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The Topors protein is a tumor suppressor in humans that associates with and regulates a number of cell cycle regulators that include Topoisomerase I and p53. It possesses both ubiquitin and SUMO ligase activity and its mutation or downregulation has been associated with some human cancers and diseases. The Drosophila homologue, dTopors, presents structural and functional similarities to human Topors. Both proteins have four conserved domains: a RING finger domain, an RS region, PEST domains and a consensus sequence unique to Topors homologs. To gain insight into the role(s) of these domains in subcellular localization of the protein, we generated transgenic flies expressing dTopors peptides fused to Green Fluorescent Protein. These included full length dTopors protein, and truncations containing amino acids 1-967, 1-367, 1-182, 368-1038, and 968-1038. Using confocal microscopy on living cells, we localized the expressed proteins in two different cell types where dTopors function had been previously examined, salivary glands and spermatocytes. We identified and mapped two nuclear localization sequences (between aa183-367 and aa368-968), a chromosome-binding domain (aa1-367), two nuclear lamina localization domains (between aa1-367 and aa368-967), and a domain responsible for targeting dTopors to punctate nuclear spots (aa368-968). We observed some tissue-specific differences in localization patterns, suggesting that tissue-specific modifications and/or binding partners may be important for dTopors localization, and presumably, its function. These findings constitute a first step to understanding the relationship between dTopors localization and its function.

IDENTIFICATION OF DTOPORS DOMAINS REQUIRED

FOR SUBCELLULAR LOCALIZATION

By

Fidele Byungura

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Approved by

_____
Committee Chair

To my wife Veronique, my children Yasmine, Francis and Daisy and my parents.

APPROVAL PAGE

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

Committee Chair _____

Committee Members _____

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Date of Acceptance by Committee

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Date of Final Oral Examination

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CHAPTER I

INTRODUCTION

The Topoisomerase I-binding Arginine Serine-rich protein, Topors, was originally identified as a protein that interacts with N-terminal 250 amino acids of human Topoisomerase I (TopoI) in a two-yeast hybrid/*in vitro* binding screen (Haluska, Saleem et al. 1999). Further studies showed that it is not only a binding partner of Topo1 but also a binding partner of many other proteins including p53 (Zhou, Wen et al. 1999), Dj-1(Shinbo, Taira et al. 2005) and NKX3.1 (Guan, Pungaliya et al. 2008). It is located in nuclear speckles associated with PML (Promyelocytic leukemia) bodies, nuclear structures implicated in transcription, DNA repair, viral defense, stress, cell cycle regulation, proteolysis and apoptosis (Rasheed, Saleem et al. 2002). It is the only protein to date that has been identified that has both E3 ubiquitin and SUMO-1(Small ubiquitin-like modifier) ligase activity (Rajendra, Malegaonkar et al. 2004; Weger, Hammer et al. 2005).

Topors is expressed in a wide variety of tissues including the germ line (Haluska, Saleem et al. 1999). The Topors protein contains 4 conserved domains; a RING (Really Interesting New Gene) finger domain at the N terminus, an RS/SR domain (arginine and serine-rich domain) 5 PEST sequences (proline (P), glutamic acid (E), serine (S), and threonine (T) rich sequences) and two bipartite nuclear localization sequences close to

the middle of the protein (Haluska, Saleem et al. 1999; Zhou, Wen et al. 1999). Comparison of Topors homologues has also revealed a consensus domain located next to the RING finger (Tomkiel unpublished). RING finger domains are known to be involved in multiple functions including transcriptional control and post-translational protein modification (Satijn, Gunster et al. 1997; Schoorlemmer, Marcos-Gutierrez et al. 1997; Wang, Lybarger et al. 2004; Rajendra, Malegaonkar et al. 2004) and PEST domains are involved in protein degradation (Rogers, Inselman et al. 2004). RS domains are commonly found in RNA-binding proteins, particularly in proteins involved in pre-mRNA splicing (Zahler, Lane et al. 1992), but the function of the RS domain in Topors is unknown.

Topors has an important role in cell cycle control as it directs the modification of a number of cell cycle regulatory proteins that include the tumor suppressor p53 (Zhou, Wen et al. 1999), Topo I (Haluska, Saleem et al. 1999), DJ-1(Shinbo, Taira et al. 2005) and NKX3.1(Guan, Pungaliya et al. 2008). The increasing interest in Topors is driven by the crucial role of its binding partners in cell cycle regulation. Topo1 is a nuclear enzyme that binds DNA and regulates its topology by causing a single strand DNA break followed by religation, leading to removal of supercoiling and DNA relaxation as it unwinds the DNA during replication (Haluska, Saleem et al. 1999). Because of its essential role in DNA replication and transcription (Merino, Madden et al. 1993), Topo1 has been used as a target for the development of antineoplastic drugs, including camptothecins, an important class of antitumor compounds which are associated with increased Topors expression (Lin, Ozaki et al. 2005). Topors also enhances the

formation of high molecular weight SUMO-1 conjugates of Topo1 in a reconstituted *in vitro* system and also in human osteosarcoma cells (Hammer, Heilbronn et al. 2007). The N-terminus of Topo1 that interacts with Topors has been shown to participate in binding to helicases and other proteins including TATA-binding proteins (Merino, Madden et al. 1993), SV40 T (Haluska, Saleem et al. 1999; Simmons, Melendy et al. 1996) and nucleolin (Bharti, Olson et al. 1996) which may suggest a role of Topors in regulating Topo1 interaction with its binding partners.

p53 is a tumor suppressor that is ubiquitously expressed in vertebrates and has important functions in cell cycle control, cell differentiation, apoptosis, gene regulation and tumor suppression (Zhou, Wen et al. 1999). It is particularly important in the DNA damage response where it induces cell cycle arrest or apoptosis by transcriptional activation of genes involved in these processes. Its role in tumor suppression is highlighted by the findings that more than 50% of human cancers contain mutations in p53 gene (Levine, Chang et al. 1994). This protein has been identified both *in vitro* and *in vivo* as a ubiquitination substrate of Topors (Rajendra, Malegaonkar et al. 2004), as well as a sumoylation substrate of Topors (Weger, Hammer et al. 2005).

NKX3.1 is a putative prostate tumor suppressor that has been extensively studied for its role in prostate development and carcinogenesis, and has been also identified as a ubiquitination substrate of Topors (Guan, Pungaliya et al. 2008).

The dual ubiquitin/SUMO-1 ligase activity of Topors makes the characterization of the precise mechanism of Topors effect on cell cycle regulation difficult, as these two functions can have opposite effects on a target protein. Poly-ubiquitination targets

modified proteins for proteosome-mediated degradation while sumoylation does not. Instead, it may regulate protein localization (Matunis, Coutavas et al. 1996), transcription factor activity (Girdwood, Tatham et al. 2004) or oppose protein ubiquitination (Desterro, Rodriguez et al. 1998). p53 is an example of a protein both ubiquitinated and sumoylated by Topors, resulting in either a proteosome-dependent decrease in p53 (Rajendra, Malegaonkar et al. 2004) or an increase in endogenous p53 (Weger, Hammer et al. 2005), respectively.

While the mechanism of action of Topors appears complex and is not fully understood, many observations have led to the suggestion that Topors acts as a tumor suppressor, as it can negatively regulate cell growth and this is believed to be independent of its ubiquitination activity. Lin, Ozaki et al. (2005) have shown that Topors acts as a coactivator of p53 in growth suppression induced by DNA damage. Their studies in mouse cells showed that overexpression of Topors stabilizes p53, enhancing p53-dependent transcriptional activities of MDM2, P21 and Bax promoters and elevating endogenous p21 mRNA levels. Topors overexpression in mouse cells leads to apoptosis or cell cycle arrest. Also, Topors expression is induced by camptothecin (topoisomerase 1 inhibitor) and cisplastin, both potent anticancer drugs (Lin, Ozaki et al. 2005).

A survey of 51 non-small cell lung cancer patients showed that *topors* mRNA was significantly lower than in control patients, and decreased expression was correlated with both development and progression of cancer (Oyanagi, Takenaka et al. 2004). Exogenous expression of Topors causes an accumulation of cells in G0/G1 by inhibiting

cell cycle progression (Saleem, Dutta et al. 2004). Also Topors expression is down-regulated in human adenocarcinomas, associated with increased methylation of a CpG island in the *topors* gene promoter (Saleem, Dutta et al. 2004). Loss of heterozygosity in the location of human *topors* on chromosome 9p21 is frequently observed in many different malignancies that include bladder, lung, lacrimal cancers and melanoma (Mistry, Taylor et al. 2005; Peng, Shibata et al. 2005; Shibata, Uryu et al. 2005; Trkova, Babjuk et al. 2006; Tse, Finkelstein et al. 2006) . Interestingly, mutations in *topors* also cause a form of autosomal dominant retinitis pigmentosa with perivascular retinal pigment epithelium atrophy (Chakarova, Papaioannou et al. 2007). It is unknown, however, if the targets of Topors in the retina differ from those involved in cell cycle regulation.

The Drosophila Topors homologue, dTopors, has incited great interest as Drosophila has proven to be an informative model for characterizing functions of this conserved protein. The dTopors protein was first identified as an interactor with and ubiquitinator of the transcriptional repressor, Hairy, that is required for proper segmentation in early embryonic development (Secombe and Parkhurst 2004). Like human Topors, dTopors interacts with Drosophila Topoisomerase I and p53 (Dmp53) *in vitro* (Secombe and Parkhurst 2004).

Topors and dTopors proteins share several structural motifs, including the N-terminal RING finger, the region rich in RS/SR dipeptide repeats, bipartite nuclear localization sequences and PEST sequences (Secombe and Parkhurst 2004). Secombe and Parkhurst (2004) described 17 RS/SR dipeptides repeats in dTopors. These SR/RS-rich

regions are observed in mRNA splicing factors, where they are targeted for regulated phosphorylation (Furuyama and Bruzik 2002; Graveley 2000). However, relative to these RNA-binding proteins, dTopors has a reduced number of RS repeats. It is also missing the consensus RNA-binding domain that characterizes RS-domain splicing factors. This suggests that dTopors is unlikely to be a direct regulator of mRNA splicing, even though its SR region may be involved in protein-protein interactions or be phosphorylated (Secombe and Parkhurst 2004). Also, given the presence of four bipartite nuclear localization sequences, and four PEST sequences which signal proteolysis of different cellular proteins (Rechsteiner and Rogers 1996), dTopors is likely to be a short-lived nuclear protein.

Despite these similarities between Human and Drosophila Topors, there may also be some important differences. Unlike mammalian Topors, dTopors does not appear to ubiquitinate p53 (Secombe and Parkhurst 2004). Thus it is currently unclear which aspects of dTopors function are conserved.

In flies, several novel roles of dTopors in transcriptional regulation and nuclear organization have been discovered. The first series of studies found that dTopors interacts with proteins of the gypsy insulator complex, and is required for gypsy insulator function (Capelson and Corces 2005). Chromatin insulators are proposed gene regulatory elements involved in the establishment of independent chromatin domains, and are thought to play an important role in regulating the proper expression of independent gene units. This is hypothesized to be achieved by organizing the chromatin into structural

domains that enable the autonomy of gene activity (Geyer and Corces 1992). Chromatin insulators have been shown to have enhancer-blocking activity, as they are able to oppose promoter-enhancer communication (Geyer and Corces 1992; Kellum and Schedl 1992) and barrier activity as they protect incorporated transgenes from the possible influence of the neighboring chromatin (Chung, Whiteley et al. 1993; Kellum and Schedl 1991). The gypsy insulator of *D. melanogaster* is a protein complex that consists of three known components, Suppressor of Hairy wing (Su(Hw)), Modifier of mdg4 2.2 (Mod(mdg4)2.2), (Ghosh, Gerasimova et al. 2001; Gause, Morcillo et al. 2001), and Centrosomal Protein 190 (CP190) (Pai, Lei et al. 2004). Analysis of Drosophila polytene chromosomes shows that insulator complexes are found at multiple endogenous sites dispersed throughout the fly genome (Gerasimova and Corces 1998).

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

Based on knowledge of the Topors and dTopors activity as E3 ubiquitin ligases (Rajendra, Malegaonkar et al. 2004; Secombe and Parkhurst 2004) and Topors as an E3 SUMO ligase (Weger et al, 2003, Capelson and Corces (2005; 2006) explored the possibility that dTopors ubiquitination or sumoylation activity may be involved in gypsy insulator activity regulation. They generated a *dtopors* transgenic construct carrying a point mutation, which changes a highly conserved cysteine of the RING domain to a serine (C118S). Mutation of this conserved residue has been demonstrated to disrupt the ubiquitin ligase activity of MDM2, a mammalian RING finger protein (Honda and Yasuda 2000). This mutation disrupted insulator function. None of the known insulator complex proteins, however, could be demonstrated to be ubiquitinated by dTopors.

Further experiments showed, both *in vivo* and *in vitro*, that dTopors may negatively regulate the sumoylation of CP190 and Mod(mdg4)2.2. In an *in vitro* experiment, Su(Hw), Mod(mdg4)2.2 and CP190 were used as substrates in a sumoylation reaction with or without dTopors. All three insulator proteins are potential targets for sumoylation as they possess lysines that are located in a SUMO modification consensus motif ψKxE (Capelson and Corces 2006). Each reaction consisted of incubating the E1, E2 enzymes, SUMO, with *in vitro*-transcribed and -translated ^{35}S-labeled substrate protein and *in vitro*-generated or -purified recombinant dTopors. The results showed that CP190 and Mod(mdg4)2.2 were SUMO-modified, characterized by higher molecular weight bands in presence of sumoylation machinery. However, adding dTopors decreased CP190 and Mod(mdg4)2.2 sumoylation instead of enhancing it (Capelson and Corces 2006). In an *in vivo* experiment, Capelson and Corces (2005) overexpressed

dTopors in larvae using a *UAS-dtopors* transgenic construct driven by an *actin-GAL4* (*ActGAL4*) promoter. Western blot analysis of protein extracts from larvae showed a decrease in sumoylated forms of Mod(mdg4)2.2 and CP190 when dTopors was induced compared to uninduced. Furthermore, mutations in components of the SUMO conjugation pathway improved the enhancer-blocking function of a partially active insulator (Capelson and Corces 2006). Based on these findings, it was proposed that SUMO modification of insulator complex proteins negatively regulates the activity of the gypsy insulator, and dTopors was proposed to regulate the gypsy insulator activity by downregulating insulator sumoylation.

In unrelated studies, dTopors was also found to be required for proper chromosome segregation, chromosome condensation, normal nuclear lamina formation and the regulation of centrosome duplication in the male germ line (M. Matsui, K. Sharma and J. Tomkiel, unpublished). In spermatocytes, dTopors localizes to the nuclear lamina and to intranuclear spots reminiscent of PML bodies. Mutations in *dtopors* disrupt the localization of dTopors to these nuclear structures, alter the centrosome duplication cycle and cause germ-line genomic instability and the production of aneuploid gametes (M. Matsui, K. Sharma and J. Tomkiel, unpublished). Drosophila males have a unique meiotic system that does not involve recombination to maintain homologs together during prophase I and these findings showing the requirement of dTopors in successful meiosis are another piece of puzzle toward understanding the molecular basis of meiosis in this organism.

Meiosis is a specialized cell division that results in reducing the chromosome number of diploid organisms by half to produce haploid gametes (eggs and sperm). The meiotic process is essential for sexual reproduction as two haploid gametes are fused to form a euploid zygote. Meiosis consists of two rounds of cell division. In Meiosis I, homologous chromosomes segregate into two daughter cells, and in Meiosis II, sister chromatids of each homolog separate then segregate into two cells. Proper homolog pairing is an important event in meiosis for congression to the metaphase plate and proper segregation in anaphase I (Vazquez, Belmont et al. 2002). Homolog pairing and segregation is a conserved process during which each homolog locates and joins its partner. This is followed by the establishment of some form of adhesion and/or connection between the two homologs to keep them together as they congress to the metaphase plate. There, the adhesion and connection between homologs is removed and pairing released so that partners can segregate to opposite poles at anaphase I (Vazquez, Belmont et al. 2002).

Most meiotic systems utilize homologous recombination to interlock the homologs in a way that ensures they are properly segregated (Hawley and Waring 1988). In this recombination-dependent pathway homologs are unpaired when entering meiosis (McKee 2004) and recombination between homologs is accomplished by a conserved process involving double strand breaks (DSBs) mediated by the Spo11 endonuclease (Keeney 2001) and subsequent DNA repair. This follows homolog alignment, pairing and formation of a synaptonemal complex (SC), which has been described as a zipper-

like structure that connects aligned homologs from end to end (von Wettstein, Rasmussen et al. 1984; Page and Hawley 2004).

While most meiotic systems rely on recombination to maintain homologs together during prophase I, male Drosophila has a poorly understood achiasmate system. To understand the dynamics behind meiotic homolog pairing in male Drosophila, Vazquez et al. (2002) conducted an experiment that allowed them to follow specific chromosomal loci in living spermatocytes throughout all stages of meiosis, using a GFP-Lac repressor(GFP-Lac I)/lac operator(LacO) system as a tag. In this experiment, an array of LacO sequences were inserted at specific chromosomal locations and labeled using a GFP-Lac I fusion protein expressed in the male germ line. The chromosomes were then tracked live from mitotically dividing spermatogonia to the mature spermatocytes. Their results showed that most homologs are already paired before entering meiosis, the pairing frequency increases as cells transit from spermatogonia to spermatocytes and this pairing is observed for 13 different euchromatic lacO inserts tested. The pairing is sustained until mid-prophase I. At this stage, chromosomes reorganize and bivalents separate into nuclear territories (Cenci, Bonaccorsi et al. 1994). Shortly after the bivalents have separated into territories, both homologous pairing and sister chromatid cohesion appear to be released all along the euchromatic regions (Vazquez, Belmont et al. 2002).

While maintenance of the homologous pairing in male Drosophila is still not well understood, models suggest that it may be mediated by homolog chromosome entanglements (Duplantier, Jannink et al. 1995; Vazquez, Belmont et al. 2002), via

cohesion in the heterochromatin or by the establishment of chromosome domains in spermatocyte nucleus (Vazquez, Belmont et al. 2002).

Although it is unclear how pairing is first established, genetic studies have identified a number of genes involved in regulating pairing maintenance. Tomkiel et al. (2000) have genetically and cytologically characterized a gene, *teflon*, specifically involved in the maintenance of autosome pairing. Analysis of four ethyl methanesulfonate (EMS)-induced mutations in this gene revealed autosomal nondisjunction in meiosis1 specifically in males. They did not see a measurable effect on sex chromosomes which suggests that sex chromosomes and autosomes segregation are at least in part controlled by different genetic pathways. These results led to the conclusion that *teflon* is involved in mediating or regulating the maintenance of autosomal homolog pairing in Drosophila male meiosis1 (Tomkiel, Wakimoto et al. 2001).

Modifier of mdg4 in Meiosis (mnm) and *Stromalin in Meiosis (snm)* are other genes that were subsequently shown to be involved in maintenance of homolog pairing in male Drosophila (Thomas, Soltani-Bejnood et al. 2005). The two gene products colocalize to sex chromosomes during prophase I and metaphase I, but are absent at anaphase I. Mutations in either gene result in both sex and autosomal nondisjunction, leading to conclusion that they function in stabilizing homolog pairing (Thomas, Soltani-Bejnood et al. 2005). Thomas et al. (2005) also found that Teflon is required for MNM localization to autosomes, and presumably for its function in autosome pairing. They found that mutations in *teflon* do not affect the localization of MNM and SNM on sex

chromosomes while they eliminate the localization of MNM on autosomes. In later studies, a genetic screen for *teflon* modifiers identified *mnm* mutations, but not *snm* mutations, as enhancers of *teflon* (Thomas et al., 2007). This supports the model that Teflon recruits and stabilizes MNM to paired autosomes where the two proteins may interact to secure the connection between the autosomal bivalents (Thomas, Soltani-Bejnood et al. 2005), while a different factor may be required to recruit and stabilize SNM and MNM on paired sex chromosomes.

The *mnm* mutations specifically affect one of 31 isoforms encoded by the *mod(mdg4)* locus, the same gene that encodes the insulator component *mod(mdg4)2.2*. The observations that Mod(mdg4)2.2 is part of the gypsy insulator which is regulated by dTopors, and that both dTopors and MNM are independently found to regulate proper homolog segregation in male Drosophila, raises the possibility of a similar function for dTopors at insulators and in meiosis. Whether or not dTopors function in meiosis is associated with the function of MNM is not yet known.

The involvement of Topors in regulatory processes makes this gene very interesting on multiple levels and all these observations suggest that Topors activities may be context-dependent and/or modified by the presence of tissue or organismal-specific factors. Understanding different structural and functional aspects of dTopors in Drosophila, with an ultimate goal of identifying precisely which domains are required for a given function, may significantly contribute to the understanding of conserved activities of human Topors in tumor suppression and retinitis pigmentosa.

In this study, we examine the role of dTopors conserved domains with respect to the protein localization in two different tissue types, testis and salivary glands. In particular, we assay which domain(s) is/are required for nuclear, lamina and chromosome localization.

CHAPTER II

MATERIALS AND METHODS

Competent cells

Competent cells were prepared following Scott-Simanis transformation protocol (M. Montiero, personal communication). On day 1, a frozen stock of DH5α *E.coli* cells was used to streak a Ψa plate (Bacto-yeast extract, Bactotryptone, $MgSO_4*7H_2O$, pH 7.6 , BactoAgar) and grown overnight. On day 2, a single fresh colony was used to inoculate 5 ml Ψ_β medium (Bacto-yeast extract; Bactotryptone, $MgSO_4*7H_2O$, pH 7.6) and grown overnight at 37°C at 250 rpm. On day 3, the 5ml overnight culture was transferred into 500ml Ψ_β medium and further grown at 37°C at 250rpm for a few hours until OD590=~0.48. Cells were cooled on ice for 5 min. then spun down at 6k for 5 min. at 4°C. Using pre-chilled pipettes, cells were gently resuspended in 100ml of ice cold TfbI (30mM potassium acetate, 100mM $RbCl_2$, 10mM $CaCl_2$ * $2H_20$, 50mM $MnCl_2$ * $4H_20$, 15% glycerol (v/v), pH 5.8), left on ice for 5' then spun down at 6K for 5' at 4°C. The cell pellet was then resuspended in 12.5ml of ice cold TfbII (10mM MOPS, 75mM CaCl2*$2H_20$, 10mM $RbCl_2$, pH 6.5, 15% glycerol (v/v)) and incubated on ice for 10 to 15 more min. 200 µl aliquots were made and snap frozen in liquid nitrogen before being stored at -80°C.

Bacterial transformation

Competent DH5α cells were thawed and placed on ice. 100µl of cells were used per transformation and different dilutions of pCaSpeR hs plasmid DNA containing an ampicillin-resistance gene were added to cells and incubated on ice for 20 min. The cells were heat shocked at 42°C for 2 min. then returned on ice for 2 min. before adding 1ml LB (Bacto-Tryptone, Bacto-yeast extract, 10g NaCl, pH 7.0) and incubating for 1hr at 37°C. Cells were then plated on LB amp plates (Bacto-tryptone, Bacto-yeast extract, NaCl, Bacto-agar, 1000x Ampicillin (100mg/ml in stock), pH 7.0) and incubated at37°C overnight.

Vector preparation

pCaSpeRhs fly vector that expresses the *white* gene and contains an *hsp70* heat shock promoter upstream from a multiple cloning site (figure1) was transformed into DH5alpha competent *E. coli* as described above. A single fresh colony from the transformation plate was used to inoculate 500ml LB amp medium and incubated at 37°C overnight at 250rpm. Cells were harvested and pCaSpeRhs DNA purified using a plasmid maxi kit (Qiagen, Valencia, CA). A sample of the purified plasmid (5µl) was run on agarose gel to estimate the DNA yield and verified integrity. DNA was quantified by absorbance at 260nm.

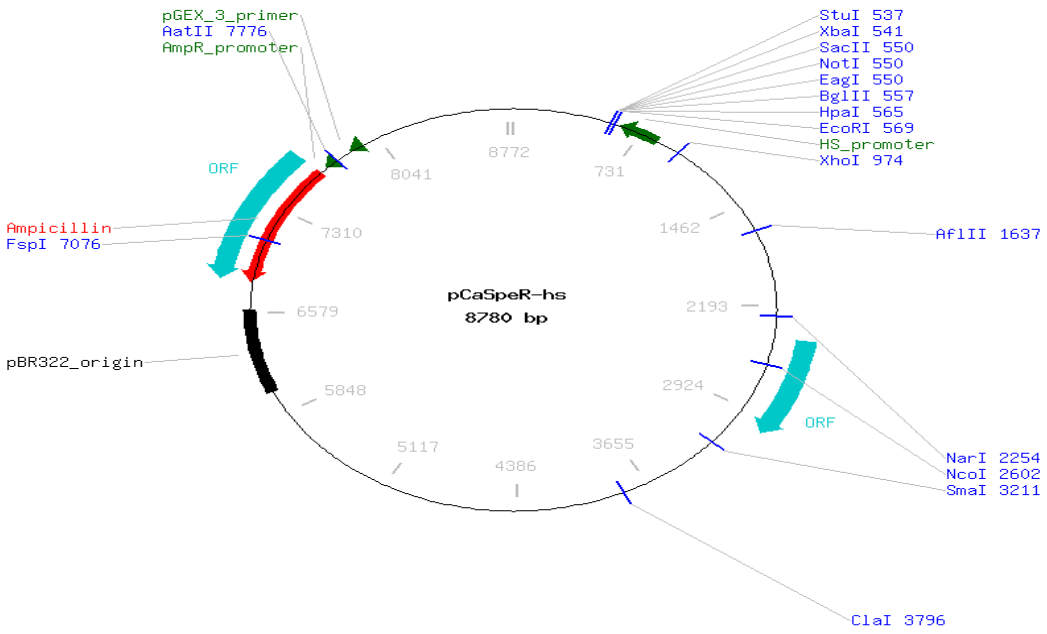


Figure 1: pCaSpeRhs vector (www.addgene.org)

PCR amplification of cDNA and generation of *dtopors* fragments

A plasmid containing the *dTopors* cDNA was purchased from the Drosophila Genomics Resource Center (https://dgrc.cgb.indiana.edu, stock# LD43109). The full length *dtopors* cDNA (1-1038) and its various fragments (expressing amino acids 1-182, 1-367, 1-967, 183-1038, 368-1038, 968-1038) were amplified from the plasmid DNA by PCR and the *Enhanced Green Fluorescent Protein* (EGFP) cDNA was PCR-amplified from an existing *EGFP* –containing plasmid (pCaSpeR tefEGFP331-end). Upstream and downstream specific oligonucleotide primers containing respectively *Bgl*II and *Xba*I restriction sites for *EGFP* and *Hpa*I and *Bgl*II for *dtopors* fragments were used for these

amplifications (Table 1). PCR amplification cycling conditions were set at 30 cycles of denaturation at 95°C for 30 sec, annealing at 58°C for 30 sec and elongation at 72°C for 30 sec.

EGFP primers:

*Bgl*IIGFPF: 5'GC**AGATCT**ATGGTGAGCAAGGGCGAG 3'

*Xba*IGFPR: 5'CC**TCTAGA**TTACTTGTACAGCTCGT 3'

dtopors full length primers:

*Hpa*I Dt1038F: 5′ CT**GTTAAC**ATGGCGGAGGAGAATCCC 3′

*Bgl*Dt1038R: 5' CC**AGATCTA**TACGGCAGTAGTCCCTGAT 3'

dtopors aa1-182 primers:

*Hpa*I Dt1038F: 5′ CT**GTTAAC**ATGGCGGAGGAGAATCCC 3′

*Bgl*Dt182R: 5'CC**AGATCT**ACGCCTCACAATGTGGTAACG 3'

dtopors aa1-367 primers:

*Hpa*I Dt1038F: 5′ CT**GTTAAC**ATGGCGGAGGAGAATCCC 3′

*Bgl*Dt367R: 5' CC**AGATCT**GTAACCGTTTATGTCATACGG 3'

dtopors aa1-967 primers:

*Hpa*I Dt1038F: 5′ CT**GTTAAC**ATGGCGGAGGAGAATCCC 3′

*Bgl*Dt967R: 5’ CC**AGATCT**TGCGGCCTCCAGCGAATAGGC 3’

dtopors aa 183-1038 primers:

*Hpa*IDt183F: 5’CT**GTTAAC**ATGCCCAGGTACACGCCGCTGGTG 3’

*Bgl*Dt1038R: 5’ CC**AGATCTA**TACGGCAGTAGTCCCTGAT 3’

dtopors aa368-1038 primers:

*Hpa*IDt368F: 5’CT**GTTAAC**ATGGATCATGTGGTGCAGTATTCG 3’

*Bgl*Dt1038R: 5’ CC**AGATCTA**TACGGCAGTAGTCCCTGAT 3’

dtopors aa968-1038 primers:

*Hpa*IDt968F: 5’CT**GTTAAC**ATGATCGATGTAGTTGGCGAATCA 3’

*Bgl*Dt1038R: 5’ CC**AGATCTA**TACGGCAGTAGTCCCTGAT 3’

Table 1: Forward and reverse oligonucleotide primers used for PCR amplification of *EGFP*, *dtopors* full length and *dtopors* fragments. Restriction sites are bold and underlined.

All primers were purchased from MWG biotech (Highpoint, NC). PCR products were purified using Qia-quick kit (Quiagen, Valencia, CA) and each DNA product was verified by agarose gel electrophoresis before and after purification.

Gene cloning

EGFP cloned into pCaSpeRhs vector

EGFP and pCaSpeRhs purified DNAs were digested with *Xba*I and *Bgl*II restriction enzymes (Promega, Madison, WI). The two digestions were done separately, first with *Xba*I. Digest products were purified using Qia-quick kit (Quiagen, Valencia, CA) and yield was estimated using agarose gel electrophoresis. Digested *EGFP* was ligated into the digested vector downstream from the hs promoter (Figure 2) using 10x ligase buffer and T4 ligase (Promega, Madison, WI). Ligation products were transformed into DH5α competent cells and grown overnight at 37°C on LB amp plates. Resulting colonies were used to inoculate 5ml LB amp media and were grown overnight at 37°C in shaker at 250 rpm. The plasmid DNA from each clone was extracted and purified following the mini-prep protocol (Sambrook 1989) and the DNA yield was estimated using agarose gel electrophoresis. The mini-prep DNA was digested with *Pvu*I and the resulting products were separated by agarose gel electrophoresis to test for the presence of the *EGFP* insert. One colony containing the insert was selected then used to inoculate 500ml LB amp medium, and were grown at 37°C overnight at 250 rpm. The plasmid DNA was extracted and purified using a maxi-prep DNA purification kit (Quiagen, Valencia, CA). After confirming the purification yield by gel electrophoresis

and absorbance at 260 nm, the plasmid DNA was sent to MWG Biotech (Highpoint, NC) for DNA sequencing.

DtoporsFL (full length) and dtopors fragments cloned into pCaSpeRhsEGFP

This cloning was designed to generate a vector that expresses a dTopors-EGFP fusion protein driven by a heat shock promoter (figure 2). Both pCaSpeRhsEGFP vector and full-length and *dtopors* fragments cDNA were digested with *Hpa*I and *Bgl*II. The two digests were also done separately, first with *Hpa*I where 5µl of DNA, 10 µl multicore buffer, 84 µl ddH_2O and 1 µl *Hpa*I were mixed and incubated at 37°C overnight. The digest products were run on 0.7% agarose gel and purified using Qia-quick DNA gel extraction kit (Quiagen, Valencia CA). After verification of DNA yield using agarose gel electrophoresis, the purified DNA was then digested with BglII, where 43 µl DNA, 10 µl Buffer D, 46µl ddH_2O and 1 µl *Bgl*II were mixed in a microcentrifuge tube and incubated at 37°C overnight. The digestion products were purified using Qia-quick kit (Quiagen, Valencia CA) and the purification was verified using agarose gel electrophoresis. Ligations, transformations, and DNA purification were performed as described above. Agarose gel electrophoresis was used to verify the presence of inserts in the clones after digestion with *Pvu*I. Plasmid DNA was sent to MWG Biotech (Highpoint, NC) for DNA sequencing. Each final clone contained *dtopors-EGFP* in-frame fusion cDNA inserted downstream from the hs promoter (Figure 2).

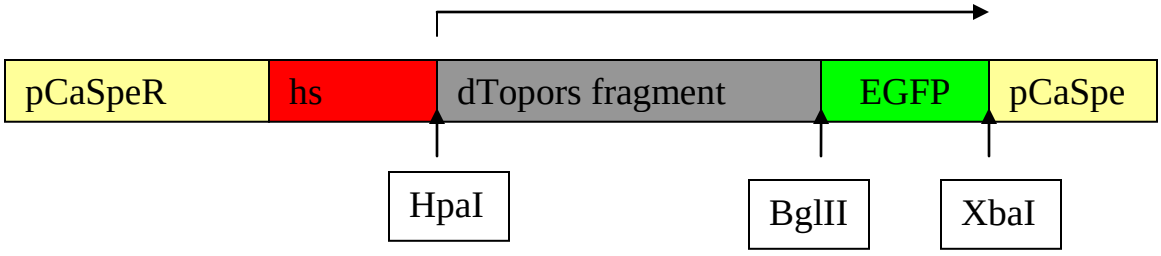


Figure 2: pCaSpeRhs vector expressing a heat shock promoter driven dTopors-EGFP fusion protein.

Drosophila culture and stocks

The fly lines w^{1118}, *yw/ Dp(1;Y)y[+]; tef* ^{k15914}*/Cy; Sb/TM3, Ser;* spa^{pol} , *yw/ Dp(1;Y)y[+]; dtopors* $^{[f05115]/}$*CyO;* spa^{pol}, and *yw/yw; dtopors*^{Z1837}*/Cy; Sb/TM3,Ser;* spa^{pol} were used for this experiment. The *dtopors* transgenic lines were generated as described below (see transgenic flies). All flies were grown on standard cornmeal, molasses, yeast, agar medium at room temperature (25°C).

Transgenic flies stocks

After the constructs were verified by DNA sequencing, plasmid DNA was sent to a commercial fly injection company to be injected in the fly embryos (Genetics Services, Inc., Salisbury, MA). We received 200 injected embryos per clone which gave our first generation (G_0) of transgenic flies. From the injected embryos only a few of them survived (Table 3). Each G_0 male fly was crossed with 5 w^{1118} virgin females while each virgin G_0 female was crossed with 3 w^{1118} males. From our 2nd generation (G_1), only w^+

flies were collected from each G_0 parent, and each of these flies was used to generate an independent transgenic fly line. Each G_1 fly selected was crossed to w^{1118} flies, and the resulting [w+] G_2 offspring were intercrossed. Homozygous transgenic flies were selected based on the eye color intensity and were used to establish, stable transgenic flies stocks for each line.

Transgene mapping

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

Localization of dTopors/EGFP fusion proteins

Homozygous flies and larvae were heat-shocked by incubation at 37°C for 1hr each eight hrs throughout development. Male flies were dissected to collect testis, and larvae were dissected to collect salivary glands. Dissections were performed in Schneider's media (GIBCO BRL, Gaithersburg, MD) to keep the tissue alive. Collected tissues were incubated in Hoechst 33258 at 1/500 dilution (Ashburner et al., 1989) for five min. Tissues were transferred to a fresh drop of Phosphate Buffered Saline (PBS;

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

CHAPTER III

RESULTS

Generation of Vectors for expressing dTopors-EGFP Fusion Proteins

DTopors protein is a 1038 amino-acid protein that has a RING finger domain at the N terminus end, four PEST sequences, an RS rich region and four bipartite nuclear localization sequences (Secombe and Parkhurst 2004). An additional PEST sequence at N-terminus and a consensus sequence shared by Topors homologs adjacent to the RING finger were more recently identified by homology searches (J.Tomkiel, unpublished). As these conserved domains have been associated with specific functions, we used them as landmarks in designing a collection of vector to express truncated forms of the protein. We used PCR to generate six cDNA fragments that encode dTopors polypeptides, including full length (aa1-1038), aa1-182, aa1-367, aa1-967, aa368-1038 and aa968-1038 (Figure 3). The correct sizes of the expected PCR products were verified by agarose gel electrophoresis (Figure 4).

Constructs expressing DTopors-EGFP fusion proteins were generated by subcloning both the *dtopors* cDNAs and the *EGFP* cDNA into the pCaSpeRhs transformation and expression vector. DNA fragments were cloned in a way that *dtopors* cDNAs were linked in frame with *EGFP* at the C-terminus. This vector contains

the *hsp70* promoter that drives expression of the fusion protein upon heat shock (Figure 2). The clones were transformed into DH5α cells and plasmid DNA purified (see Materials and Methods). The purified DNAs were digested with *Pvu*I restriction enzyme and separated by agarose gel electrophoresis to verify the presence and sizes of the inserts. The expected fragment sizes were confirmed (Figure 5). To ensure that no mutations had been introduced by PCR the insert of each clone was verified by DNA sequencing.

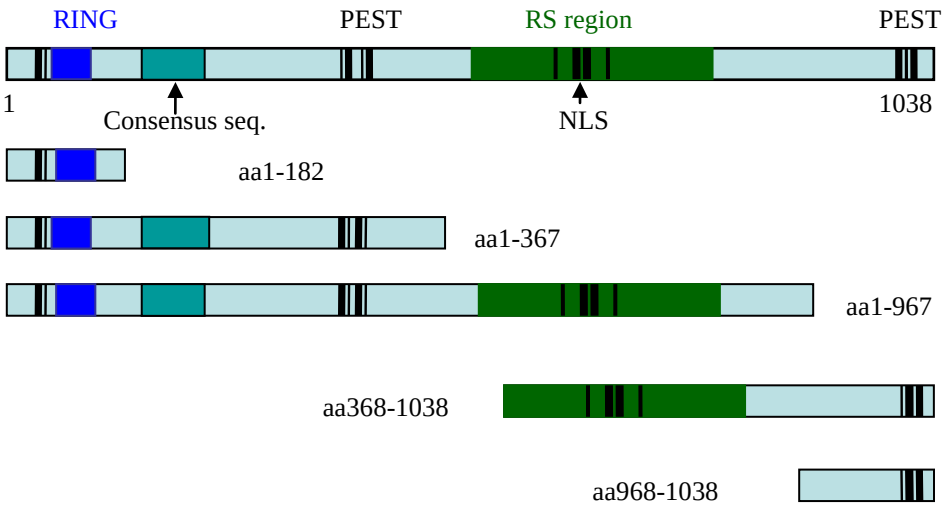


Figure 3. Schematic representation of dTopors showing the polypeptides (bars) that were expressed with EGFP fused at C-termini. (Modified from Secombe and Parkhurst 2004). Structural features include a RING finger domain at the N terminus, a Topors consensus sequence (J.Tomkiel, unpublished), five PEST sequences, four putative bipartite nuclear localization signals and an arginine/serine (RS) rich region.

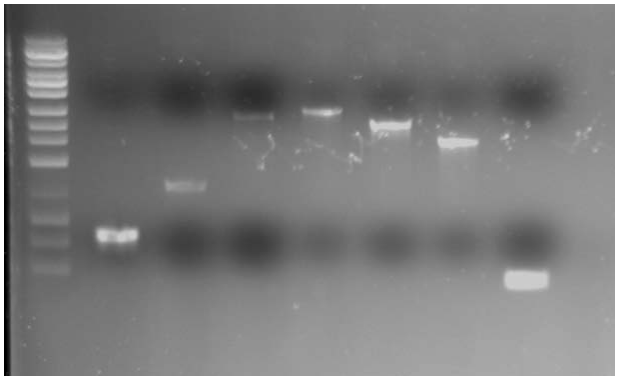
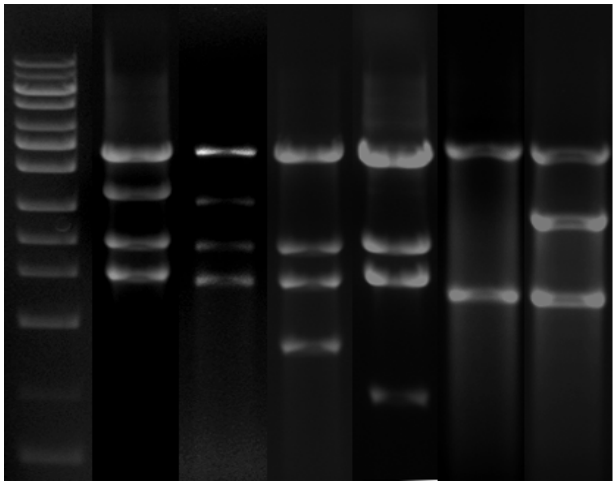


Figure 4. PCR amplification of *dtopors* cDNA fragments . Agarose gel electrophoresis of PCR-amplified *dtopors* cDNA. Shown here are fragments 1-546bp (encoding aa1-182), 1-1101bp (aa1-367), 1-2901bp (aa1-967), 1-3117bp (aa1-1038), 567-3117bp (aa183-1038), 1102-3117bp (aa368-1038), 2902-3117bp (aa968-1038).



Fugure5. *pCaSpeRhs::dtopors-EGFP* clones. Agarose gel electrophoresis of purified plasmid DNA from each *dtopors -EGFP* clone digested with *Pvu*I , confirming the insertion of the various *dtopors-EGFP* sequences into the vector. Shown here are digestion products for fragments 1-3117bp (aa1-1038), 1-2901bp (aa1-967), 1-1101bp (aa1-367), 1-546bp (encoding aa1-182), 1102-3117bp (aa368-1038), 2902-3117bp (aa968-1038).

Generation of transgenic flies

To generate *phs::dtopors-EGFP* transgenic flies, the purified DNA of each clone was sent to a commercial fly injection company to be injected in the fly embryos (Genetics Services, Inc., Salisbury, MA). Two hundred injected embryos were received for each clone, but only a few of them survived to give our first generation (G_0) of transgenic flies (Table 2). Each G_0 fly was crossed with 5 w^{1118} flies (see Materials and Methods) to produce the second generation (G_1). From each G_0 cross, a maximum of two transgenic w^+ G_1 flies were collected, and each of these flies was used to generate an independent transgenic fly line. Ultimately, only a single line was kept from each G_0 parent to insure that each line established was independent. Each G_1 fly selected was individually crossed to w^{1118} flies, and the resulting w+ G_2 offspring collected. Homozygous transgenic flies were selected based on the eye color intensity and were used to establish stable transgenic flies stocks for each line.

Amino acids of dTopors encoded by transgene	# of injected embryos	# of G_0 flies	# of G_1 w+ flies (# of transgenic fly lines)
full-length aa1-1038	200	116	20 (9♀, 11♂)
aa1-967	200	104	16 (7♀, 9 ♂)
aa1-367	200	98	22 (9♀, 13♂)
aa1-182	200	96	24 (6♀, 18♂)
aa368-1038	200	42	7 (3♀, 4♂)
aa968-1038	200	125	25 (9♀, 16♂)

Table 2: Injected embryos and transgenic lines. Shown are number of injected embryos per transgene, number of surviving G_0 adults and number of G_1 germ-line transformants which were used to establish independent transgenic lines.

Transgene mapping

As transgenes were randomly inserted into chromosomes, we had to identify which chromosome was carrying the transgene for each of the transgenic lines. This was accomplished by setting up crosses that enabled us to score the patterns of segregation of the [*w+*] transgene from second and third chromosome dominant markers (see Materials and Methods). The mapping results are shown in Table 3.

Amino acids of dTopors encoded by transgene	Line #	Chromosome
Full-length aa1-1038	5, 9, 11	X
	1, 2, 3, 7, 12, 13, 15, 16, 18, 19	2
	4, 6, 8, 14, 17, 20	3
	10	4
aa1-967	16	X
	1, 2, 4, 5, 7, 8, 10, 12	2
	3, 6, 11, 13, 14	3
aa1-367	7, 11, 20	X
	1, 3, 4, 9, 12, 14, 16, 19, 21, 22	2
	6, 8, 10, 13, 15, 17, 18	3
aa1-182	13, 15, 17, 23	X
	6, 12, 18, 19, 20, 22,	2
	1, 2, 3, 4, 5, 7, 8, 9, 10, 14, 16, 21, 24	3
aa368-1038	1, 2, 3, 7	2
	4, 5	3
aa968-1038	5, 15, 18	X
	4, 9, 11, 12, 14, 16, 17, 19, 24	2
	1, 2, 3, 6, 7, 8, 10, 13, 20, 21, 22, 23, 25	3

Table 3: Chromosomal mapping of transgene insertion sites. Transgenic lines were numbered and are grouped based on the chromosome that is carrying the transgene. (Note: Not all lines were mapped))

Expression of transgenes and transgenic protein localization

In order to investigate the role of each dTopors conserved domains in the protein's subcellular localization, we examined the localization of the transgenic protein in living tissues. To induce the expression of the transgene, the transgenic flies were heat-shocked at 37°C for 1 hr every 8 hrs throughout their life cycle (~10 d). Larvae were dissected to collect salivary glands and young adult males were dissected to collect testes. Both salivary gland and testis cells were examined live using confocal microscopy. The results show a variety of subcellular localization patterns and reveal differences between transgenes, and to some extent, between the two tissue types examined.

In salivary gland, the DNA was visualized in living cells by staining with Hoechst33258. The full length transgenic protein localized to the nuclear lamina, in some spots on chromosomes and throughout the nucleus except the nucleolus. The aa1-967 and 368-1038 fragments showed similar patterns of localization to the nuclear lamina and in the nucleus, but unlike the full-length protein they were excluded from the chromosomes and the nucleolus. The aa1-367 fragment localized extensively and exclusively to the chromosomes while the aa1-182 fragment was completely excluded from the nucleus and localized in vesicles in the cytoplasm. The last fragment, aa968-1038, localized in the nucleus but was excluded from nuclear lamina, chromosomes and nucleolus (Figure 6). In testis, the DNA could not be visualized as the Hoechst staining did not penetrate the testis sheath. However, the position of the chromosomes is well characterized at the various stages of spermatocyte development, and the nucleolus could

be visualized by phase contrast optics. The full-length transgenic protein localized to the nuclear lamina, and in the nucleolus as well as in cytoplasm. A faint GFP signal was detected in the nucleoplasm but was otherwise not localized to the chromosomes. The aa1-967 protein localized to the nuclear lamina and in the nucleoplasm, but was excluded from the chromosomes and the nucleolus. The aa1-367 fragment showed a marked localization to the nuclear lamina and nuclear structures consistent with the positions of the chromosomes. The aa1-182 fragment was excluded from the nucleus and localized in the cytoplasm, possibly in vesicles, similar to its localization in salivary glands. The aa368-1038 fragment showed an interesting localization pattern to nuclear spots, but no evidence of nuclear lamina localization could be detected. The last fragment, aa968-1038 localized in cytoplasm, and was excluded from the nucleus (Figure 7).

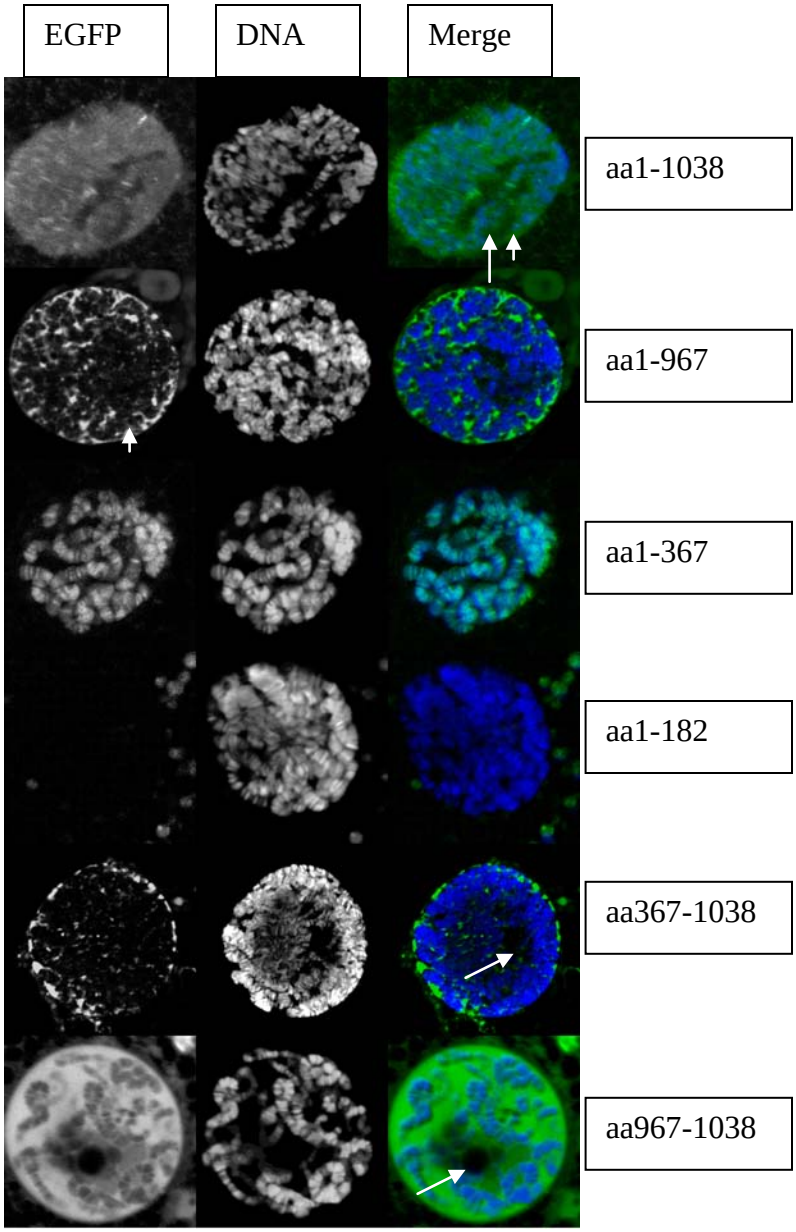


Figure 6. Confocal analysis of localizations of dTopors-EGFP fusion proteins in live salivary gland cells. DNA is stained with Hoechst 33258. The dTopors amino acids encoded by each transgene are indicated to the right, long arrows show the nucleolus and short arrows show the nuclear lamina.

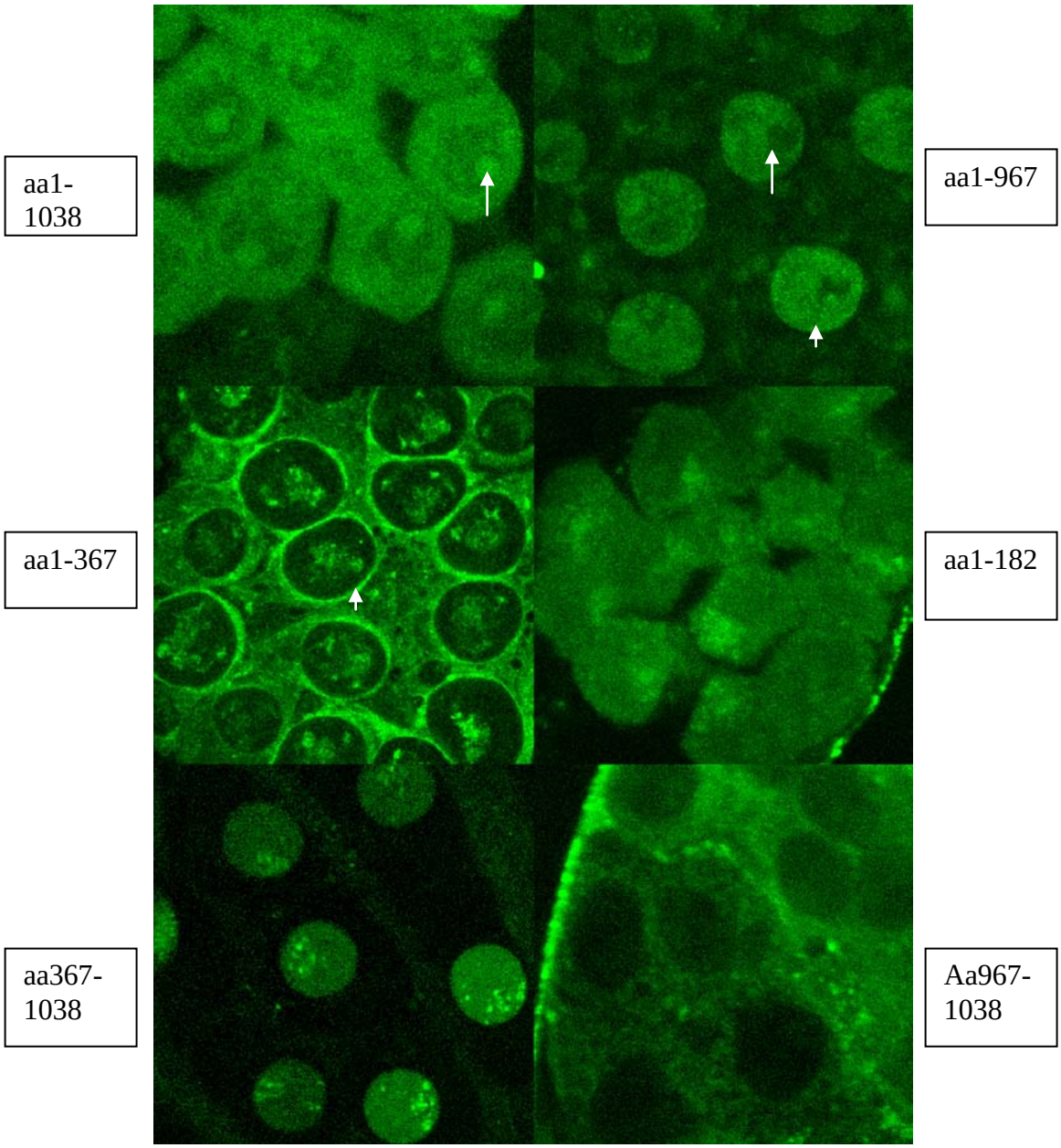


Figure 7. Confocal analysis of localizations of dTopors-EGFP fusion proteins in live spermatocytes. The dTopors amino acids encoded by each transgene are indicated to the right or left, long arrows show the nucleolus and short arrows show the nuclear lamina.

From these observations, we can map a vesicle localization sequence between aa1-182, two nuclear localization sequences, one between aa183-367 and the other

between aa368-967, a chromosome binding domain between aa1-367 and two nuclear lamina localization domains, one between aa182-367 and one between aa368-967. The fact that the aa968-1038 localized in nucleus of the salivary gland and not in nucleus of the spermatocytes leads us to suggest that the aa967-1038 peptide does not contain a nuclear localization sequence but may be carried into the nucleus by a binding partner specific to salivary tissue and then retained in the nucleus.

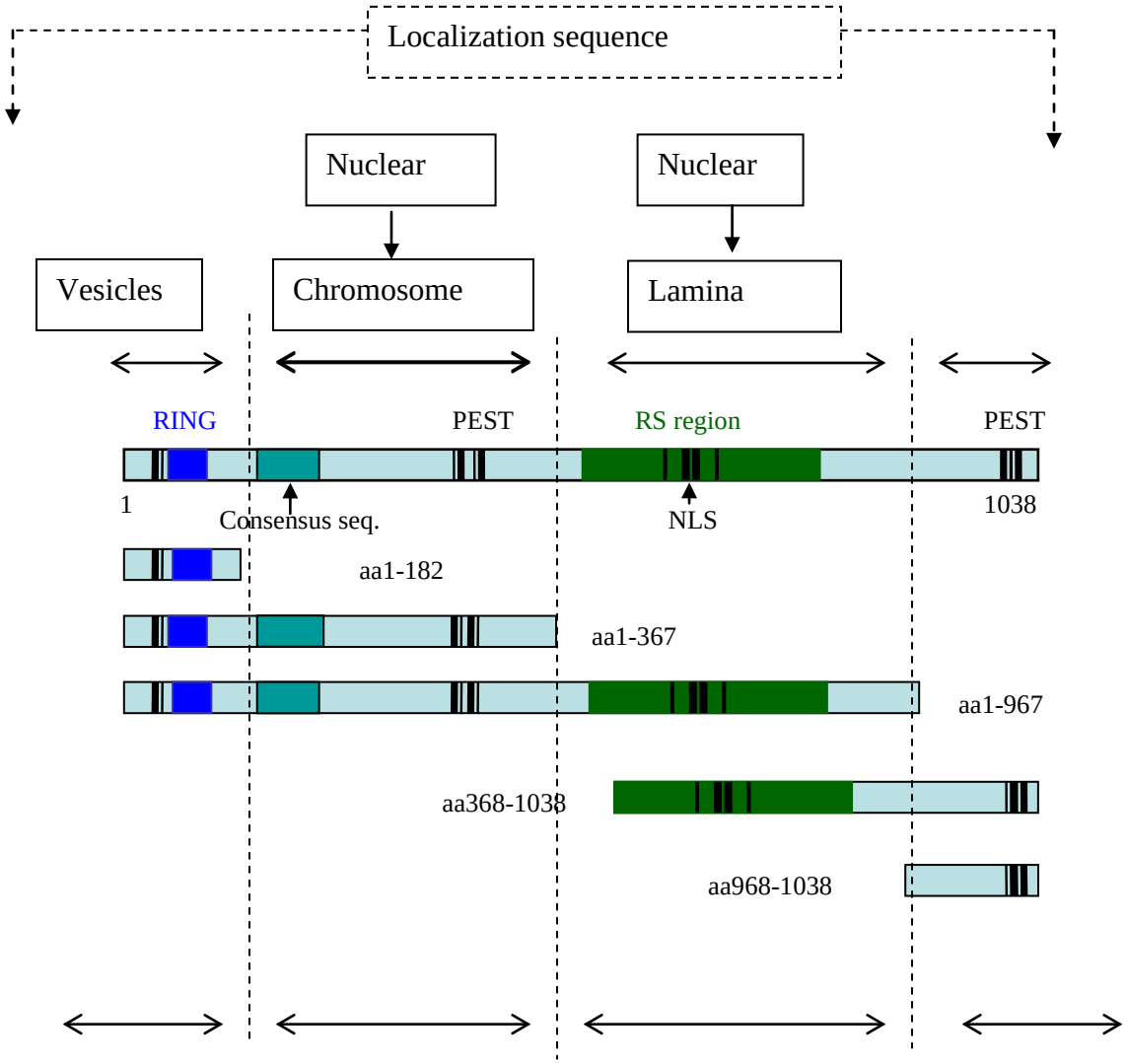


Figure 8. Summary of dTopors localization domain map in salivary gland cells.

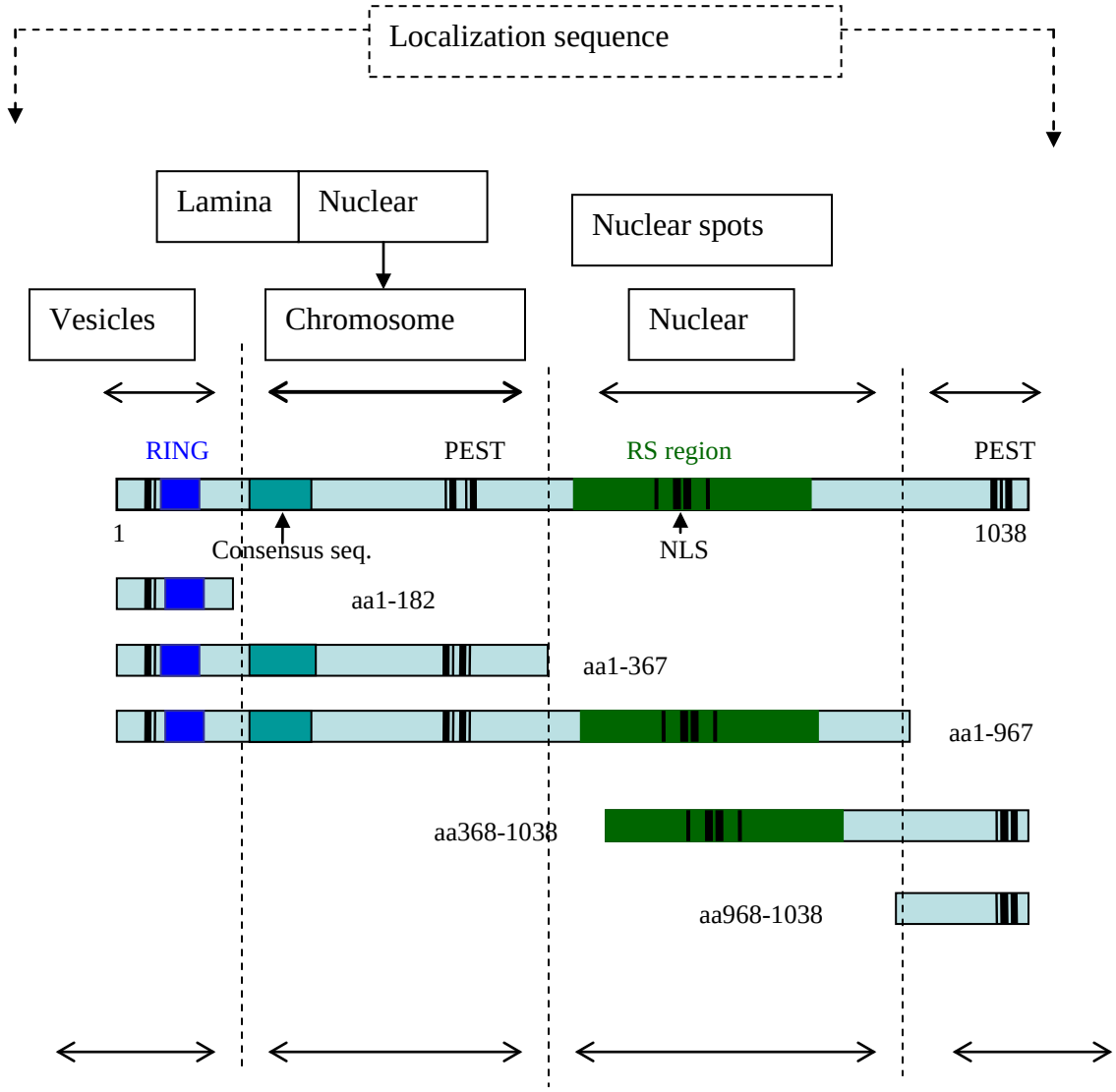


Figure 9. Summary of dTopors localization domain map in spermatocytes.

	DTopors peptides	Full-length aa1-1038	aa1-967	aa1-367	aa1-182	aa368-1038	aa968-1038
Salivary gland cells	Nucleus	+	+	+	-	+	+
	Nucleolus	-	-	-	-	-	-
	Nuclear spots	-	-	-	-	-	-
	Chromosomes	+	-	+	-	-	-
	Lamina	+	+	-	-	+	-
	Vesicles	-	-	-	+	-	-
Spermatocytes	Nucleus	+	+	+	-	+	-
	Nucleolus	+	-	-	-	-	-
	Nuclear spots	-	-	-	-	+	-
	Chromosomes	-	-	+	-	-	-
	Lamina	+	+	+	-	-	-
	Vesicles	-	-	-	+	-	-

Table 4: Summary of dTopors localization domain map in spermatocytes and salivary gland cells.

CHAPTER IV

DISCUSSION

DTopors is a homologue of a human tumor suppressor, Topors. It is a 1038 amino-acid protein that contains a RING finger domain at the N terminus end, five PEST sequences, an RS rich region, four bipartite nuclear localization sequences and a Topors consensus sequence, shared by Topors homologs, adjacent to the RING finger (Secombe and Parkhurst 2004; J.Tomkiel, unpublished). Previous studies have shown that this protein localizes to the nuclear lamina and in nuclear spots (Capelson, Corces ,2005; J.Tomkiel, unpublished). Except the four, almost overlapping, bipartite nuclear localization sequences in the C-terminus half of the dTopors protein identified by sequence homology search (Secombe and Parkhurst 2004), no studies have identified which domains are required to target the dTopors protein to these structures until now. Human Topors structure, however, has been substantially investigated at multiple levels.

Both Haluska et al. (1999) and Zhou et al. (1999) showed that Topors localizes in nuclear spots. Subsequently Rasheed et al. (2002) showed that those nuclear spots were associated with PML bodies and identified the domains required for Topors localization to the PML bodies. They generated Topors-EGFP fusion proteins with EGFP at either the N- or C- terminus, and expressed them in both Hela cells and H1299 lung carcinoma cells to control for tissue-specific localization. The fragments expressed included full-length Topors, aa231-1045, aa539-1045, aa704-1045, aa1-539, aa1-705 and GFP alone.

They observed that, while all the fragments localized in the nucleus, only the full-length protein, aa231-1045 and aa539-1045 fragments localized in distinct nuclear speckles. The aa1-705 fragment localized in microspeckles throughout the nucleus. From these observations, they determined that the aa539-704 and aa704-1045 regions were required for localization in nuclear speckles.

Similarly to Rasheed et al. (2002), we generated different fusion peptides with dTopors fragments fused at the C-terminus to EGFP and generated transgenic flies expressing these fragments. Our confocal microscopy analysis of live cells, both salivary glands and spermatocytes, showed a variety of subcellular localization patterns revealing differences between transgenes, and to some extent, between the two tissue types examined.

In salivary glands, full-length dTopors localized in the nucleus, to the nuclear lamina and to chromosomes as expected based on previous studies. In contrast, the aa1-182 fragment containing the RING finger and a PEST domain was completely excluded from the nucleus and appeared to be in cytoplasmic vesicles, suggesting that it does not contain a nuclear targeting domain. The aa1-367 fragment, containing the RING finger and the Topors consensus sequence, localized exclusively to the chromosomes suggesting that it not only contains a nuclear targeting domain but also a chromosome binding domain. Thus, we can map this nuclear targeting sequence between aa183-367 which corresponds to the Topors consensus domain, the aa1-182 fragment having failed to localize in the nucleus. Further analysis, however, is required to determine if the aa183-367 sequence contains the chromosome binding domain, given that there is a possibility

that the aa1-182 fragment may bind to chromosomes once in the nucleus as some RING finger motifs have shown to bind DNA (Laity, Lee et al. 2001). This could be tested by fusing a known nuclear localization sequence to the 1-182 fragment and asking if it binds chromosomes.

The aa1-967 and aa368-1038 fragments similarly localized to the nucleus and on the nuclear lamina, but not on the chromosomes. This suggests that both fragments contain a lamina localization sequence that could be mapped between aa368-967. It also suggests that another nuclear localization sequence maps between aa368-1038. The fact that the aa1-967 fragment did not localize on chromosomes while the aa1-367 did indicates that localization is not simply determined by the presence of localization sequences. Other conditions, such as protein folding, interdomain competition or post-translational modification may play roles in determining the ultimate localization of the protein.

The C terminal aa968-1038 fragment localized throughout the nucleus but not on nuclear lamina nor on chromosomes. However, this same fragment failed to localize in spermatocyte nuclei. This may mean that the aa967-1038 fragment does not have a canonical nuclear localization sequence, but rather is carried into the nucleus by a binding partner specific to salivary gland tissue, then sequestered there.

In spermatocytes, the localization patterns showed differences to those in salivary gland cells for the full-length, the aa1-367, the aa368-1038 and the aa968-1038 transgenic proteins. Unlike in the salivary gland cells, the full length protein was concentrated at the nucleolus and also showed a significant cytoplasmic signal, the aa1-

367 fragment showed a marked localization to the nuclear lamina, the aa368-1038 fragment showed an interesting localization pattern to nuclear spots and the aa968-1038 fragment was completely excluded from the nucleus. The other fragments, aa1-182 and aa1-967 showed the same localization pattern in both cell types. Similarly to the salivary glands, localization pattern difference between some peptides that otherwise contain some identical sequences were observed with the most striking difference between the aa1-967 and the aa1-367 fragments.

The tissue-dependent localization patterns suggest that there are other conditions, which may be tissue-specific interacting partners and/or tissue-specific functions that direct the cellular localization of these peptides. The salivary glands go through repeated cycles of endoreplication (DNA replication without mitosis) which may be relevant to the difference in the protein localization pattern between the salivary gland and testis. The difference in DNA content may affect localization via competition between binding targets as it is shown with the aa1-367 fragment where the abundance of endoreplicated DNA in the salivary gland (~1000 C) may outcompete its lamina binding. On the other hand, salivary glands are highly transcriptionally active which may indicate the localization of dTopors to the lamina to participate in insulator complexes necessary for transcription regulation (Capelson, Corces; 2005), whereas decreased transcription in spermatocytes might not require this assembly, and dTopors may have different roles in meiosis which could be reflected by the difference in localizations. The nuclear spots seen in spermatocytes, only appear in late prophase (S4-S6) just before division (J.

Tomkiel et al., unpublished) and may indicate that this dTopors localization in nuclear spots may be related to cellular division process.

The observed differences in localization patterns between some peptides that otherwise contain some identical sequences (as shown with dTopors full length, aa1-967 and aa1-367 fragments) can be further explained. We suggest an influential role of other conditions such as protein folding that can eliminate access to a given domain, interdomain competition where one domain have a stronger attraction to its target than other domain, and/or the presence of domain-specific binding partners which may differ from fragment to fragment and may target the fragments to different localizations. Proper protein folding is critical in protein synthesis as it is the basis not only of the protein half-life but also of its function. It is likely that some of the isolated dTopors fragments will fail to fold properly out of the context of the full length protein. Improperly folded domains may fail to attract binding partners which may influence their cellular localization and function. Also, post-translation processes are important in determining the fate of a protein and we believe that they may play a role in the localization and function differences between these fragments. Sumoylation is one of the post translation processes that modifies human Topors (Weger, Hammer et al. 2003) and that has been shown to direct protein subcellular location and function (Matunis, Coutavas et al. 1996; Girdwood, Tatham et al. 2004). It is possible that some of these fragments may be sumoylated differently which can also explain certain differences in localization patterns and function.

Our results are comparable to that of Rasheed et al. (2002) as far as the nuclear localization is concerned. In both studies all fragments localized in the nucleus except the dTopors N terminus aa1-182 fragment. Both the human and fly proteins have a nuclear localization sequence in the amino-terminal half in addition to the bipartite nuclear localization sequence identified in the C-terminus half, at aa616-644 for human topors (Haluska, Saleem et al. 1999; Chu, Kakazu et al. 2001) and after aa600 for dTopors (Secombe and Parkhurst 2004). However finer mapping will be required to determine if this nuclear localization motif is conserved at the amino acid level.

We were also able to see the localization of the aa368-1038 fragment in nuclear spots, only in spermatocytes. This pattern is similar to that revealed by staining fixed spermatocytes with anti-dTopors antibodies (J. Tomkiel unpublished observations) and similar to the localization pattern of the same fragment for human Topors (Rasheed, Saleem et al. 2002). This region corresponds to the RS rich region in both proteins which suggests that the RS rich domain may be involved in targeting dTopors in nuclear spots. These nuclear spots were found to be associated with PML bodies in human (Rasheed, Saleem et al. 2002) and the nuclear structure associated with the dTopors nuclear spots in Drosophila may be comparable to human PML body. These results must be interpreted with caution, however, as none of the other dTopors fragments, including the full length protein, showed this intranuclear spot localization pattern.

From our results, we identified and mapped two nuclear localization sequences, one in the consensus domain, between aa183-367, that was not previously identified and another in RS rich domain, between aa368-967, that correspond to the bipartite nuclear

localization signal previously identified by Secombe and Parkhust (2003). The chromosome-binding domain between aa1-367 that requires a finer mapping as described above. The two nuclear lamina localization domains, one between aa368-967 another between aa1-368, can also be mapped more precisely by expressing fragments contained within these two domains. A domain responsible for targeting dTopors to punctate nuclear spots maps between aa367-967 also corresponding to the RS rich domain.

Our study is a start toward a complete structural and functional analysis of dTopors. For future experiments, we suggest that the aa183-367 and aa368-967 fragments be further investigated to determine whether or not they can localize independently to the chromosome and the nuclear lamina respectively. The first fragment contains the consensus domain with a conserved sequence (aa348-361) and may be required for the chromosome and/or the nuclear lamina binding. The second fragment contains a nuclear localization sequence identified by sequence homology, and it would be interesting to test if this sequence is responsible for nuclear localization. In addition this fragment holds the RS rich domain which may target it to a specific nuclear structure.

For future experiments, it will be important to verify the sizes and stabilities of the expressed proteins by western blot analysis to make sure that the right protein is being investigated.

The transgenic flies generate here will be useful tools to analyze the function of each of the fragments and determine which ones are required for chromosome segregation, centriole duplication, lamina assembly and insulator function. For this, rescue experiments involving transgene expression in *dtopors* mutant flies will be useful

to determine which fragments will rescue the various meiotic and insulator phenotypes. It may also be informative to examine phenotypic consequences of overexpressing these proteins. A cytological analysis of spermatocytes could determine if overexpression of any of these fragments causes nuclear blebs and/or nondisjunction as some of the overexpressed fragments may interfere with the wild type protein and lead to dominant negative phenotypes. These transgene lines will also be useful tools to determine which fragments of dTopors may be sumoylated or otherwise post-translationally modified. In combination with dtopors mutants, it may also be possible to determine if dTopors sumoylates itself.

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