

**Identification and functional analysis of novel
genes involved in *Drosophila* germ cell
development**

Summary of the Ph.D. thesis

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Introduction

The fruit fly (*Drosophila melanogaster*) is an extensively used model organism for the research of germ cell development. The establishment of the germ line takes place in a very early stage of embryogenesis. The formation of primordial germ cells (PGCs) at the posterior pole of the early embryo is governed by maternal factors such as mRNAs and proteins. During gastrulation, PGCs attach to the underlying midgut anlage and are passively transported into the midgut primordium, then they pass through the midgut wall. Subsequently, PGCs migrate in the body cavity governed by chemotactic signals and coalesce with the mesodermal somatic gonadal precursor cells (SGPs) to form a pair of compact embryonic testis or ovaries. The development of gonads and the differentiation of the germ line continues during larval and pupal life. In the adult gonads, germ line stem cells (GSCs) divide and differentiate to generate mature eggs and sperms.

PGCs are zygotically quiescent until gastrulation when the zygotic transcription is progressively activated. PGCs require both maternally provided and zygotic factors for their further development. RNA-induced gene silencing (RNAi) is an ideal method to investigate *Drosophila* germ line development because both maternally provided and zygotic mRNAs can be inactivated. Using this approach, the loss-of-function phenotypes of germ line-specific genes can be examined in the embryos.

Research objectives

- The intensive development of biological databases enables us to collect genes that are specifically expressed in the *Drosophila* embryonic germline. The aim of our study was to perform an RNAi-based reverse genetic screen to reveal the role of these genes in germline development.
- We aimed to validate the genes identified in the RNAi screen in a sensitized genetic background to confirm their involvement in germ cell development.
- Furthermore, we intended to analyze the evolutionary conservation and biological functions of the genes identified in the RNAi screen.
- To examine whether our candidate genes act in a germline- or soma-specific manner, we intended to silence them using germline-specific gene silencing methods.
- In this work, we also examined the degree of functional redundancy among similar proteins in the germline.
- We also aimed at giving a detailed genetic analysis of a few promising candidate genes.

Methods

- Identification of genes expressed in the embryonic germ line based on gene expression databases and an EST-collection from embryonic gonads.
- Design and synthesis of gene silencing double-stranded RNAs (dsRNAs) by *in vitro* transcription.
- Silencing germ line-specific genes via the microinjection of dsRNA into embryos that have germ cells expressing fluorescent protein (nos-Moe-GFP).
- *In vivo* phenotypic analysis using fluorescent time-lapse microscopy on injected embryos. Categorization of abnormal PGC phenotypes.
- Raising the examined embryos to adulthood and assaying their adult gonads for phenotypic defects.
- Testing mutant alleles of the identified genes in a sensitized genetic background to confirm their involvement in germ cell development.
- Evolutionary conservation and gene ontology analysis of candidate genes with the help of FlyBase and Ensemble databases.
- Determination of the functional redundancy of the germ line transcriptome by systematically co-silencing germ line-specific genes that encode paralogs or similar proteins.
- Germ line-specific silencing of the novel identified genes to test their germ cell-dependent contribution to germ cell development. Analysis of the abnormal phenotypes via immunostaining and confocal microscopy.
- A detailed genetic analysis of the selected genes (RNAi, mutant analysis, immunostaining, confocal microscopy)

Results

We screened for genes expressed in the embryonic germ line or gonads in publicly available databases and a gonad EST-collection. Using this approach, 502 genes were selected, and their germline-dependent functions were analyzed by microinjecting gene-specific dsRNAs into syncytial embryos expressing a fluorescent protein in their germ line. Fluorescently marked germ cells were monitored until the end of embryonic development using fluorescent time-lapse microscopy. Recorded movies were analyzed by visual inspection; the RNAi phenotypes were categorized and their penetrances were determined. As a secondary screen, the examined embryos were raised to adulthood, and their adult gonads were assayed for phenotypic defects. In this way, 48 genes displaying reproducible abnormal germ cell phenotypes at various stages of development were identified.

To confirm the involvement of the newly identified genes in germ cell development, their loss-of-function alleles were tested for dominant genetic interactions with a sensitized genetic background. The sensitized line used in these genetic tests carries loss-of-function alleles of three genes known to be involved in germ line establishment leading to a germ cell-deficient phenotype with low penetrance. We demonstrated that more than 70% of the genes we analyzed significantly enhanced the penetrance of the germ cell-less phenotype of the sensitized genetic background.

Analyzing the evolutionary conservation of our candidates, we found that 72% of the genes have orthologs in vertebrates, and several of them have

been previously annotated with a function in gamete generation. One of the most important results of our work is the identification of 29 – mostly evolutionarily conserved - genes that have not been implicated in germ cell development yet.

In our experiments, when silenced, one tenth of the genes examined caused abnormal phenotypes in germline development in a reproducible way. This low rate of genes displaying RNAi phenotypes could be explained by a functional redundancy between similar proteins. In other organisms, paralogous proteins and proteins with similar domain structures have been demonstrated to substitute for each other thus ensuring the robustness of development. To determine the functional redundancy among similar proteins in the embryonic germline of *Drosophila*, we performed a large-scale combinatorial gene silencing experiment. We co-silenced the gene-pairs that are paralogs or encode proteins with similar amino acid sequences or domains. Only two genes (*trio* and *Gap1*) have been identified as showing genetic interaction with each other. Based on our results, we suggest that the redundancy of the germ line transcriptome is low.

In our RNAi experimental setup, we cannot discriminate between the PGC-specific and the somatic effects of the silenced genes. To examine whether our candidate genes act in a germ line- or soma-specific manner, we silenced them by expressing gene-specific short hairpin RNAs (shRNAs) in the germ line. We demonstrated that the majority of the genes silenced act in a germ cell-specific manner. The effect of the germline-specific expression of transgenic gene silencing shRNAs can be studied primarily in larval and

adult gonads, therefore, this technique supplements well the gene silencing experiments performed in the embryo.

The analysis of the role of pleiotropic genes in germline development is rather difficult, but the RNAi technique provides an ideal tool since it enables the generation of a series of phenotypes of different strength. Using this approach, we were able to detect genes essential for germline development. One of our identified essential genes is the RhoGEF protein encoding *pebble* (*pbl*). Silencing of *pbl* results in the decrease of PGC number, the loss of PGCs during migration, the abnormal compaction of the embryonic gonads and, as a result of these, the absence of gonads. These phenotypes were verified using loss-of-function *pbl* alleles as well. We also demonstrate that it is not only the PGCs but also the somatic gonadal precursor cells (SGPs) that are in abnormal position in the *pbl*-silenced embryo. These results suggest that the *pbl* phenotypes are caused, at least partially, by defects in somatic cells. Germline-specific silencing of *pbl* revealed its germline-dependent function in germ cell maintenance. The silencing of another essential gene, *fascetto* (*feo*), did not lead to abnormal phenotypes in embryonic stages, but in the larval gonads, the PGCs were abnormally large and low in number. This dsRNA-induced phenotype was verified with a loss-of-function allele and germline-specific silencing as well. When we examined Feo protein expression in larval germ cells, we found that the protein localizes to the midzone of the mitotic spindles. Based on these results, we suggest that Feo regulates the mitotic division of larval germ cells.

The *mei-P26* gene encodes a zinc ion binding, TRIM-NHL domain-containing protein. Mei-P26 has been shown to be an essential regulator of germline development in adult *Drosophila*: it has a critical function in germline cyst differentiation, and it is also required for germline stem cell (GSC) self-renewal during oogenesis. We found that *mei-P26* plays a role in germ cell development in earlier developmental stages as well. In Mei-P26-deficient embryos, the PGC number progressively decreases, and migrating PGCs express the CycB protein which is a marker of dividing somatic cells in this developmental stage. This result suggests that *mei-P26* plays a role in the suppression of somatic cell fate in PGCs. We also demonstrated the requirement of *mei-P26* in the larval germ line, where it participates in PGC maintenance.

Sumoylation is a conserved posttranslational modification mechanism to alter the activity, localization, or stability of target proteins. In our RNAi screen, we identified the SUMO-activator subunit, *Aos1*, and the SUMO-ligase, *Suppressor of variegation 2-10* (*Su(var)2-10*). Silencing of both genes induced the formation of vestigial adult gonads. To reveal the role of sumoylation in germline development, we performed germline-specific silencing of these genes and also that of *Uba2*, which encodes the other subunit of the SUMO-activator complex, and *smt3*, which encodes the SUMO protein. We found that sumoylation is essential for GSC maintenance in both sexes and for the differentiation of the germline in female gonads.

In summary, our screening strategy enabled the identification of 48 genes that play a role in germ cell development. We found pebble and

fascetto to be essential for germ cell migration and germ cell division, respectively. Our data reveal a previously unanticipated role of *mei-P26* in the maintenance of embryonic germ cell fate. We also demonstrated the requirement of sumoylation in GSC maintenance and differentiation. We performed systematic co-RNAi experiments in which we found a low rate of functional redundancy among homologous gene pairs. As our data indicate a high degree of evolutionary conservation in the genetic regulation of germ cell development, they are likely to provide valuable insights into the biology of the germ line in general.

Publications

Publication used in the Thesis:

Jankovics Ferenc, Henn László, Bujna Ágnes, Vilmos Péter, Spirohn Kerstin, Boutros Michael, Erdélyi Miklós (2014) Functional analysis of the Drosophila embryonic germ cell transcriptome by RNA interference. PLoS One 9: e98579.

IF: 3,730

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IF: 0,447

Total IF: 24,809