Role of sumoylation in germ cell development of Drosophila (PD 124446)

The aim of the work

In the course of a large scale RNAi screen, we have identified the SUMO-ligase Su(var)2- 10 gene as a key regulator of gonadal development in *Drosophila melanogaster*. Our purpose was to reveal the cell biological and molecular functions of Su(var)2-10 in germ cell development.

Results

1. *In the first year we aimed to determine which cell types and which stages of oogenesis are affected by the Su(var)2-10 gene.*

We applied loss-of-function experiments using the GAL4>UASshRNA system combined with the temperature-sensitive GAL80 which enables a precise spatial and temporal control of RNAi. Furthermore, we utilized FLP/FRT-based mitotic recombination to generate Su(var) 2-10 mutant mitotic clones in the germarium. The loss-of-function phenotypes was followed by immunostaining of specific markers and was analysed by confocal laser scanning microscope.
First, we depleted the *Su(var)2-10* mRNA in the germline during the early developmental stages. This resulted in a highly rudimentary adult ovary. Immunostainings revealed that the germarium does not contain germline stem cells (GSCs) and differentiating germ cells. Next, we turned to apply clone analysis experiments which allowed to examine the effect of a lethal Su(var)2-10 mutation in an intact ovary. Su(var)2-10 mutant clones were generated at the adult stage and the ovary of the flies was analysed one and two weeks after clone induction. According to our results, the percentage of the Su(var)2-10 mutant germline stem cell clones was significantly lower compared to the percentage of wild type clones. Contrarily, percentage of the Su(var)2-10 mutant cytocysts was comparable to the wild type. These results indicate that the Su(var)2-10 is required for the initial stage of germline development and it is essential for the stem cell maintenance but it is dispensable for differentiation of cystocytes.
To further investigate the role of the Su(var)2-10 in the germarium, we depleted its mRNA in the developing cytocysts in the meiotic region. In this case, the oogenesis process appeared normal, however we observed increased accumulation of DNA double strand breaks in the Su(var)2-10 depleted ovary. This result indicates that the Su(var)2-10 is essential for the repair process during the meiotic recombination.
Silencing of the Su(var)2-10 mRNA after the germarium stage, in the developing egg chambers did not influence the oogenesis and the flies were fertile, indicating that the Su(var)2-10 is not essential for the later stages.
Next, we aimed to determine the role of the Su(var)2-10 gene in the development of somatic cells of the ovary. Su(var)2-10 mRNA was depleted in a cell-type-specific manner in the niche and follicle cells.
Our results revealed that depletion of Su(var)2-10 does not affect the proper development of somatic niche cells. Germline stem cells also developed properly, as we could detect GSCs with spherical-shaped spectrosome and differentiating CBs with elongated fusomes as well. These results indicate that Su(var)2-10 is not required for the development of somatic niche cells and soma-to-germline signalling.
Contrarily, depletion of Su(var)2-10 in follicle cells resulted in abnormal germaria. Although the niche cells and the differentiating germline cells appeared normal, the cysts failed to be encapsulated by follicle cells. Immunostaining with follicle cell marker (FasIII) indicated that follicle cells are abnormally localized and their cell number drastically decreased. For further analysis, we generated homozygous Su(var)2-10 mutant clones of the lethal Su(var)2-10 allele

in the follicle cells, using FLP/FRT-based mitotic recombination. The ovary of the flies was analyzed one and two weeks after clone induction, and differentiation of the follicle cells was followed by FasIII. According to our results, the percentage of the mutant follicle clones was much lower compared to the wild type. In addition, the size of the mutant clones was also smaller compared to the wild type clones. These results indicate that the Su(var)2-10 is essential for the proper development and viability of follicle cells.

2. In the second year we characterized the Su(var)2-10 isoforms and we identified PIWI as a direct binding partner of the Su(var)2-10 protein.

According to the Flybase database, the Su(var)2-10 gene possess 14 annotated transcripts which encode 14 unique polypeptides. To determine which splice variants are expressed in the ovary, we applied RT-PCR with transcript specific primers. The PCR products with the expected size were sequenced. With the exception of the RG and RK variant, we were able to identify all other transcripts. Moreover, we find a novel transcript which is not predicted by the Flybase.
To explore the potential functions of these isoforms, we performed in silico analysis using InterPro and GPS-SUMO programs. According to this analysis, the shared region of the isoforms possesses SAP, PINIT and SP-RING domains, which are characteristics of the PIAS protein family. This suggests that each isoform has SUMO-ligase activity and DNA-binding ability. However, the isoforms differ in the number of the predicted SIM motifs: SIM1 and SIM2 can be found in all isoforms, while the SIM3 motif is localized exclusively at the last exon of the PJ and PL variants. This suggest that the PJ and PL variants have additional functions compared to the other isoforms. Therefore, we carried out protein binding assays to validate the SUMO-binding activity of the potential SIM motifs. To achieve this, I generated full length GST-Su(var)2-10-PJ isoform and I introduced mutations in each SIM motif individually and in combination. GST pull-down experiments with recombinant His-SUMO protein revealed that elimination of the SIM motifs individually decreases the SUMO binding activity of Su(var)2-10-PJ protein. Simultaneous mutations of all three motifs resulted in a more prominent loss of SUMO binding. These results indicate that the predicted SIM motifs are functional.
In the further studies, we aimed to identify Su(var)2-10 protein targets in the ovary. For this purpose, we achieved mass spectrometry analysis, which revealed more AGO proteins (AGO2, Aubergine, PIWI) as a possible binding partner of the Su(var)2-10 protein. Sahoo MR et al. described that the AGO family proteins have SUMO-like domains with which they are able to directly bind to the SIM motifs of various SUMO-binding proteins. In addition, it is also known that the Su(var)2-10 have a role in piRNA-mediated transposon silencing in the Drosophila ovary (Czech B et al, 2013; Ninova M et al, 2019). Based on these observations, I hypothesized that the Su(var)2-10 may able to directly bind to the PIWI protein through its SUMO-like domain. To test this hypothesis, I performed protein binding assays. As the PIWI protein was poorly produced by E.coli, it was expressed in a wheat germ in vitro translation system. The GST pull-down experiment revealed binding between the GST-Su(var)2-10-PJ and the V5-PIWI protein. As the wheat germ translation system hasn't got sumoylation activity, indeed the molecular weight of the translated PIWI protein was equal to the calculated non-sumoylated molecular weight, it is suggested that the Su(var)2-10 directly binds to the PIWI protein.
As the PIAS proteins are able to take part in the target recognition, it is possible, that the PIWI protein is sumoylated by the Su(var)2-10 in vivo. To test whether the PIWI protein can be a sumoylation target, we applied in vitro sumoylation assay. According to the result, the PIWI protein is not sumoylated in this assay. Furthermore, the molecular weight of the V5-PIWI produced by fly and the wheat germ system was identical. This finding also indicates that the PIWI protein is not post-translationaly modified by a SUMO moiety.

3. *In the third year we performed detailed characterization of the PIWI and Su(var)2-10 interaction.*

To gain further insights into the recognition of Piwi by Su(var)2-10, we applied GST pull-down experiments with the truncated forms of the proteins. According to our results, the CTD domain of the Su(var)2-10 is essential and sufficient for the PIWI binding. The CTD domain of PIAS proteins is located around the SP-RING domain and it take part in the donor SUMO binding through SIM-like motifs. Our results indicate, that the CTD domain of the Drosophila Su(var)2-10 possesses weak SUMO binding ability. We performed mutagenesis assays for detailed analysis of the CTD domain. Mutation in the SIM-like motif did not affect the expression of the CTD domain, however it abolished both SUMO and PIWI binding. This result supposes a SUMO-SIM-like interaction between the Su(var)2-10 and PIWI protein. However, the canonical SIM motifs in the C-terminal region did not take part in the PIWI binding.
To analyse the effect of the CTD mutation in vivo, we generated transgenic *Drosophila* lines, expressing the wild type and mutant form of the Su(var)2-10. As we expected, the wild type protein was localized in the nucleus, however the mutant Su(var)2-10 was enriched in the cytoplasm. As the mutation did not affect NLS, it is possible that, the PIWI or other target binding is responsible for the nuclear localization of the Su(var)2-10 protein. To answer this question, we plan further experiments.
Next, we performed GST pull-down assays to identify the Su(var)2-10 interaction site on the PIWI protein. For this experiment, the four domain of the PIWI protein (N, PAZ, MID, PIWI) was expressed in a wheat germ extract. The results revealed, that the Su(var)2-10 interacts PAZ, MID and PIWI domain. As the MID domain produced the most prominent binding, it was chosen for further investigations. The analysis of the protein structure of the MID domain revealed that it possesses a potential SUMO-like motif. To check whether the Su(var)2-10 interaction is mediated by this motif, mutagenesis assays will be performed.

In summary, our study revealed, that the Su(var)2-10 gene is essential for the maintenance of the germline and follicle stem cells of the Drosophila ovary. We have proved, that the Su(var)2-10 directly binds to the PIWI protein through its CTD domain. This binding is required for the localization of the Su(var)2-10 to the site of co-transcriptional silencing of transposons. The possible role of Su(var)2-10 is the sumoylation of proteins taking part in the heterochromatinization. Furthermore, the multi-SIM domain at the C-terminal region of Su(var)2-10 may provide additional regulation of the sumoylated proteins and stabilize the silencing complex.
Our results revealed for the first time that the CTD domain of a PIAS protein is involved in the target recognition and this domain is responsible for the nuclear localization of the protein as well. As the domain composition of PIWI protein family and PIAS proteins are evolutionally conserved, the structural basis of PIWI and PIAS interaction may be common in eukaryotes. Therefore, we aim to describe this interaction in detail and to identify the key motif on the PIWI protein responsible for Su(var)2-10 binding. After these experiments, we will publish our study in an international journal.