Ph.D. thesis summary

Protein interactions on telomeric retrotransposons in Drosophila

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Introduction

The *proliferation disrupter* gene (*prod*) of the fruit fly encodes a 346-amino-acid DNA-binding chromosomal protein that localizes strongly to the centric heterochromatin of the second and third chromosomes, as well as to >400 euchromatic sites, and to all telomeres. The Prod protein strongly binds to the heterochromatic regions neighbouring the centromeres of the second and third chromosomes, more specifically to a 1.686 g/cm^3 10-bp satellite (ProdSat), where it is indispensable for normal chromosome condensation. Prod cooperatively binds DNA directly on the heterochromatin, with low affinity and sequence specificity. The protein does not have a well-defined DNA-binding domain, 2/3 of the protein is needed for adequate binding. Besides ProdSat, the presence of Prod can be shown by immunostaining at over 400 euchromatic loci, as well as all telomeres. The euchromatic role of Prod is so far unknown. In my work, I primarily investigated the telomeric role of the Prod protein.

Eukaryotic telomeres are nucleoprotein complexes that guard the ends of the linear chromosomes from degradation and fusion, while also balancing the natural shortening of chromosomes resulting from imperfect replication. Instead of telomerase generated repeats, the chromosomal ends of the fruit fly (*Drosophila melanogaster*) contain telomere-specific retrotransposons. The

Drosophila is totally devoid of a telomerase enzyme, instead, the telomere increases in length through the directed transposition to the chromosomal ends and the homologue recombination between three non-LTR retrotransposons (*HeT-A*, *TART*, and *TAHRE*, abbreviated as HTT). Fruit fly telomeres can be divided into three structural domains: the free end of the chromosomal DNA molecule, the retrotransposon repeat (HTT), and the subtelomeric heterochromatin region, the TAS. Although the sequences of the telomeric regions are quite well known, our knowledge about the protein elements are far from complete. The terminal capping complex, consisting of multiple proteins, binds to the chromosomal ends without sequence specificity and prevents the DNA repair mechanisms of the cell from recognizing the ends as a double-stranded break. The HTT domains have a more open, euchromatin-like structure, in which the retroelements align in a head-to-tail orientation, with their oligo(A) tail always towards to the centromere. Depending on the chromosome and the strain of the fruit fly, there can be major differences in the length and composition of the retrotransposon regions, however to a certain degree, the length of the telomeres in the fruit fly are also genetically regulated. The relevance and possible consequences of the various lengths are not fully understood, but it is known that a too long HTT region decreases fertility. The subtelomeric repeat region (TAS), which is situated directly neighbouring the HTT domain in a proximal direction, has a length of 15-26 kb, and consists of

complex repetitive blocks that show slight differences in sequence from chromosome to chromosome. The TAS region has been shown to consist of tightly packed heterochromatin, thus allowing it to regulate the activity of the HTT retrotransposons.

The telomeric domains described above not only differ in their DNA sequences, but also in their interacting chromatin proteins. To understand the mechanisms regulating telomere length, it is necessary to identify all proteins comprising the telomeric chromatin. Identifying the proteins interacting with the HTT region is however difficult, because unlike the telomere fusion phenotype of capping protein mutants or the PEV altering phenotype of TAS-associated protein mutants, the genes encoding for HTT-interacting proteins do not have a well-predictable phenotype, which is a prerequisite for any effective genetic screen. Therefore the best available strategy is the detailed investigation of known chromosomal proteins that show telomeric localization with immunostaining. In this manner, three proteins have been identified, besides Prod, they are Z4 and JIL-1. It has also been shown that although in a small concentration, the capping protein HP1 can be detected with immunoprecipitation in the HTT and TAS domains. While the roles of HP1 and Prod at the HTT region have been partially identified, less is known about Z4 and JIL-1.

Aims

The goal of our work was to identify the role of Prod at euchromatic loci and telomeres. We analysed the precise location of Prod within telomeres using cell biological, molecular biological and biochemical methods. Because Prod, in all likelihood, is found in the telomeric chromatin as part of a complex, we studied the protein-interaction partners of Prod. For this, we used a yeast two-hybrid screen and attempted to validate positive hits using biochemical and immunochemical techniques.

Methods

- Chromosome immunostainings
- UV cross-linking, formaldehyde cross-linking and chromatin purification
- GST-pulldown
- BAC filters and Southern blot hibridization
- RNA- and DNA extraction, cDNA syntesis, qPCR
- Yeast two-hybrid screen
- Immunoprecipitation

Results

The immunofluorescent staining of polytene chromosomes of larval fruit fly salivary glands showed that besides numerous euchromatic loci, the stained Prod proteins can clearly be found in every telomeric region. The Prod staining pattern of the telomeres is identical to the *in situ* hybridization pattern of the *HeT-A* probes, indicating a Prod-*HeT-A* interacting relationship. This by itself does not exclude the possibility of Prod binding to other telomeric sequences. The Prod immunostaining of the *yellow* strain, which has the TAS region deleted from its X chromosome, shows Prod binding to the telomere, even in the absence of TAS. This by itself does not exclude the possibility of Prod being capable of binding to TAS. A strain carrying the dominant *Tel* mutation has an HTT domain almost ten times longer than wild-type, owing mostly to the increased copy number of *HeT-A* retrotransposons. Crossing this line with a wild-type Oregon-R line produces heterozygote telomeres, where the *Tel* homologue with the long HTT domain far overextends the shorter Oregon-R homologue. Immunostaining of Prod shows the protein present on the entire length of the HTT overhang, indicating that Prod binds to the HTT domain. In mutants where in addition to TAS, the terminal retrotransposons are also deleted, the telomeric presence of Prod cannot be shown. Because in such

mutants the terminal cap remains intact, it can be concluded that the Prod protein is not part of the terminal capping complex.

In addition to the cytological evidence, we demonstrated the interaction between the Prod protein and *HeT-A* sequences using chromatin immunoprecipitation. We radiolabeled the UV- and FA-cross-linked embryonic chromatin with ^{32}P and hybridized to a Southern blot that carried the entire *HeT-A* sequence in the form of subclones each with identified positions. Based on this experiment, we were able to conclude that Prod binds directly upstream of the *HeT-A* promoter, suggesting a role for Prod in the regulation of *HeT-A* transcription.

Next, using quantitative PCR (qPCR), we examined whether the *HeT-A* copy number changes in *prod/CyO* flies. From these experiments, we could conclude that 50% decrease in the gene dosage of *prod* does not change the frequency of transpositions. We next wanted to know if the transcription level of *HeT-A* is different in these heterozygotes compared to the wild type. We examined this using quantitative reverse transcription-PCR (qRT-PCR), and found that in the case of *prodk^{08810}/CyO*, the *HeT-A* transcript was 6.5 times the amount found in the Oregon-R control, while in the case of *prodH/CyO*, this transcript was 12.5 times the amount present in the Oregon-R control. Based on these results, we can say that the wild type Prod protein represses the

transcriptional activity of *HeT-A*, but a 50% decrease in its expression does not increase the frequency of transpositions.

Using the yeast two-hybrid assay, with the complete *prod* cDNA acting as the bait, we were able to identify the protein interacting partners of Prod, which included Prod itself, indicating that Prod binds to DNA as a multimer. We were also able to identify the two main members of the sumoylation pathway, indicating that either the Prod protein itself is sumoylated, or that it binds the sumoylation complex for the modification of other Prod interactors. The interaction of Prod with *Z*4 and Chromator is a clear indicator of Prod's telomeric role. Z4 is one of the previously identified proteins that bind to the HTT domain of telomeres, while Chromator was identified through its interaction with Z4, and is known to be present at the X and 2L telomeres.

Using co-immunoprecipitation done in S2 cells, we were able to show that Chromator binds to Prod *in vivo*. Based on the known interaction between Chromator and Z4, we can surmise a common protein complex with all three proteins as members, at least at those positions on the chromosome where the three proteins colocalize.

We performed immunofluorescent staining on *Tel*/Oregon-R hybrid polytene chromosomes and found that Z4 binds to the HTT region, and that Chromator is present at all telomeres. Further staining showed that within telomeres, both proteins bind to the HTT region, from which we can conclude

that the HTT domain can be the primary target of the potential Z4/Chromator/Prod protein complex.

Based on the yeast two-hybrid assay results, Prod interacts with the two most important members of the sumoylation enzyme cascade, the Uba2 and lwr proteins. Because Smt3 is the only SUMO homologue found in the fruit fly, sumoylated proteins on the polytene chromosomes can be identified using an anti-Smt3 antibody staining. Using this technique, we investigated the presence and distribution of sumoylation at telomeric sites. The telomeric localization of Smt3 indicates that the HTT region is strongly sumoylated. The sumoylation pattern of telomeres coincides with Prod localization, that is Smt3 can be found throughout the HTT region, but it does not bind to the TAS region or to the telomeric cap.

The chromatin state of the HTT domain can influence telomere length through the regulation of retrotransposition and gene conversion. In heterozygote mutants, the protein concentration is diminished by 50%, which can lead to a mutant phenotype if the phenotype is sensitive to gene dosage. In this case, in strains heterozygotic for telomeric proteins, the length of the telomeres is modified. To investigate this further, we used quantitative PCR to measure the *HeT-A* content of the heterozygote mutants and compared to the wild type. We found that a decreased level of Prod and the Chromator null allele slightly increase telomere length compared to the wild-type, while a decrease in

the level of Z4 and SUMO do not have effects on telomere length. Thus the changes in chromatin structure or sumoylation of HTT proteins is not sufficient to increase either transposition frequency or gene conversion at telomeres.

Based on prior knowledge and our own experiments, we were able to construct a working model of the telomeric HTT domains of *Drosophila*. The central mediator of the protein interaction at the HTT domain is likely to be the Chromator protein, as it directly binds to the Prod, JIL-1, and Z4 proteins. Z4 is proposed to be a part of the DREF complex, while Prod, in multimer form, specifically binds to the region directly upstream of the *HeT-A* promoter. Prod likely recruits the sumoylation complex, therefore it is able to modify the binding properties of the other members of the complex. The sumoylated Eggless protein methylates the lysine in the 9^{th} position of the H3 histone, which is then recognized by the HP1 protein, allowing it to bind to this site, ultimately leading to the inhibition of transcription.

List of publications

Török, T., Benitez, C., Takács, S., és Biessmann, H. (2007). **The protein encoded by the gene proliferation disrupter (prod) is associated with the telomeric retrotransposon array in Drosophila melanogaster.** Chromosoma ***116***, 185-195. IF: 4.337

Takács, S., Biessmann, H., Reddy, H.M., Mason, J.M., Török, T. (2012) **Protein interactions on telomeric retrotransposons in Drosophila.** Int J Biol Sci **8(7)**:1055-61. IF: 3.168