recognize various E3s, and additional components unique to certain E2s may increase specificity towards an E3 ligase (Ye & Rape, 2009). Topors was shown to interact with several E2 ligases (UbcH5a, UbcH5c, and UbcH6) *in vitro* (Rajendra et al., 2004), and it is unclear if those interactions are biologically meaningful as proteins are present in high concentrations in these assays. For future work, the hypomorphic *Df(114-118)* allele would serve as a great tool to screen for potential E2-dTopors interactions *in vivo,* where possible interactions between *Df(114-118)* and specific E2 ligases are expected to exacerbate the mutant phenotype.

CHAPTER III: IDENTIFYING TARGETS OF DTOPORS SUMOYLATION

Introduction

Post-translational modifications are important components for regulation of protein function and are essential for cell survival. After translation, most proteins undergo adjustments to their amino acid side chains or to one of their termini in form of a covalently attached group, often determining the protein's fate, or effecting functional activation. Ubiquitination and sumoylation are types of post-translational modification in which a small peptide is covalently conjugated to lysine residues of a target protein via a glycine-lysine isopeptide bond. Although these processes are very similar, both involving an E1 activating enzyme, E2 conjugating enzyme and E3 ligases, the outcomes for the target proteins are quite different. In contrast to ubiquitination where a (Ub) tag usually marks proteins for degradation, the addition of a SUMO (Small Ubiquitin-like Modifier) group does not lead to protein degradation, but may stabilize proteins by competing for lysine residues with ubiquitin (Desterro et al., 1999). SUMO was first identified as SMT3 (Suppressor of Mif Two 3) in *S. cerevisiae* (Meluh & Koshland, 1995a, 1995b) and although its 3D structure resembles Ubiquitin, its amino acid sequence is less than 20% identical to ubiquitin (Bayer et al., 1998; Bernier-Villamor et al., 2002; Mossessova & Lima, 2000). The human *SUMO* gene family encodes for four SUMO proteins paralogs (SUMO 1-4) (Gareau & Lima, 2010), whereas other organisms like yeast (Lapenta et al., 1997), *C. elegans* (Jones & Candido, 1993) and *D. melanogaster* (Lehembre et al., 2000) have a single SUMO gene, *smt3*.

The Sumoylation Pathway

To initiate the sumoylation pathway in mammals, SUMO proteins need to be activated by means of a C-terminal cleavage of amino acids via SENP (Sentrin/SUMO-specific protease), which results in an exposed di-glycine motif, ready to be conjugate to the lysine residue of a target protein (S.-J. Li & Hochstrasser, 1999). Afterwards sumoylation commences in three steps: first, SUMO is attached to the heterodimeric SUMO E1 activating enzyme (SAE 1/2), then it is passed to an E2 conjugating enzyme (Ubc9), which catalyzes the SUMO transfer to the target protein mediated by an E3 SUMO ligase (Hochstrasser, 2000). Interestingly, in comparison to the ubiquitin pathway which utilizes dozens of E2 conjugating enzymes, only one E2 enzyme (Ubc9) is known to exist for the SUMO pathway (Gill, 2004). To date, several SUMO E3 ligases have been discovered including the nucleoporin RanBP2 (Pichler et al., 2002), members of the PIAS (Protein Inhibitors of Activated STATs) family (Hochstrasser, 2000), polycomb complex protein Pc2(Kagey et al., 2003), Mms21 (Zhao & Blobel, 2005) and Topors (Weger et al., 2005).

The Impact of Sumoylation in Human Cells

Sumoylation plays an important role in many nuclear and subnuclear processes in the cell. Localization of proteins may change with sumoylation as is the case with the small GTPase activating protein RanGAP, the first identified target of SUMO (Matunis et al., 1996). Prior to sumoylation RanGAP is cytoplasmic, however, once sumoylated, it localizes to the nuclear pore complex and associates with RanBP2 (Matunis et al., 1996), a nucleoporin and SUMO E3 ligase (Pichler et al., 2002).

Sumoylation also plays a role in epigenetic modifications of Histones. Histone 4 was the first shown to be modified by SUMO, and *in vitro* studies showed that the N-terminal tail serves

as a substrate for SUMO modification (Shiio & Eisenman, 2003). Since then, the other Histones were found to be targets of cross-talks between sumoylation and various other post-translational modifications (Ryu & Hochstrasser, 2021). In general, sumoylation of Histones is associated with chromatin compaction and transcriptional repression, which result in chromatin structure modifications and gene silencing (Ryu & Hochstrasser, 2021). However, research suggests that in yeast, Histone-SUMO conjugates may also be involved in DNA damage repair response and chromosome segregation (Ryu & Hochstrasser, 2021).

Cellular signaling pathways are also linked to sumoylation of several components like HSF1, PML, p53 and IKB (Inhibitor of NFKB). Signal-dependent phosphorylation of HSF1 (Heat Shock factor 1) in response to heat induced stress increases sumoylation of HSF1 (Hietakangas et al., 2003), while phosphorylation appears to decrease sumoylation of PML (Muller, 1998; Everett et al., 1999), p53 (Müller et al., 2000), and IKB (Desterro et al., 1999). Interestingly, phosphorylation of IKB inhibits its sumoylation, thus allowing ubiquitination to occur on the same residue, K21 (Desterro et al., 1999). Ubiquitinated IKB undergoes proteasomal degradation and as a result, NFKB is released from its inactive state. Considering the substrates involved, just like ubiquitination, sumoylation is expected to be influential in human cancers. The oncoprotein Mdm2 is a RING finger E3 ubiquitin ligase known to ubiquitinate p53 for degradation (Ellison-Zelski & Alarid, 2010; Haupt et al., 1997; Honda & Yasuda, n.d.; Kubbutat et al., 1997) and if needed, Mdm2 can also self-ubiquitinate and thus help regulate p53 levels. Ubc9-mediated sumoylation of Mdm2 causes a reduction of self-ubiquitination levels and thus an increase of Mdm2 ubiquitination activity of p53 (Buschmann et al., 2001). The mammalian homolog of the yeast repressor Sin3A (switch independent 3) is transcriptional repressor of key apoptotic genes whose expression promotes breast cancer cell

growth by inhibiting apoptosis (Zelski et al., 2010). The Sin3A complex localizes to the PML body where it serves as a scaffold to recruit other corepressors into a complex (Ayer et al., 1995; Khan et al., 2001). In the PML body, Sin3A and the corepressors (c-Ski, N-CoR) recruit HDAC1 (histone deacetylase 1) and interact with PML and initiate transcriptional repression (Khan et al., 2001). SAP130 (SinA3 Associated Protein 130) is part of the mammalian Sin3A complex and interacts with the Sin3A-HDAC complex for transcriptional repression (Marshall et al., 2010). Human Sin3A is sumoylated by Topors, suggesting a potential role of Topors as a tumor suppressor by regulating Sin3A in HeLa cells (Pungaliya et al., 2007). Topors also sumoylates TOP1 (DNA Topoisomerase I) in osteosarcoma cells (Hammer et al., 2007) and p53 *in vitro* and *in vivo* in HeLa cells (Weger et al., 2005).

The Impact of Sumoylation in Flies

Sumoylation also occurs in a variety of tissues in *D. melanogaster* and plays a role in multiple biological functions. SUMO involvement can be detected among others in immune signaling, central nervous system development, chromatin structure and chromosome segregation in mitotic and meiotic cells. As part of the fly immune response to infection, RSS (Arginyl tRNA Synthetase) which is a member of the MARS (multi-aminoacyl tRNA complex) immune signaling complex, is sumoylated (Nayak et al., 2021). In the central nervous system, sumoylation of SoxN (SRY-related HMG-box Neural, a transcription factor) is required for proper CNS development *in vivo* to avoid severe CNS defects (Savare et al., 2005). Sumoylation also contributes to chromatin modifications and regulates heterochromatic silencing such as PEV (Position Effect Variegation), which can be suppressed by SUMO modification of Su(var)2-10 (a SUMO E3 ligase and member of PIAS protein family) (Hari et al., 2001) or enhanced by sumoylation of SU(VAR)3-7 (Reo et al., 2010). Chromosome segregation in mitotic and meiotic

cells is also partly regulated by SUMO modification of components involved. In mitotic cells, sumoylation of various targets is required for progression through the cell cycle (Nie et al., 2009). In meiosis, the SUMO E2 conjugation enzyme Ubc9 mediates homolog separation during female meiosis I and the Ubc9 mutant *lesswright* displays chromosome segregation defects (Apionishev et al., 2001). Based on prior observation of mammalian Topors localization to PML bodies containing increased concentrations of proteins involved in the sumoylation pathway (Rasheed et al., 2002) and because dTopors is a dual E3-ubiquitin/SUMO-1 ligase (Rajendra et al., 2004; Weger et al., 2003, 2005), Corces et al., explored the possibility of the sumoylation function of dTopors on the components of the *gypsy* insulator in fly somatic cells. *In vivo* and *in vitro* results showed two components of the gypsy insulator (Mod(mdg4)2.2 and CP190) are sumoylated in the absence of dTopors indicating that dTopors may inhibit the sumoylation of these proteins (Capelson & Corces, 2006). In contrast to human Topors, which is known to interact with Ubc9 and sumoylate several targets, there is no known interaction between dTopors and Ubc9 in flies.

Aim of Study

Sumoylating activities of Topors were described in detail in human somatic cells, but it is not known if dTopors mediates sumoylation in Drosophila. In yeast and human cells, the SUMO E2 conjugating ligase Ubc9 interacts with the E1 ligase and the SUMO group for sumoylation of substrates (Desterro et al., 1999; Johnson & Blobel, 1997; Saitoh et al., 1998; Schwarz et al., 1998). The Ubc9 protein (i.e., 99% identical to human protein in mouse, 87% in flies, 78% *C. elegans*, >60% in yeast), acts as an E2 sumoylating enzyme and sumoylates targets in concert with Uba2 (E1) and SUMO. In humans, Topors interacts with Ubc9 to sumoylate targets (Pungaliya et al., 2007). Although direct evidence for a role of dTopors in sumoylation is

lacking, Ubc 9 and dTopors interaction were shown in a yeast-two-hybrid assay (Capelson & Corces, 2006), suggesting dTopors likely acts as an E3 SUMO ligase. However, even if dTopors is not a Sumo ligase, evidence indicates it can regulate sumoylation of other proteins, and it would be interesting to identify which proteins are regulated in this manner. As such, direct targets of dTopors sumoylation need to be identified and dTopors effects on the activities of these substrates characterized. Based on the dual ligase activity of dTopors, it is possible that dTopors could regulate the numerous targets alternatively by ubiquitination and sumoylation, as it is the case with p53 and hTopors, or perhaps the two functions have entirely separate targets. Very few proteins in humans are both ubiquitinated and sumoylated by Topors, and it is not known if these are exceptional cases or if the modification of many proteins by both sumoylation and ubiquitination is regulated by Topors. To explore these possibilities, and to identify proteins for which the sumoylation status is affected by dTopors in *D. melanogaster* male meiosis, we immunoprecipitated SUMO-conjugated proteins from testes lysates of a *dtopors* null mutant versus wildtype males and analyzed the recovered peptides via mass spectrometry.

Materials and Methods

Mass Spectrometry

Tissue Preparation. For each mass spectrometry analysis two thousand pairs of testes of 1–3-day old males of either yw/$y^{+}Y$; spa^{pol} and $yw/y^{+}Y$; $dtopors^{fsF114SX19}$; spa^{pol} genotypes were collected in Schneider's Drosophila medium (GIBCO BRL, Gaithersburg, MD). Testes were centrifuged at 1700 rpm for 2 minutes, Schneiders was removed, and testes were gently washed in 200µl 1X PBS (Phosphate Buffer Saline pH 7.4). The centrifugation was repeated, and the PBS was removed. An Inhibitor Cocktail (IC, 0.1 mM MG132, 80 mM NEM and 0.4 mM IAA (Sigma-Aldrich, St Louis, MO), and 0.1 M Halt 100X to final concentration of 1X (Thermo

Scientific, Rockford, IL) was added in amounts of 1 µl per 2 pair of testes. The tissues were sonicated in short 20 second intervals for a total of 10 repeats and a standard Bradford assay was done to quantitate protein concentrations (Thermo Scientific, Rockford, IL). Mass spectrometry analysis was performed by Creative Proteomics, Shirley, NY. Briefly, the samples were digested by α-Lytic Protease and the resulted diglycine remnant containing peptides were enriched through Anti-Ubiquitin Remnant Motif (K-ε-GG) Antibody conjugated agarose beads and then identified by applying a nano-LC-MS/MS platform.

Protein Preparation. Disulfide bridges were reduced by 10 mM Tris(2-carboxyethyl) phosphine (TCEP) at 56℃ for 1 hour. Reduced cysteine residues were alkylated by 20 mM iodoacetamide (IAA) in the dark at room temperature for 30 min. The lysates were then diluted to < 1M urea by addition of 50 mM Tris-HCl, pH 8.2 before digestion. For the analysis, 6.85 mg protein was digested by 50 ug α-Lytic Protease. After overnight digestion at 37℃, TFA was added to 1% final concentration. Any precipitate was removed by centrifugation at 1,780g for 15 min.

Peptide Purification. Purification of peptides was performed at room temperature on C18 reversed-phase columns. The columns were first conditioned by 100% ACN followed by 0.1% TFA, 50% ACN and then equalized by 0.1% TFA. The acidified and cleared digest were then loaded into column. After the wash with 0.1% TFA, the peptides were eluted from the column by 0.1%TFA, 50% CAN and dried using a SpeedVac.

Anti- K-ε-GG Peptide Enrichment and Fractionation. Before incubation, the beads were washed in pre-chilled PBS 4 times. The peptides were resuspended in IAP buffer (50 mM MOPS, pH 7.2, 10 mM sodium phosphate, 50 mM NaCl). The peptide solution was then added into the vial containing motif antibody beads and incubated on a rotator for 2 hr at 4°C. After incubation, the beads were washed with pre-chilled IAP buffer 2 times and HPLC grade water 3 times. The enriched peptides were eluted from the beads by 0.15% TFA and were separated into 3 fractions using an Ultimate 3000 nano UHPLC system (ThermoFisher Scientific, USA) with a trapping column (PepMap C18, 100Å, 100 μm × 2 cm, 5 μm) and an analytical column (PepMap C18, 100Å, 75 μm × 50 cm, 2μm). A sample volume of 1 μg was loaded along a mobile phase made of A: 0.1% formic acid in water; B: 0.1% formic acid in 80% acetonitrile. The total flow rate for 250 nL/min LC linear gradient was from 2 to 8% buffer B in 3 min, from 8% to 20% buffer B in 50 min, from 20% to 40% buffer B in 26 min, then from 40% to 90% buffer B in 4 min.

Analysis of Mass Spectrometry Data. The full scan was performed between 300-1,650 m/z at the resolution 60,000 at 200 m/z, the automatic gain control target for the full scan was set to 3e6. The MS/MS scan was operated in Top 20 mode using the following settings: resolution 15,000 at 200 m/z; automatic gain control target 1e5; maximum injection time 19ms; normalized collision energy at 28%; isolation window of 1.4 Th; charge sate exclusion: unassigned, 1, > 6; dynamic exclusion 30 s. Raw MS files were analyzed and searched against *Drosophila melanogaster* protein database based on the species of the samples using Maxquant (1.6.2.14). The parameters were set as follows: the protein modifications were Carbamidomethylation (C), oxidation (M) (variables), GlyGly(K); Lys-C α-Lytic Protease was set as the enzyme for SUMO-proteomic analysis. The maximum missed cleavage was set to 4, the precursor ion mass

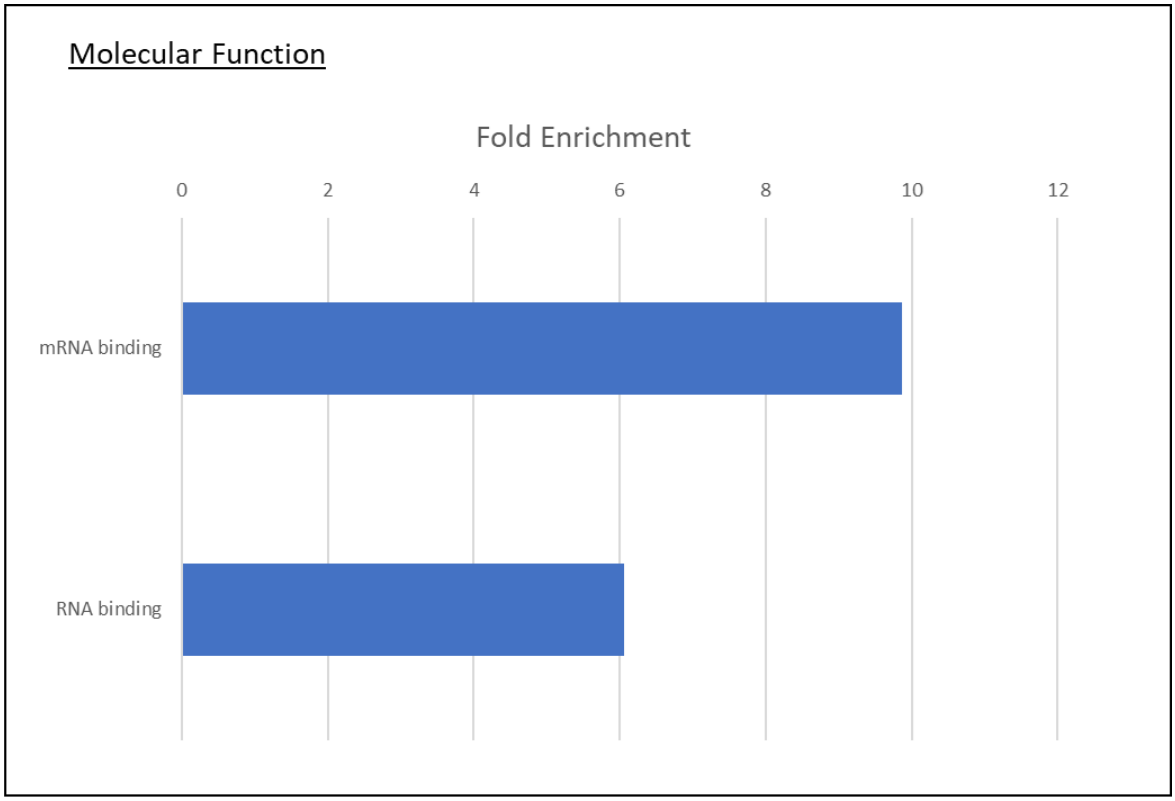
tolerance was set to 10 ppm, and MS/MS tolerance was 0.6 Da. The data was sorted by gene ontology enrichment analysis and categorized for molecular function and cellular components (geneontology.org).

Results

To identify targets of dTopors sumoylation, I made use of the *FS* (*fsF114SX19*) mutant I recovered from the CRISPR-Cas9 experiment. This mutant was genetically confirmed to be a *dtopors null* allele and thus give me an opportunity to look for targets of sumoylation in the presence and absence of dTopors in the male germline. To identify such potential downstream targets of dTopors sumoylation I made lysates of whole testis dissected from *wildtype* and *FS* young adults and immunoprecipitated ubiquitinated peptides using antibodies directed against the diglycine-lysine remnant (K-ε-GG) derived from digestion with α-Lytic Protease. SUMO is conjugated to lysine residues of its target peptides by the -TGG (threonine-glycine-glycine) residue sequence, resulting in a SUMO-TGG-K bond. α-Lytic Protease cleaves the bond between threonine and glycine, thus exposing an -GGK epitope for antibody binding and subsequent immunoprecipitation. Recovered immunoprecipitants were analyzed by tandem mass spectrometry (see Methods section).

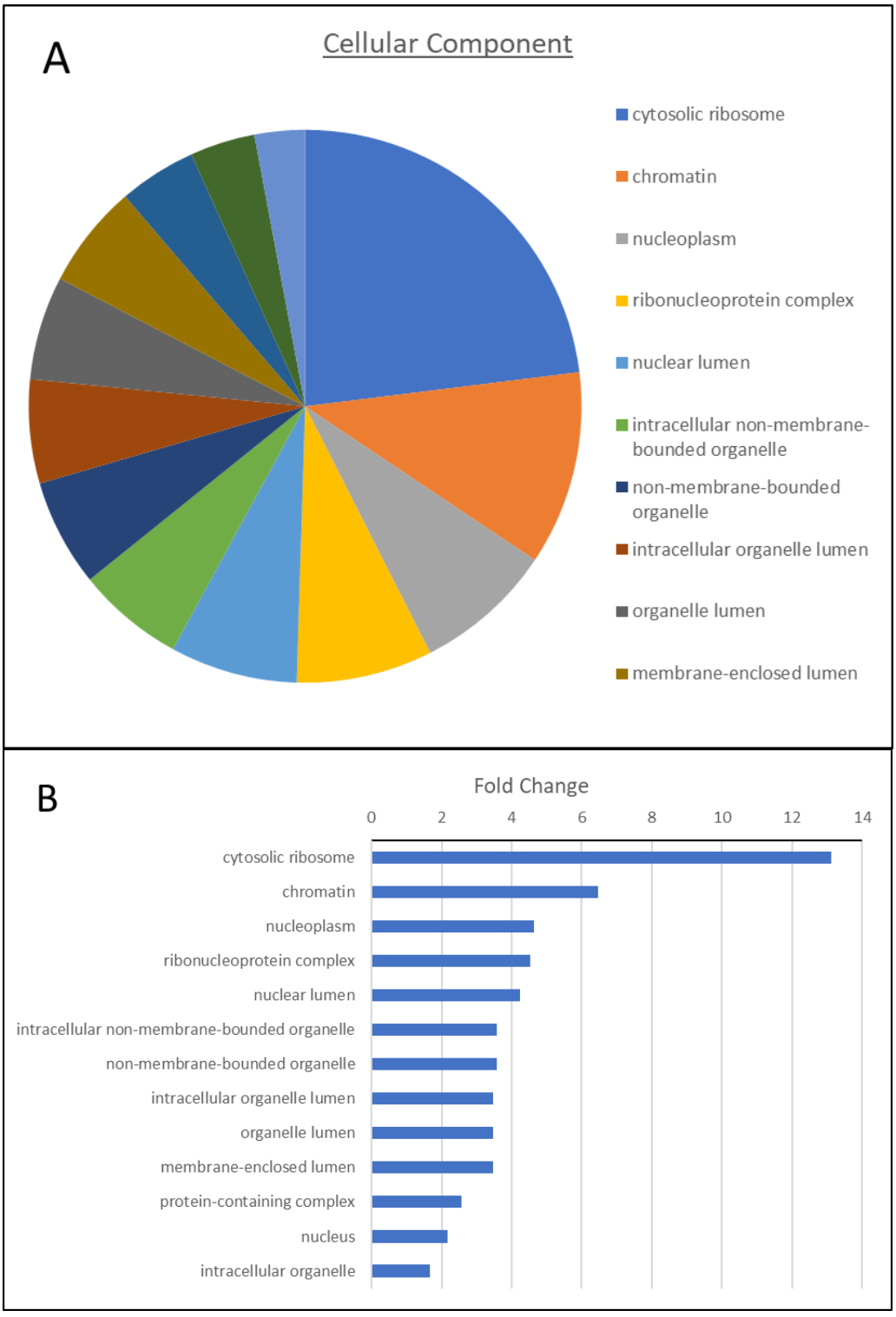
I identified 59 differentially sumoylated peptides, of which 33 were upregulated (FC >1.5) and 14 were downregulated (FC<1/1.5) when compared to the *wildtype* (supplementary data). When sorted for molecular function, I found that all targets grouped into only two gene ontology groups, mRNA, and RNA binding (Figure 10). This suggests that the molecular function of dTopors sumoylation targets may be association with RNA.

Figure 10. Gene Ontology Groups for Molecular Function of Targets with Altered Sumoylation.



When sorted for cellular components, the two largest ontology groups were ribosome and chromatin related (Figure 11), suggesting that there may be targets dTopors at the chromatin that are differentially sumoylated in the presence or absence of dTopors.

Figure 11. Gene Ontology Groups for Cellular Components of Targets with Altered Sumoylation. In the presence or absence of dTopors (A) and fold change of these GO groups(B).



To identify these targets, I performed additional analysis of the 59 peptides and chose candidates that had a 2-fold difference for both in up or down regulation. From these I selected a subset of sumoylated peptides candidates that were either present in wildtype but undetected in the mutant and those that were undetected in the wildtype but are present in the mutant. Peptides absent in the mutant precipitate but present in wildtype are the most likely direct targets of dTopors sumoylation, I will refer to these as dTopors-dependent targets of sumoylation. Peptides present in the mutant precipitate but absent in the wildtype suggest suppression of sumoylation by dTopors and I will refer to these as dTopors-suppressed targets of sumoylation.Interesting candidates in the dTopors-suppressed class include *Su(var)3-7* in the Chromatin/Transcription associated ontology group and *smt3* and *Uba2* in the sumoylation associated group (Table 3).

Table 3. Gene Products Differentially Sumoyalted in *dtopors.* (*sumoylated in the presence and absence of dTopors, **sumoylated and ubiquitinated).

Gene Ontology	dTopors-dependent	dTopors-suppressed
Chromatin/Transcription	*Sap130, mle, CG9899*	*Su(var)3-7, Nipped-B, Saf-B***
Chromosome segregation	*n/a*	*Plp*
ER association	*CG11190*	*n/a*
Membrane association	*CG9702, CG5549*	*RyR*
Metabolism	*CG8673, l(2)k01209*	*Sk2*
Ribosome/Translation	*Aats-glupro**	*Aats-asp, Aats-glupro*, RpL13*
RNA association	*n/a*	*Sym, DIP1*
Signaling	*DIP2, Spn, l(2)01289*	*n/a*
Sperm	*n/a*	***heph****
Sumoylation	*n/a*	*smt3, Uba2*
Ubiquitin-mediated proteolysis	*n/a*	*RpL40*

SU(VAR)3-7 (*Suppressor of variegation 3-7*) associates with the heterochromatin and increased expression triggers heterochromatin expansion and gene silencing (Cléard et al., 1997). PEV

(Position Effect Variegation) is enhanced by sumoylation of *SU(VAR)3-7* (Reo et al., 2010) and here we show that in absence of dTopors, *SU(VAR)3-7* is sumoylated, suggesting an indirect role of dTopors in the sumoylation pathway of *SU(VAR)3-7*. What this role is in *SU(VAR)3-7* sumoylation and how it affects PEV should be further investigated. Surprisingly, we also found two components of the sumoylation pathway *smt3* (Drosophila SUMO group) and Uba2 (Ubiquitin-like activating enzyme 2, SUMO E1 ligase) among the candidates of dTopors-suppressed groups, suggesting that dTopors may be a regulator of the sumoylation pathway (Table 3).

Among the dTopors-dependent ontology groups, the Chromatin/Transcription group has three candidate genes, *Sap 130, mle and CG9899* (Table 3). SAP130 (Sin3A-Associated Protein) is a subunit of the HDAC1-dependent Sin3A corepressor complex involved in transcriptional repression (Marshall et al., 2010). Human Sin3A was shown to be sumoylated by Topors (Pungaliya et al., 2007) and here I present evidence that the SAP130 subunit of the Drosophila Sin3A (dSin3A) complex is sumoylated in the presence of dTopors, suggesting that analogous to human Topors, dTopors may regulate sumoylation of the Sin3A complex in flies. *CG9899* has not yet been fully described but based on its protein structure, it is predicted to enable the binding of RNA Polymerase II complex to DNA and thus facilitating transcription of target genes by RNA Pol II, and to associate with transcriptionally active chromatin by regulating histone H3-K4 methylation (FBgn0034829). The last gene in the Chromatin/Transcription group, *mle* (*maleless*), encodes a helicase and is a member of the MSL chromatin modifying complex (male-specific-lethal) responsible for X chromosome dosage compensation in male flies involving the two-fold increase of transcription of the X-chromosome genes (Kuroda et al., 1991). The presence of these in the Chromatin/Transcription associated dTopors-dependent

group supports dTopors' sumoylation role in chromatin structure remodeling and transcription regulation. In a similar experiment Pungaliya et al., found that chromatin-modifying/transcriptional regulators to be the largest ontology for human Topors and showed that human Topors sumoylates Sin3A (Pungaliya et al., 2007). I did not find Sin3A itself in this screen nor p53 (Weger et al., 2005), however, I detected Saf-B which was also identified in the same screen by Pungaliya et al., as sumoylation target of TOPORS (Pungaliya et al., 2007). It is possible that Sin3A and p53 are not targets of sumoyaltion by dTopors in flies, or they may be present at levels below our level of detection.

I found only two targets for which both sumoylation and ubiquitination is regulated by dTopors, *heph* (*Hephaestus*) and *saf-B* (*Scaffold attachment factor B*). *Hephaestus* encodes the protein PTB (Polypyrimidine Tract-binding Protein), involved in spermatid individualization, and its male germline-specific expression is correlated with male fertility (M. Robida et al., 2010). In a *heph* mutant, spermatid differentiation is disrupted and cysts of elongated spermatids form without fully separated motile sperm (M. D. Robida & Singh, 2003). Our results show that PTB is modified on the same lysine residue K27, when ubiquitinated in the presence of dTopors or sumoylated in the absence of dTopors (**NIGVK(gl)R**GSDELLS). This suggests that dTopors regulates PTB ubiquitination, and in the absence of ubiquitination this residue may be sumoylated. Thus, these two modifications may be competing for the same residue and may result in alternative outcomes on PTB function.

Saf-B is the other protein differentially regulated by dTopors. Human and mouse studies showed that Saf-B binds to SAR/MAR (Scaffold/Matrix Attachment Region) sequences on chromosomes (Renz & Fackelmayer, 1996) and aides the assembly of a transcriptosome complex for actively transcribed genes, i.e. Hsp27 (heatshock protein 27) (Weighardt, et al.,

1999). Chromatid interactions with SAR/MAR link the chromosomes to the nuclear scaffold and serve as attachment points for nuclear matrix tethering (Nayler et al., 1998). Saf-B is also an estrogen receptor corepressor (Townson et al., 2000) and has been implicated in breast cancer tumorigenesis (Oesterreich, 2003; Townson et al., 2000). Pungaliya et al., also detected Saf-B2 (SAF-B paralog) as a target of dTopors sumoylation in an *in vitro* assay when searching for Topors sumoylation targets (Pungaliya et al., 2007). The Drosophila homolog of Saf-B contains all conserved domains and motifs of the human Saf-B, it is a component of the nuclear matrix important for connecting the nuclear matrix to gene expression (Alfonso-Parra & Maggert, 2010). Drosophila Saf-B (dSAF-B) localizes in intranuclear punctate spots and interacts with RNA polymerase II and with SR (serine-arginine-rich) proteins (Nayler et al., 1998; Nikolakaki et al., 2001). Akin to Heph, Saf-B is also ubiquitinated in the presence of dTopors and is sumoylated in the absence of dTopors, however, in contrary to Heph where both modifications happen at the same lysine residue, Saf-B modifications target different lysine residues (K341-VIGAK(gl)VVTNTR for dTopors-dependent ubiquitination; K89-IK(gl)EEPIDANEEEVK(gl)V for dTopors-suppressed sumoylation.

Discussion

Chromatin modifications are critical regulators of gene expression. Histone acetylation is addition of an acetyl group to a histone lysines by HATs (Histone Acetyl Transferases) generally leading to open chromatin, while deacetylation by HDACs removes the acetyl group and closes the chromatin structure. These processes make genes either accessible or inaccessible for transcription. HATs and HDACs are also SUMO modified, affecting localization and/or function of these enzymes (Girdwood et al., 2003; Kirsh et al., 2002; David et al., 2002). One of the proteins we found to be sumoylated in the presence of dTopors is SAP130 (Sin3A-Associated

Protein), a subunit of the HDAC1-dependent Sin3A corepressor complex involved in transcriptional repression (Marshall et al., 2010). The Sin3A complex localizes to the PML body where along with other corepressors recruits HDAC1 and interacts with PML and initiate transcriptional repression (Khan et al., 2001). In HeLa cells, Topors also localizes to PML-NBs via PML protein binding (Rasheed et al., 2002) and sumoylates Sin3A (Pungaliya et al., 2007). . In vitro sumoylation assays with human Topors found many targets of dTopors mediated sumoylation of proteins involved in chromatin modification and transcriptional regulation, including Sin3A (Pungaliya et al., 2007). Although I did not find the fly homolog of Sin3A in my screen, I found evidence that the SAP130 subunit of the Drosophila Sin3A (dSin3A) complex is sumoylated in the presence of dTopors. This suggests that dTopors may regulate the dSin3A complex in flies by sumoylation of its subunit SAP130.

Sumoylation also contributes to chromatin modifications and regulates phenotypes associated with heterochromatic silencing, such as PEV, which may be suppressed by SUMO modification of Su(var)2-10 (Hari et al., 2001) or enhanced by sumoylation of SU(VAR)3-7 (Reo et al., 2010). *SU(VAR)3-7* (*Suppressor of variegation 3-7*) that associates with the heterochromatin and increased expression triggers heterochromatin expansion and gene silencing (Cléard et al., 1997). In flies, *SU(VAR)3-7* interacts with *mle* and these proteins mutually suppress each other (Spierer et al., 2008). The morphology of the X chromosome is sensitive to amounts of SU(VAR)3-7 expressed, and changes in SU(VAR)3-7 expression levels affect H4K16 acetylation patterns (Spierer et al., 2008). Acetylation of Histone 4 enhances its sumoylation (Shiio & Eisenman, 2003) and the attached SUMO groups may lead to transcriptional repression (Spierer et al., 2008). Our screen revealed that *mle* is sumoylated in the presence of dTopors suggesting that it may be a potential direct target of dTopors. Given that

MLE and SU(VAR)3-7 are mutually repressive, it is interesting to note that one is sumoylated in the presence of dTopors (*mle*) while the other is sumoylated in the absence of dTopors (*SU(VAR)3-7*)). An explanation could be that when dTopors is present, MLE is sumoylated and represses SU(VAR)3-7. Changes in SU(VAR)3-7 expression levels affect H4K16 acetylation, thus altering chromatin structure affecting gene transcription. CG9899 is also sumoylated in presence of dTopors and although it has not yet been fully described, it is predicted to associate with transcriptionally active chromatin by regulating histone H3-K4 methylation (FBgn0034829). Taken together these results support the role of dTopors in regulating chromatin structure and transcription regulation in flies.

Among the candidates sumoylated in the absence of dTopors were the SUMO-1 ligase Uba2 and Drosophila SUMO encoded by *smt3*. It was surprising to find that in the absence of dTopors two components of the sumoylation pathway are sumoylated. It is possible that dTopors may prevent sumoylation of Uba2 and of SUMO by ubiquitinating upstream targets, thus regulating of the sumoylation pathway.

Can dTopors Regulate Sumoylation and Ubiquitination of the Same Targets?

Automodification by SUMO can interfere with an E3 ligase's activity as is the case of Mdm2, a RING finger E3 ubiquitin ligase and oncoprotein. Mdm2 regulates p53 levels by directly ubiquitinating p53 for degradation or by ubiquitinating itself to stabilize p53 levels (Haupt et al., 1997; Honda & Yasuda, n.d.; Kubbutat et al., 1997). The SUMO E2 conjugating ligase, Ubc9 (*lesswright*), sumoylates Mdm2, thus causing a competition with Mdm2's auto-ubiquitination. This leads to increased Mdm2 levels and thus to increased p53 ubiquitination (Buschmann et al., 2000). In a different case, Topors automodifications can act as binary switch function and influence its own activities. This is evident in Topors regulation of p53 alternatively

by ubiquitination and sumoylation. When Topors is phosphorylated, p53 sumoylation is downregulated and p53 ubiquitination is induced (Müller et al., 2000; Yang et al., 2009). Topors ubiquitinates (Rajendra et al., 2004) and sumoylates (Shinbo et al., 2005; Weger et al., 2005) *in vitro* and *in vivo*.

In this screen we identified two proteins, PTB and SAF-B, for which sumoylation and ubiquitination are both regulated by dTopors. Both PTB and Saf-B are ubiquitinated in the presence of dTopors and sumoylated in the absence of dTopors, however, whereas in PTB both modifications occur at the same lysine residue (K27), SAF-B modifications target different lysine residues (K341 for ubiquitination; K89 for sumoylation). Targeting of the same residue suggests competing post-translational modifications, perhaps due to differential regulation of targets in different pathways. It is possible that sumoylation stabilizes the protein by preventing its ubiquitination. Ubiquitination of the protein may ensure turnover after its function during spermatid individualization has been completed. In an *in vitro* screen for Topors sumoylation targets in human cells, Pungaliya et al., also detected SAF-B2 (SAF-B paralog). In humans, SAF-B interacts with its paralog SAF-B2 to bind to the estrogen receptor and suppress *ERα* expression (Chan et al., 2007; Hashimoto et al., 2012; Oesterreich, 2003), and they are also involved with alternative splicing complexes (Sergeant et al., 2007). dTopors mediated SAF-B modifications at different lysine residues (342K for ubiquitination; 90K for sumoylation) supports the idea of differential regulation of this protein. This is important because human SAF-B binds to DNA and regulates many genes (Hammerich-Hille et al., 2010), suggesting of a direct link between nuclear matrix and chromosomes. Based on dSAF-B localization, its DNA binding domain (SAP-BOX), its RNA recognition motif (RRM) and its interaction with RNA pol II, Alphonso-Parra et al., proposed that dSAF-B links the nuclear matrix to chromosomes and

influences transcriptional activity (Alfonso-Parra & Maggert, 2010). Ubiquitination of SAF-B in the presence of dTopors would suggests a role for dTopors at the nuclear matrix and transcription.

While the mass spec screen revealed some interesting targets for dTopors sumoylation, they are only preliminary results, and these interactions must be confirmed by direct biochemical tests of sumoylation to determine if dTopors can act as an E3 SUMO ligase. My work identifying potential sumoylation targets provided candidate substrates for such experiments.

CHAPTER IV: IDENTIFICATION OF E2 UBIQUITIN LIGASE PARTNERS OF DTOPORS

Introduction

Ubiquitin is a small regulatory peptide found everywhere in eukaryotic tissues. As a form of post-translational modification, Ubiquitin (Ub) is covalently attached to a target protein, altering the protein's function or destiny. Ubiquitin is highly conserved among many species, however, the number of genes coding for Ub varies (Ozkaynak et al., 1984; Wiborg et al., 1985). In humans, four genes (UBB, UBC, UBA52 and RPS27A) code for Ub (Kimura & Tanaka, 2010), while in D. melanogaster there are two genes Ubi-p5E (X chromosome) and DmUbi-p63E (chromosome 3), and three additional genes coding for ribosomal proteins with tandem repeats of Ub (RpL40, RpS27A and RpS30), from which, the Ub groups are produced via post-translational cleavage (Barrio et al., 1994; Spradling et al., 1999). Ub conjugation is a major pathway of protein degradation, and it involves three types of enzymes: a Ubiquitin activating enzyme (E1), a Ubiquitin conjugating enzyme (E2) and a Ubiquitin ligase (E3). In humans there is only one E1 ubiquitin activating enzyme known to date (Uba1) shared by all E2 ligases, while there are about 40 E2 and over 600 E3 ligases (Hou et al., 2012; W. Li et al., 2008). *D. melanogaster* also has a single E1 ubiquitinating enzyme (Uba1), that interacts with 28 E2 ligases and about 150 E3 ligases. However, one of the E2 ligases, Taf1 (TBP-associated factor 1) can function as an E1 or E2 ligase. Taf1 encodes a component of the evolutionarily conserved general transcription factor TFIID and also acts as a HAT (Histone Acetyl Transferase). There are different types of E2 enzymes: Ubc and UEV. Ubc type E2 ligases catalyze the attachment of polyubiquitin chains to proteins which marks them for degradation, while UEV type E2 ligases attach polyubiquitin chains to proteins but these do not automatically cause target degradation

(Pickart, 2000). E3 ligases fall into three categories depending on their enzymatic domains: RING (Really Interesting New Gene, domain located near the N terminus of protein), HECT (Homologous to the E6-AP Carboxyl Terminus, domain located at the C terminus of protein), and U-box (an E4 ligase and ubiquitin chain elongation factors, mediate ubiquitination in absence of HECT and RING ligases, or elongate chains previously set up by RING or HECT E3 ligases).

Ubiquitination is a three-step process and starts with the ATP-mediated activation of Ub via E1 ubiquitin ligase. This step is then followed by conjugation of Ub to an E2 conjugating enzyme, which then complexes with an E3 ligase. The E3 ligase recognizes the substrate and catalyzes the Ub transfer to a lysine residue on the target protein, causing its degradation by the 26S proteosome (Wilkinson et al., 1995).The ubiquitination process may repeat itself and some targets may become polyubiquitinated like HDAC1 by IFIXalpha (Yamaguchi et al., 2008) and p53 by the histone acetyltransferase p300 and CBP (CREB-binding protein) (Grossman et al., 1998, 2003; Lill et al., 1997). Polyubiquination is a conserved aspect of targeting proteins for degradation, and also occurs in Drosophila (Secombe & Parkhurst, 2004). Generally, Ub groups are attached to lysine residues, and the exact location of the lysine to which a ubiquitin chain is added can be a decisive factor in determining the proteins fate. Ubiquitin groups are linked into a chain through the K48 residue and polyubiquitination leads to 26S proteasome mediated degradation, while polyubiquitination through K63 regulates signaling (Thrower et al., 2000). Although ubiquitination is mostly utilized to mark proteins for degradation via the 26S proteasome, ubiquitination is also involved in many other cellular processes, most notably the DNA damage response, NF-κB signaling pathway and cancer.

Dual E3 Ubiquitin SUMO-1 Ligase Topors is Implicated in Cancer

E3 ligases have pleiotropic roles in a variety of tissues and due to their ubiquitin ligase activity, many of them are implicated in different types of cancer. Topors, a E3 ligase with a RING domain, is expressed in both soma and germline, and was the first Ub ligase identified to also have SUMO-1 ligase activity (Chu et al., 2001; Rajendra et al., 2004; Weger et al., 2003). The RING domain has been shown to be required for DNA-binding and ubiquitin ligase activity (Rajendra et al., 2004) but not for sumoylating activity (Weger et al., 2003). The complexity of Topors enzymatic activity is not yet fully understood. The expression levels of Topors differ in various cancers suggesting alternate targets, tissue specificity or possibly different E2 ligase interactions. In colon adenocarcinomas, *topors* expression is downregulated, however, when upregulated, *topors* halts the cell cycle, trapping cells in G0/G1 phase, suggesting a possible role as a tumor suppressor (Saleem et al., 2004). In prostate cancer, *topors* is overexpressed and ubiquitinates the tumor suppressor NKX3-1 for proteasomal degradation (Guan et al., 2008), while in ovarian cancer elevated levels of TOPORS-AS1 showed disruption of Wnt/β-catenin signaling pathway and correlated to more favorable disease outcome (Fu et al., 2021).

dTopors Role in Drosophila Soma and Male Germline

In flies, E3 RING ligases are involved in protein turnover, thus regulating many pathways in the cell. For instance, the E3 RING ubiquitin ligase Bre1 monoubiquitinates H2B and influences the transcription of RNA pol II mediated transcription of genes (Bray et al., 2005). dTopors, which is also an E3 RING ligase (Haluska, 1999b; Rasheed et al., 2002), is involved in early embryonic patterning as it regulates the transcriptional repressor Hairy (Secombe & Parkhurst, 2004). In wildtype embryos, Hairy in concert with several cofactors (including dTopors), regulates the repression of Fushi tarazu (*ftz*) gene, resulting in seven stripes during

early development (Dearolf et al., 1989; Secombe & Parkhurst, 2004). In a transheterozygous *hairy* mutant, *ftz* is only partially repressed and the stripes are wider (Secombe & Parkhurst, 2004). Dosage reduction of dTopors in the *hairy* transheterozygous mutant improves the stripes, suggesting that polyubiquitination of Hairy by dTopors causes degradation of Hairy, thus improving *ftz* expression (Secombe & Parkhurst, 2004).

When compared to its human homolog, dTopors possesses many similarities structurally and functionally, however, there are also some important differences in their targets and subcellular localizations. In flies, dTopors colocalizes with Lamins (Dm0 and C) at the nuclear lamina in both spermatocytes (Matsui et al., 2011) and in somatic cells (Capelson & Corces, 2005), whereas a laminar localization of human Topors is not observed (Haluska, 1999a; Rasheed et al., 2002; Weger et al., 2003). At the somatic nuclear lamina, dTopors colocalizes with components of the *gypsy* insulator complex and is required for proper insulator function, but does not ubiquitinate insulator components, suggesting an alternative function for dTopors at the insulator complex (Capelson & Corces, 2005). In spermatocytes dTopors also accumulates in punctate intranuclear spots (Matsui et al., 2011) that may be analogous to Topors accumulation in PML bodies in humans (Rasheed et al., 2002).

Analogous to human Topors, dTopors ubiquitination activity requires its RING domain (Secombe & Parkhurst, 2004). Secombe et al., (2004) created a ubiquitin null mutant (*His_6-$dTopors^{C102A}$*) and tested ubiquitin ligase activity of dTopors in the presence of both E1 and the human E2 enzyme UbcH5a. Ubiquitin chains formed in the presence of His_6-dTopors but none with His_6-dToporsC102A, confirming that dTopors ubiquitin ligase activity requires the RING domain (Secombe & Parkhurst, 2004).

My research shows that the nuclear morphology phenotype and chromosome segregation phenotype seems to have disparate responses to distinct *topors* mutations, both the hypomorph *Df(114-118)* and the ubiquitin null *C102A* had extensive nuclear blebbing, while chromosome NDJ was significantly different, raising the possibility of separate targets, and perhaps involvement of distinct E2s. Could the nuclear structure and chromosome segregation defects result from dTopors ubiquitinating different targets and using different E2 ligases? Generally, E3-E2 interactions are determined by specificity of an E2 to an E3, however, a single E2 can interact with different E3 ligases, as seen with Ube2D/UbcH5 and the ubiquitination of a target depends on which E3 is chosen by an E2 (Ye & Rape, 2009). E2s utilize different residues to recognize various E3s, and additional components unique to certain E2s may increase specificity towards an E3 ligase (Ye & Rape, 2009). In flies, the only E2 ligase to date shown to interact with dTopors *in vitro* was UbcH5a, the human version of the fly E2 conjugating enzyme UbcD1 (*effete)* (Capelson & Corces, 2005; Secombe & Parkhurst, 2004), while human Topors appears to be promiscuous and interacts with more than one E2 ligase (UbcH5a, UbcH5c and UbcH6) (Rajendra et al., 2004). However, it is unclear if these interactions are biologically meaningful as proteins were present in high concentrations in each of these assays and do not reveal which interactions are relevant in vivo with respect to developmental and/or tissue specificities. Are there other E2 ligases that would interact with dTopors and would their tissue- and temporal-specific expression patterns determine the nature of these interactions with dTopors?

Aim of Study

It is not known which E2 ligases dTopors interacts with *in vivo* and in different developmental and tissue-specific contexts. In this study, we aimed to identify which E2 ligases are required *in vivo* for meiotic chromosome segregation and/or for proper nuclear structure. To

achieve this, we knocked down the expression of several E2 ligases in the male germ using RNAi and observed effects on meiotic chromosome segregation and spermatocyte nuclear structure. As a secondary screen, we assayed deletions of E2 ligase genes for their ability to dominantly enhance the phenotypes of *Df(114-118)*. This allele behaves as a hypomorph with respect to chromosome segregation, and we speculate that the phenotype may result from a partial disruption of its interaction with an E2 ligase. Thus, enhancement of the mutant phenotype might be expected if the abundance of the relevant interacting E2 were reduced by 50%. We also developed an assay to quantify the severity of the nuclear blebbing phenotype to test if a similar enhancement might be observed.

Materials and Methods

Drosophila Stocks

Drosophila were reared at room temperature on a standard diet of cornmeal, molasses, agar, and yeast with propionic acid and tegosept added to prevent mold. Fly stocks were purchased from Bloomington Drosophila Stock Center at Indiana University (BDSC, NIH P40OD018537) and from Vienna Drosophila Resource Center (VDRC, www.vdrc.at). The following stocks were obtained from BDSC and are described on Flybase (http://flybase.bio.indiana.edu): BDSC6998 P{GawB}T110 (T110), BDSC6998(nanos), P{bam-GAL4:VP16}1, BDSC6978 P{GawB}C135 (C135), BDSC28721 P{TRiP.JF03148}attP2 (ben), BDSC35431 P{TRiP.GL00355}attP2 (effete), BDSC77416 P{TRiP.HMC06548}attP2 (Ubc6), BDSC76066 P{TRiP.HMS05878}attP2 (Uba1), BDSC67934 P{TRiP.HMS05766}attP40 (Taf1), BDSC35489 P{TRiP.GL00418}attP2 (CG4502), BDSC51410 P{TRiP.GLC01776}attP40 (UbcE2H), BDSC51681 P{TRiP.GLC01774}attP40 (CG2924), BDSC51687 P{TRiP.GLC01787}attP40 (CG10254), BDSC51814 P{TRiP.HMC03385}attP2

(Bruce), BDSC64032 P{TRiP.HMS05368}attP40 (CG40045), BDSC64035 P{TRiP.HMS05371}attP40 (Ubc4), BDSC(65871 P{TRiP.GLC01873}attP40 (CG8188). VDRC stocks used in the RNAi crosses were described by Dietzl et al [Nature 448,151-156,12 Jul 2007] and are listed as: VDR105725 P{KK107231}VIE-260B (CG2574), VDR104207 P{KK104360}VIE-260B (CG3473), VDR100099 P{KK103640}VIE-260B (CG5440), VDR108292 P{KK101280}VIE-260B (CG5823), VDR104478 P{KK109208}VIE-260B (CG7220), VDR100791P{KK108673}VIE-260B (CG7656), VDR101113 P{KK106941}VIE-260B (CG10862), VDR105594 P{KK105908}VIE-260B (CG14739), VDR108804 P{KK107234}VIE-260B (CG17030), VDR101755 P{KK109433}VIE-260B (CG46338), VDR330352 P{KK109433}VIE-260B (Ubc2), VDR104440 P{KK108858}VIE-260B (Ubc7), VDR100570 P{KK108273}VIE-260B (Ubc10), VDR106363 P{KK110243}VIE-260B (Ubc84D), VDR102641 P{KK103525}VIE-260B (Ubc87F), VDR107720 P{KK102343}VIE-260B (vihar).

RNAi Mediated Knockdown of E2 Ubiquitin Conjugating Enzymes in the Male Germline

Test males (*GAL4/RNAi-E2 ligase*) were generated bearing one of four different Gal4 drivers and a UAS-RNAi construct targeting an E2 ligase. In this system, the transcription factor GAL4 is cloned downstream of a tissue-specific promoter (either bam, nanos, T110 or C135) to allow for expression of GAL4 at different stages during spermatogenesis while the RNAi hairpin repeat is cloned downstream of a the Gal4 binding Upstream Activating Sequence (UAS).

Cytological Assessment of Nondisjunction and Nuclear Structure. To identify E2 ligases that may affect chromosome segregation and/or nuclear structure, testes of five young *GAL4/RNAi-E2 ligase* test males were dissected and one testis per slide was mounted under a coverslip in 3 μl of Schneider's tissue culture medium. The slides were immediately observed

under Nikon Eclipse E600 phase microscope and were assessed for the presence of unevenly sized nuclei, micronuclei (indicating NDJ) and blebs.

Genetics Tests of Fourth Chromosome Segregation. *GAL4/RNAi-E2 ligase* test males were crossed to *y w sn; C(4)ciey* females to detect fourth chromosome NDJ as *spa* progeny produced from *diplo-4* sperm or *ci ey* progeny from *nullo-4* sperm. I counted at least 200 progeny per line from at least 10 fathers for each of the drivers.

Genetic Assays of Meiotic Chromosome Segregation

Deficiencies of E2 ligase positives previously identified in the RNAi screen were crossed into *dtoporsDf(114-118)* and stocks were made as yw/y+Y; *Df(114-118)/CyO;Df(*)/TM3 Ser*, where (*) stands for an E2 deficiency. These stocks were made for the following deficiencies: *Df(7635), Df(7622), Df(7582), Df(9076), Df(8959), Df(26836)* and $Taf1^{1}red^{1}e^{1}$ (BDSC5300). Null alleles of E2 ligases located on chromosome 2 were recombined with *dtoporsDf(114-118)* and made into stocks: rec*Df(114-118)+P{lacW}Ubc2[k13206]/CyO, recDf(114-118)+P{GT1}Ubc10[BG00902]/CyO,* and *recDf(114-118)+ P{lacW}Uba1[s3484]/CyO.* To monitor sex and fourth chromosome NDJ, *yw/y+Y; spa* males either homozygous or heterozygous for the alleles above were mated to *y w sn; C(4)ciey* females. Normal progeny consisted of y+ w males and y w females, while progeny from sex chromosome NDJ was detected as either *y w sn* males (*nullo-XY*, which received no paternal sex chromosome) or as *y+ w* females (*diplo-XY*, which received both paternal X and Y chromosomes). Fourth chromosome NDJ was detected as *spa* progeny produced from *diplo-4* sperm or *ci ey* progeny from *nullo-4* sperm.

Cytological Analysis of the Spermatocyte Nuclear Phenotype

To identify E2 ligases that modified the *dtopors* nuclear phenotype, testes of young males *yw/y+Y; spa* either homozygous *Df(114-118),* and heterozygous for *Df(E2 ligase)* were dissected and one testis per slide was mounted under a coverslip in 3 µl of Schneider's tissue culture medium. The slides were immediately observed under Nikon Eclipse E600 phase microscope and images of S5-S6 spermatocytes were taken using a Moticam X3 WiFi color camera (Motic Scientific). Images were collected from at least 10 individual males per genotype and at least 200 cells were assessed for the presence of blebs.

Results

A screen was performed using RNAi-mediated knockdown of E2 ligases to identify those involved in both male meiotic chromosome segregation and/or in spermatocyte nuclear structure. It was expected that knockdown of an E2 conjugating enzyme important for dTopors-mediated ubiquitination would result in a phenocopy of a *dtopors* mutant. In other words, I would observe defects in meiotic chromosome segregation and/or in nuclear structure. As it was not known *a priori* when dTopors activity is required, GAL4 drivers were selected to cause RNAi knockdown of these ligases at different times during spermatogenesis. The drivers *nanos* and *bam* are germline specific, and express specifically in stem cells (nanos) or in gonial cells and early prophase spermatocytes. While *T110* and *C135* are not germline -specific, they are useful candidates for temporal expression of E2 ligases at different times during meiotic prophase. T110 expresses in the early spermatocytes (S1-S2) and C135 expresses late spermatocytes (S3-S6) (Arya et al., 2006). I used three *dtopors* RNAi lines as controls to test whether this approach was effective (Table 4). As an indication of NDJ, I expected to see micronuclei and uneven sized nuclei in spermatids (onion cells). If an E2 was necessary for nuclear structure, I expected

nuclear blebbing in spermatocytes. One RNAi line was tested for most E2 ligases, and I also included a line for Uba1 (the sole E1 ubiquitin ligase).

Cytological analysis of spermatocyte nuclei for each of the *GAL4/RNAi-E2 ligase* test lines failed to reveal blebbing, except for the *dtopors* control lines. NDJ, however, was produced in several different tests. In addition to these cytological assays, we assessed 4th chromosome NDJ frequency genetically for all drivers /*RNAi-E2 ligase* combinations. I identified several lines that gave 4th chromosome NDJ with at least one of the driver lines, and some lines (*dtopors, Bruce, CG10862, CG17030, vihar, CG4502, Ubc4, CG3473, CG2574*) had NDJ with more than one driver, and only *dtopors*, *Bruce, CG10862,* and *Ubc87F* had NDJ significantly above the background level of 0.5% (Table 4).

Table 4. Results of Fourth Chromosome Nondisjunction from RNAi Knockdown of E2 Ubiquitin Ligase Expression in the Male Germ Line. Gal4 Drivers Indicated in Table Below.

Gal4 Driver		*Bam*	*Bam*	*Nanos*	*Nanos*	*T110*	*T110*	*C135*	*C135*
Stocks	UAS-RNAi	Total flies	% 4th ndj	Total flies	% 4th ndj	Total flies	% 4th ndj	Total flies	% 4th ndj
BDSC34671	*dtopors*	50	2.00	256	0.00	251	0.00	284	0.35
BDSC10642	*dtopors*	220	0.46	350	0.29	239	0.00	231	0.00
BDSC31091	*dtopors*	429	0.23	168	0.00	523	0.00	152	0.00
BDSC51814	*Bruce*	716	1.40	391	0.00	786	0.13	591	0.00
VDR101113	*CG10862*	563	0.36	407	0.74	298	0.00	346	0.00
VDR102641	*Ubc87F*	218	0.00	256	0.00	478	0.63	552	0.00
VDR10884	*CG17030*	724	0.14	351	0.00	350	0.00	575	0.52
VDR104440	*Ubc7*	276	0.00	268	0.00	360	0.00	396	0.51
BDSC35431	*effete*	0	sterile	317	0.00	258	0.00	392	0.26
BDSC77416	*Ubc6*	0	sterile	279	0.00	lethal		lethal	

BDSC67934	*Taf1*	0	sterile	251	0.00	lethal		lethal	
BDSC76066	*Uba1*	0	sterile	262	0.00	lethal		lethal	
VDR100570	*Ubc10*	281	0.00	258	0.00	276	0.00	674	0.45
VDR330352	*Ubc2*	285	0.00	261	0.00	262	0.00	256	0.39
VDR107720	*vihar*	460	0.43	507	0.39	262	0.00	256	0.00
BDSC35489	*CG4502*	468	0.43	460	0.22	355	0.00	272	0.00
BDSC64035	*Ubc4*	306	0.33	230	0.00	478	0.42	226	0.00
VDR104207	*CG3473*	681	0.15	247	0.00	301	0.00	565	0.35
VDR105725	*CG2574*	245	0.00	573	0.17	307	0.00	431	0.23
VDR108292	*CG5823*	283	0.00	294	0.00	300	0.00	453	0.22
VDR101755	*cbx*	286	0.00	266	0.00	399	0.00	488	0.20
VDR100099	*CG5440*	447	0.00	338	0.00	358	0.00	512	0.20
VDR105594	*CG14739*	339	0.00	258	0.00	280	0.00	552	0.18
BDSC35476	*Ubc6*	422	0.00	260	0.00	604	0.17	359	0.00
VDR104478	*CG7220*	386	0.00	331	0.00	573	0.17	313	0.00
BDSC51687	*CG10254*	379	0.00	224	0.00	407	0.00	614	0.16
VDR100791	*CG7656*	380	0.00	307	0.00	331	0.00	924	0.11
BDSC28721	*ben*	267	0.00	375	0.00	322	0.00	457	0.00
BDSC35314	*Taf1*	204	0.00	239	0.00	364	0.00	324	0.00
BDSC51410	*UbcE2H*	370	0.00	312	0.00	391	0.00	400	0.00
BDSC51681	*CG2924*	430	0.00	292	0.00	375	0.00	323	0.00
BDSC64032	*CG40045*	283	0.00	349	0.00	464	0.00	320	0.00
BDSC65871	*CG8188*	224	0.00	258	0.00	285	0.00	290	0.00
VDR106363	*Ubc 84D*	321	0.00	350	0.00	303	0.00	275	0.00

These genetic tests were largely consistent with our cytological observations but were likely more sensitive at detecting NDJ, as NDJ of the tiny fourth chromosome is not apparent

cytologically. Thus, it was not surprising that we found additional lines that caused NDJ by these genetic tests. Knockdown of an E2 ligase that results in NDJ suggests its importance in chromosome segregation, but not necessarily through a dTopors pathway. Thus, each of these were candidates to be part of the dTopors pathway, but an additional assay would be necessary to determine which were. From all the *GAL4/RNAi-E2 ligase* lines tested with all drivers, *Bruce* (*BIR repeat containing ubiquitin-conjugating enzyme*) had the highest frequency of fourth chromosome NDJ. I also observed four lines (*effete, Ubc6*, *Taf1*, and *Uba1*) which were sterile with the *bam* driver. In addition, RNAi against Taf1, Uba1 and Ubc6 was lethal when expressed via T110 and C135 drivers. As the T110 and C135 are not germline-specific, the lethality reflects essential functions for these E2 ligases in the soma. Generally, homozygous mutant alleles of these lines are expected to be either lethal or sterile. I dissected the lines that were sterile when RNAi was driven by *bam GAL4* and found no cytological evidence for NDJ nor nuclear blebbing for Uba1, Ubc6 or effete knockdowns. I detected an unusual phenotype in *Taf1*, *Uba1* and *Ubc6* which had mostly spermatocytes with only a few weak sperm tails, suggesting that these were important for progression through meiosis. To identify those that might be dTopors-related, I tested a subset of the initial NDJ screen positives for enhancement of the *dtopors* hypmorphic allele, *Df(114-118).* I generated males heterozygous for deficiencies of both *Bruce* and *effete* and either homozygous or heterozygous for *Df(114-118).* These were tested in genetic assays for fourth and sex chromosome NDJ (Table 5). The *Df(114-118);+/Ser RING* hypomorph was chosen because my previous results showed relatively high levels of blebbing and at the same time reduced levels of chromosome segregation defects, especially for chromosome four which was reduced to near wildtype levels. I expected that a reduction of the E2 ligase levels would exacerbate the NDJ defect of the hypomorph if the E2 interacts with dTopors in the germline.

For controls I used *Df(114-118); +/TM3 Ser* homozygous and heterozygous *Df(114-118)/CyO; +/TM3 Ser* (see Methods).

Table 5. Sex and Fourth Chromosome Disjunctional Data from Crosses of *y/y+Y; dtopors; Df(E2 ligase)/+; spa^{pol}* Males to *y w sn; C(4)RM ci ey/0* Females

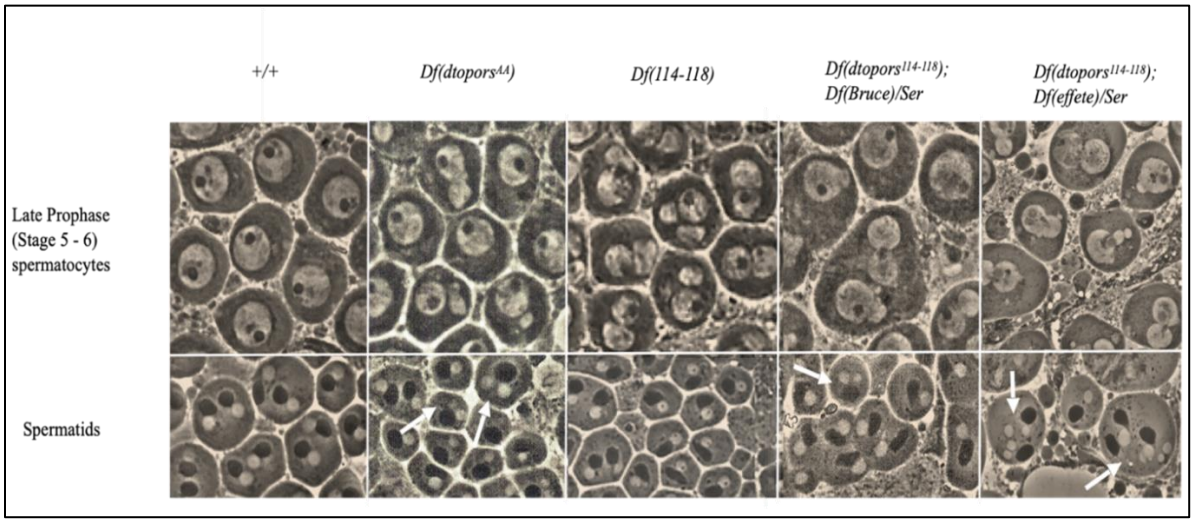
Recovered male gametes	*Y; 4*	*X; 4*	*0; 4*	*X/Y; 4*	*X; 0*	*Y; 0*	*X; 4/4*	*Y; 4/4*	*0; 0*	*0; 4/4*	*X/Y; 0*	*X/Y; 4/4*	Total
Df(dtopors^{AA})	16	15	12	12	4	7	2	10	15	8	2	2	105
Df(dtopors$^{114-118}$); +/Ser	171	377	38	9	0	0	0	0	1	0	0	0	596
Df(dtopors$^{114-118}$);Df(Bruce)/Ser	279	508	99	22	0	1	1	0	0	0	0	0	910
Df(dtopors$^{114-118}$);Df(effete)/Ser	329	567	157	53	2	6	3	2	2	4	0	2	1127
Df(dtopors^{AA})/CyO	206	342	0	0	0	0	0	2	0	0	0	0	550
Df(dtopors$^{114-118}$)/CyO; +/Ser	373	547	1	1	0	2	2	0	0	0	0	0	926
Df(dtopors$^{114-118}$)/CyO;Df(Bruce)/Ser	168	288	0	0	1	0	0	0	0	0	0	0	457
Df(dtopors$^{114-118}$)/CyO;Df(effete)/Ser	54	79	0	0	0	0	0	0	0	0	0	0	133

% Nondisjunction	*Nullo XY*	*Diplo XY*	*Nullo 4*	*Diplo 4*	*XY*	*4*
Df(dtoporsAA)	33.3	15.2	26.7	21	48.5	47.7
Df(dtopors114-118); +/Ser	6.5	1.5	0.2	0.0	8.1	0.2
Df(dtopors114-118);Df(Bruce)/Ser	10.9	2.4	0.1	0.1	13.3	0.2
Df(dtopors114-118);Df(effete)/Ser	14.5	4.9	0.9	1.0	19.3	1.9
Df(dtoporsAA)/CyO	0.0	0.0	0.0	0.4	0.0	0.4
Df(dtopors114-118)/CyO; +/Ser	0.1	0.1	0.2	0.2	0.2	0.4
Df(dtopors114-118)/CyO;Df(Bruce)/Ser	0.0	0.0	0.2	0.0	0.0	0.2
Df(dtopors114-118)/CyO;Df(effete)/Ser	0.0	0.0	0.0	0.0	0.0	0.0

I found that both the *Df(Bruce and the Df(effete)* each significantly increased sex chromosome NDJ in *Df(114-118)* males. For the *Df(Bruce)*, sex chromosome NDJ was significant (p=0.0015 at p<0.05), while no significant difference was found for the fourth chromosome NDJ (p=0.9313 at p<0.05). For the *Df(effete)*, both sex and fourth chromosome NDJ were significant (p < .00001 and p=0.005477 at p<0.05, respectively).

I further analyzed these deficiencies for cytological enhancement of *Df(114-118).* Testes of *Df(114-118); Df(Bruce)/TM3 Ser* and *Df(114-118); Df(effete)/TM3 Ser* were dissected and compared to those of the *RING* hypomorph *Df(114-118)* alone and to those form the control *wildtype* and the *dtopors* deficiency null allele (*Df(dtopors^{AA})*). I assayed NDJ in form of micronuclei in the onion stage spermatids, and nuclear blebbing in S5-S6 spermatocytes (Figure 12).

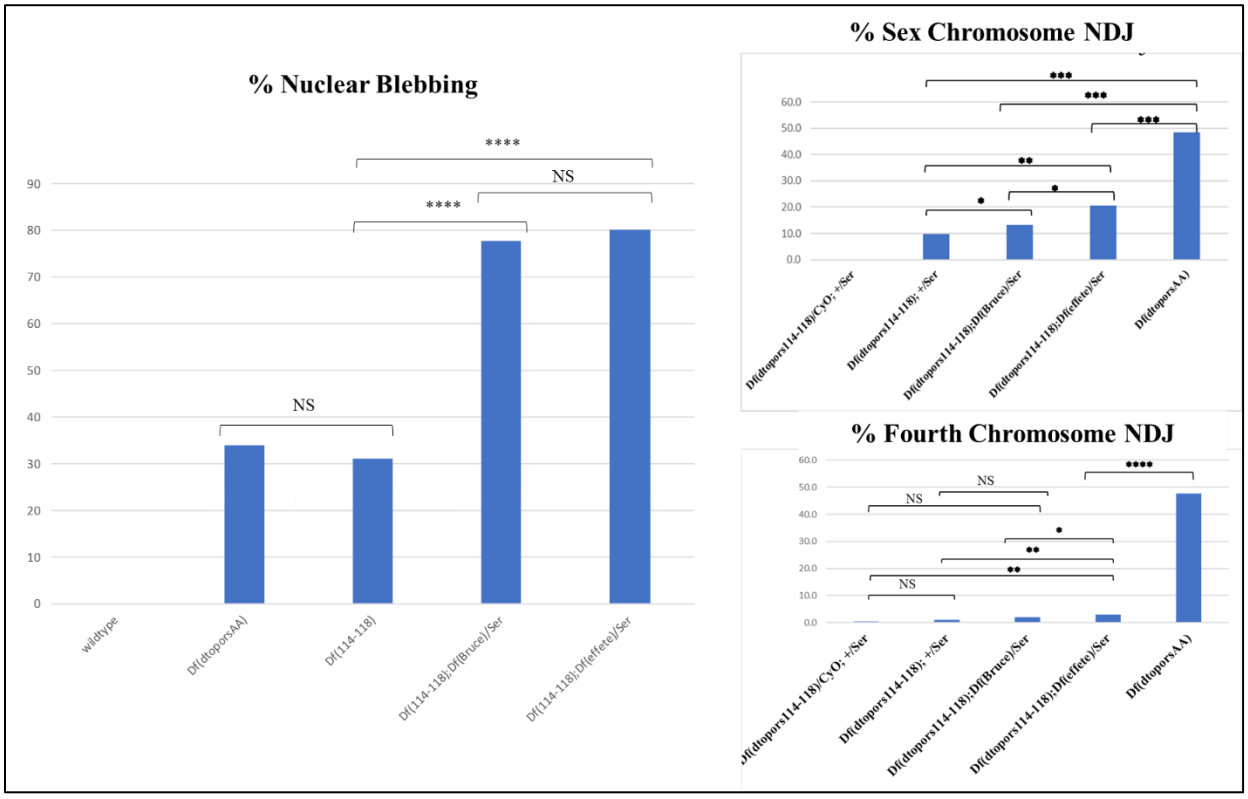
Figure 12. Phase-Contrast Images of S5-S6 Spermatocytes and Onion Stage Spermatid Nuclei. Genotypes are indicated above each column. Arrows indicate unequally sized nuclei and micronuclei resulting from nondisjunction.



I saw a few micronuclei in *Df(114-118);Df(Bruce)/TM3 Ser*, almost none in the *hypomorph*, and very high amounts in the *null*. Interestingly, *Df(114-118); Df(effete)/TM3 Ser* had also very high frequencies of NDJ, comparable to the *null*, which might explain the low fertility of *Df(114-*

118); Df(effete)/TM3 Ser males. Furthermore, in each of the mutants I saw extensive blebbing and both *Df(Bruce)* and *Df(effete)* appeared to enhance the frequency of nuclear blebbing over that of the hypomorph or the null (Figure 12).

Figure 13. Percent Blebbing and Sex and Fourth NDJ in *Df(114-188);Df(E2)/Ser* Males. Blebbing assessed by the frequency of S5-S6 spermatocyte nuclei with visible herniations using phase microscopy. NS = Not significant. *p <0.1, *** p < 0.00001.



To quantitate the blebbing phenotype, I dissected testes of at least ten males and scored at least 200 S5-S6 spermatocyte nuclei per genotype. I found a significant increase of the blebbing in both *Df(114-118);Df(Bruce)/TM3 Ser* and *Df(114-118); Df(effete)/TM3* when compared to the *Df(114-118); +/Ser* hypomorph ($p<0.00001$ for both) and to *Df(dtopors^{AA})* deficiency ($p<0.00001$ for both) (Figure 12). There was no significant difference in percent blebbing between the hypomorph and the null.

Discussion

The aim was to identify potential E2 ligases as interaction partners of dTopors in male meiosis. To identify E2 ligases involved in chromosome segregation, we knocked down E2 ligase expression by RNAi in the germ line using germline-specific drivers which expressed at specific times of spermatogenesis. I identified several E2 lines with fourth chromosome NDJ and four lines where RNAi caused either sterility or lethality for the offspring. I expected to see nuclear blebbing in one or more E2 ligases RNAi-knockdown, based on the assumption that some E2 ligase would be required at the nuclear lamina, but surprisingly, none of the knockdowns showed blebbing. The lack of blebbing may suggest that a pathway is functionally redundant: dTopors may be able to interact with multiple different E2 ligases to affect the same outcome on nuclear structure. Alternatively, the RNAi knockdown may not have sufficiently reduced E2 ligase expression in the germline, given the weak blebbing in the positive control, *dtopors*. As the male germ line has low level expression of the RNAi mediator Dicer, this screen may be improved by upregulating Dicer to enhance RNAi knockdown efficiency (Kim et al., 2005).

Based on RNAi screen NDJ positives, *Bruce (*BIR repeat containing ubiquitin-conjugating enzyme) and *effete* (UbcD1) were selected for further testing. I selected *Bruce* because it had the highest rate of NDJ and therefore was the strongest candidate. I selected *effete* because it is the fly ortholog of the human UbcH5(A,B,C), and is functionally substitutable for UbcH5 (Treier et al., 1992). Human Topors was shown to interact with UbcH5a, UbcH5c, and UbcH6 *in vitro* (Rajendra et al., 2004), furthermore human UbcH5a was also shown to function *in vitro* with dTopors to direct ubiquitination Hairy (Secombe & Parkhurst, 2004). To determine if these E2 were important for dTopors function, I examined their ability to enhance the *dtopors*

hypomorphic allele. A change in NDJ frequency or/and in nuclear blebbing would indicate that the E2 deficiency affects the *Df(114-118)* phenotypes. Halving expression levels of BRUCE and Effete significantly intensified nuclear blebbing in *Df(114-118)*, and increased NDJ frequencies in both. The NDJ frequencies, however, were not as high as in a *dtopors* deletion. Because *Bruce* and *effete* enhanced blebbing more severely than NDJ, it suggests that Bruce and Effete in concert with dTopors ubiquitinate different targets at the nuclear lamina than at chromosomes. The NDJ frequency increased greatly in the hypomorph when the amount of these E2s was reduced to half the amount. This indicates that when contrasted to chromosome segregation, there may be a dosage sensitivity for these E2s and perhaps a threshold requirement for ubiquitin ligase activity at the nuclear lamina. At wildtype E2 levels, blebbing occurs if ubiquitin ligase activity is either missing or reduced to a level where proper nuclear structure is not sustainable. The enhancement of NDJ in the hypomorph by *Bruce* was less severe than that of *effete* (13.3% versus 19.3% for sex NDJ, respectively). This suggests that these E2s might have different targets in chromosome segregation, or they target the same protein(s) with different efficiencies. In mouse soma, Bruce is an important regulator of the DNA damage response by activating checkpoints and DNA repair machinery (Ge, Che, & Du, 2015; Ge, Che, Ren, et al., 2015), while in the male germline, Bruce is required for genomic stability in spermatogonia and spermatocytes by promoting DNA damage repair induced by double strand breaks (Che et al., 2020). It is possible that reduced levels of Bruce in the *RING* hypomorph may cause increased DNA repair via NHEJ rather than HDR, therefore resulting in joining of heterologs and anaphase I bridges during meiosis I. It is known that in flies, Bruce blocks apoptosis by ubiquitinating pro-apoptotic proteins like rpr (reaper) (Domingues & Ryoo, 2012). In testis, Bruce interacts with Klhl10, a protein that recruits substrates for a testis specific Cul3-ubiquitin E3 ligase for

spermatid terminal differentiation in spermatogenesis. My proteomics results identified Klhl 10 as ubiquitinated in the absence of dTopors, and here we implicate Bruce as an E2 ligase partner of dTopors. In vitro ubiquitination assays will be necessary to determine if and how dTopors acts with Bruce to target Klh10 ubiquitination pathway.

The *Df(114-118); Df(effete)/Ser* males were nearly sterile and had very low number of offspring, this is not surprising since nearly all homozygous effete mutants are either lethal or sterile. Testes dissections showed high frequency of NDJ comparable to the *dtopors* null in the onion cells, suggesting that ubiquitin transferase activity of Effete is necessary for dTopors mediated ubiquitination of targets in chromosomes segregation. In flies, Effete is implicated in many processes including chromatin modifications (Cipressa F. & Cenci G., 2013) and heterochromatin induced PEV (Cipressa et al., 2013). In mitosis, Effete physically and genetically interacts with dAPC2, subunit of APC (Anaphase Promoting Complex, multi-subunit E3 ligase) to ubiquitinate cyclin A for degradation (Chen et al., 2009), thus allowing for completion of mitosis. In mitosis and in male meiosis, Effete is needed for proper telomere behavior (Cenci et al., 1997). Male flies mutant in *effete*, display abnormal telomere attachments in both meiotic divisions and exhibit lagging chromosomes in form of acentric chromosome fragments in both anaphase I and anaphase II (Cenci et al., 1997). There is no present research linking Effete to dTopors. Males mutant for *effete* display fused telomeres and lagging chromosomes at anaphase I (Cenci et al., 1997). Therefore, it is possible that the reduced levels of Effete in the *RING* hypomorph may be originating from abnormal telomere attachments causing anaphase bridges. If dTopors is involved in this pathway by acting with Effete to ubiquitinate a target important for telomere behavior (i.e., telomere capping), then this might also explain the anaphase bridges observed in *dtopors* mutants.

Sex but not fourth chromosome NDJ was significantly increased in *Df(114-118)* males when heterozygous for each *Df (Bruce)* and *Df(effete)*. This could suggest that the ubiquitin transferase activity of each Bruce and Effete is needed for dTopors mediated ubiquitination of targets at the chromosomes. Since the fourth chromosome NDJ was minimal and my genetic crosses did not assay for major autosome NDJ, I cannot say whether the elevated sex chromosome NDJ was due to the larger size of the chromosome perhaps requiring more enzyme than the much smaller forth chromosome. However, *effete* alleles do show a similar variation in frequencies of bridges for different chromosomes (Cenci et al., 1997).

In conclusion, these enhancements of the hypomorphic phenotype strongly suggests that these genes act in the *dtopors* pathway, and therefore are candidate E2s for a direct interaction with dTopors and should be verified by yeast-2-hybrid and coimmunoprecipitation assays.

CHAPTER V: CONCLUSIONS

My research showed that ubiquitination by dTopors is essential for proper nuclear structure and for chromosome segregation. The nuclear structure was seen to be more sensitive to perturbation by loss of ubiquitination than was chromosome segregation. Reduction of ubiquitin ligase activity had a profound effect on the nuclear structure, while only caused low NDJ. Total elimination of the ubiquitin ligase activity caused severe blebbing, but NDJ was not observed at levels comparable a dTopors deletion. Based on my data I conclude that the laminar structure has only limited influence on chromosome segregation during meiosis. This is surprising given the intimate association observed between chromosomes and the lamina throughout prophase in this organism. Furthermore, numerous examples of interactions between chromosomes and the nuclear lamina in other organisms suggests that this is a common feature of meiosis. This could mean that the unique bivalent domains that form in male flies in meiotic prophase are not dependent on direct interactions with the lamina. This role is ubiquitination and is essential at the lamina structure, while for chromosome segregation, loss of ubiquitination does not completely disrupt chromosome segregation, suggesting the involvement of another function of dTopors (perhaps sumoylations?).

In my proteomics screen, I identified an abundance of differentially ubiquitinated and/or sumoylated proteins in the presence versus absence of dTopors activity across a variety of ontology groups, highlighting the importance of dTopors in various cellular aspects. Besides the ubiquitination-related targets, many of these proteins appeared to be chromatin/transcription-related. I identified Histone affected by dTopors, including H3 epigenetic marks for chromatin regulation and other histone modifications such as H2B, previously unknown in flies. Several

other targets identified here may be implicated in conserved processes important for regulation of cell division, transcription, etc.

In my *in vivo* genetic assays, I identified E2 candidates as potential interaction partners of dTopors. Little is known about what specificity is needed for E2-E3 interaction but generally, they are determined by specificity of an E2 to an E3. A single E2 can interact with different E3 ligases, as seen with Ube2D/UbcH5 (Effete) and the ubiquitination of a target depends on which E3 is chosen by an E2 (Ye & Rape, 2009). E2s utilize different residues to recognize various E3s, but an E3 is specific to and E2 and a substrate. However, human Topors was shown to interact with several E2 ligases (UbcH5a, UbcH5c, and UbcH6) *in vitro* (Rajendra et al., 2004). Here I identified two potential E2 partners of dTopors *in vivo*, thus suggesting that dTopors may be promiscuous as well. The E2s I identified through the *in vivo* assay, combined with the list of ubiquitination targets from the mass spectrometry screen may be used to investigate and potentially discover dTopors mediated ubiquitination pathways in flies.

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