Identification and analysis of novel target genes of the TRA-1/Ci/GLI transcription factor in *Caenorhabditis elegans*

PhD Thesis

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Introduction and aims

Members of the Glioblastoma-associated (GLI) protein family act as the terminal transcription factors of the Hedgehog (Hh) signaling pathway, which control several key developmental processes in both invertebrates and vertebrates. In humans, dysregulated Hh signaling has been implicated in cancer, skeletal malformation and defective neuronal patterning.

The *C. elegans* genome encodes a single GLI ortholog, the TRA-1 transcription factor, which is the terminal regulator of the nematode sex determination pathway. Until now, only a few direct targets of TRA-1 have been identified. These target genes, all of which are repressed in XX hermaphrodite animals, determine different aspects of the sexual fate determination within a single cell or a small group of cells.

The somatic sex determination directs all of the morphological differences between the sexes in an organism. In mammals, flies, and worms, sex is determined by different mechanisms that also cause males and females to differ in the levels of X-linked gene expression. The sex-specific process which ensures the same amount of X-linked gene products in the different species is known as dosage compensation. The worm dosage compensation is controlled by the same developmental signal that establishes sex, the ratio of X chromosomes to sets of autosomes (X:A signal). Following a common step of regulation, somatic sex determination and dosage compensation are controlled by distinct genetic pathways

As studies of *tra-1* in nematodes may shed light on the role of Hedgehog signaling in human development and disease, the aim of my PhD work was to determine and analyze new transcriptional targets of the TRA-1/GLI transcription factor in order to understand better how TRA-1 controls somatic fates during development.

Materials and Methods

In silico binding site predictions

The availability of sequence information of TRA-1 binding sites in the regulatory regions of known target genes prompted me to deduce a consensus binding site from the nucleotides conserved within these sites. I then searched the *C. elegans* genome for consensus TRA-1A binding sites, considering the highly conserved nucleotides at their appropriate positions. I investigated the conservation of the predicted binding sites by comparing the regulatory regions of the orthologous genes in two closely related *Caenorhabditis* species, *C. briggsae* and *C. remanei*.

In vitro DNA binding assay

TRA-1A protein was generated by in vitro transcription and translation of full-length *tra-1* cDNA. I performed electrophoretic mobility shift assays (EMSA or bandshift experiment) to investigate whether TRA-1A is able to bind to the wild-type and mutated, putative binding sequences. As positive controls, I used the published target sites from the regulatory region of *mab-3* and *egl-1* genes.

RNAi

In those cases where I could not use mutant alleles for depleting gene function, double-stranded RNA-mediated gene silencing was used. Worms were fed with bacteria expressing double-stranded RNA against given *C. elegans* genes.

In vivo gene expression studies

I used green fluorescent protein-tagged transcriptional (promoter only) or translational (the whole length protein product fused with GFP) reporter transgenes to monitor changes in gene expression in response to decreased or elevated *tra-1* activity.

Results

Herein I summarize the results of my PhD work:

I;

- By comparing the proposed binding sites of TRA-1A targets identified previously, I have determined a new consensus binding site for the TRA-1A transcription factor: $TT^{A}/_{T}TCNNNNTG^{G}/_{T}G^{T}/_{A}GGTC$
- I have searched the *C. elegans* genome for putative consensus TRA-1A binding sites, and identified 21 novel evolutionally conserved target sites within upstream regulatory or intronic sequences.

II;

- One of these sites is located within the *xol-1* promoter, 154 bp upstream of the ATG translational initiation site. This consensus site, which is almost identical to that found in the *mab-3* regulatory region, is highly conserved in *Caenorhabditis* species.
- Using gel electrophoretic mobility shift assay, I have detected efficient binding of TRA-1A to this element. By contrast, TRA-1A was not able to bind to the oligonucleotide when the putative TRA-1A binding site was mutated in four critical positions. In addition, unlabeled wild-type, but not mutant, *xol-1* oligonucleotides were able to compete with the labeled oligonucleotides in a concentration-dependent manner.
- To monitor *xol-1* expression, I analyzed the expression of an integrated *xol-1::gfp* transcriptional fusion reporter. The *xol-1::gfp* was ectopically active in *tra-1* loss-of-function mutant embryos with XX karyotype (note that *xol-1* is normally active only in XO embryos). *tra-1* RNAi treatment strongly phenocopied this effect.
- I have also *gener*ated worms carrying extrachromosomal arrays of a modified form *of xol-1::gfp* reporter [*Ex*(pmut*xol-1::gfp)],* in which the putative TRA-1A binding site is mutated in several conserved positions. This mutant reporter was also ectopically expressed in XX em*bryos.*
- Inactivation *of tra-1* enhanced, whereas hyperactivation of *tra-1* suppressed, lethality in animals with elevated *xol-1* activity in *sex-1* and *dpy-21* loss-of-function mutants.

- The lethality of *tra-1* mutant embryos is XX specific and depends on *xol-1* activity.

III;

- The *in silico* analysis predicted *a* putative TRA-1A binding site in the regulatory region o*f lin-39* too, 1 kilobase upstream of the translational initiation site. This TRA-1A target sequence is highly conserved between *C. elegans and C. briggsae.*
- By using gel mobility shift assays, I have showed that *in vitro* translated full-length TRA-1A is able to bind to the wild-type, but not the mutant, putative TRA-1 binding site.
- A functional *lin-39::gfp* reporter, which normally accumulates at basal level in the vulval precursor cells [P(3-8).p], became excessively activated in these cells in *tra-1(RNAi)* genetic background. These results suggest that TRA-1A might repress *lin-39* expression in the VPCs before and at the time of vulval induction.
- Analyzing the phenotype caused by a hypomorph *tra-1* allele, *e1488,* and the phenocopy of various *tra-1* RNAi treatments indicate that *tra-1* influences vulval development in this organism (note that vulva is a typical sex-specific organ in *C. elegans*).
- I have found that alteration of *tra-1* activity interferes with the mutant vulva phenotype of *lin-12/Notch* gain-of-function mutant animals. *lin-12(gf)* mutant animals display a Multivulval pohenotype that is due to ectopic adoptions of 2° (secondary) vulval cell type by all VPCs. While inactivation of *tra-1* increased the expressivity of the Muv phenotype in *lin-12(gf)* animals, hyperactivtion of *tra-1* suppressed vulval induction in this genetic background.
- I have also found that TRA-1 accumulates in and regulates the fusion process of cells involved in vulval patterning.
- Inactivation of *tra-1* promoted vulval induction in *synMuv AB* (for *synthetic Multivulva Class AB*) double mutant animals as well.
- Loss of *tra-1* function caused ectopic vulval induction in *synMuv A*, but not in *synMuv B*, mutant background. This implies that *tra-1* acts as a *synMuv B* gene.

Conclusions

TRA-1A is able to bind to the regulatory region of *xol-1 in vitro* and inhibit the expression of *xol-1*in XX animals *in vivo*. Inactivation of *tra-1* enhances, whereas hyperactivation of *tra-1* suppresses, lethality in animals with elevated *xol-1* activity. Thus, *tra-1* is the first identified autosomal gene that inhibits *xol-1*. By repressing *xol-1* in XX animals, *tra-1* contributes to the maintenance of X-chromosome repression during dosage compensation. These data imply the existence of a regulatory feedback loop within the *C. elegans* sex-determination and dosage compensation gene cascade that ensures the accurate dose of X-linked genes in cells destined to adopt hermaphrodite fate. Our results might help to better understand the molecular mechanism underlying X-linked gene dosage equalization between hermaphrodites and males.

TRA-1A also binds to the regulatory region of the *Hox* gene *lin-39*, a central regulator of vulval development, and regulates its expression in the vulval precursor cells (VPCs) prior to and during vulval induction. Furthermore, *tra-1* interacts with the class B *synMuv* genes, many of which are known to be involved in chromatin-mediated transcriptional repression of cell proliferation, to specify vulval fates. *tra-1* also influences the fusion pattern of VPCs with the hypodermis and the fusion of seam cells with each other. The regulation of cell fusion is a newly identified function for GLI-like proteins. Furthermore, signaling via *tra-1* is a novel pathway that specifies cell fates during *C. elegans* vulval development.

In humans, dysregulation of Hh signaling has been implicated in cancer, skeletal malformation and defective neuronal patterning. Our results presented here may help to better understand how compromised GLI activity leads to the development of such diseases.

Publications related to the PhD thesis

15. January 2010.

Szabó E*, Hargitai B*, Regős á, Tihanyi B, Barna J, Borsos É, Takács-Vellai K, Vellai T. TRA-1/GLI controls the expression of the *Hox* gene *lin-39* during *C. elegans* vulval development. *Dev Biol*. 2009 Jun 15;330(2):339-48. (*contributed equally to the work)

Hargitai B, Kutnyánszky V, Blauwkamp TA, Steták A, Csankovszki G, Takács-Vellai K, Vellai T. *xol-1*, the master sex-switch gene in *C. elegans*, is a transcriptional target of the terminal sex-determining factor TRA-1. *Development*. 2009 Dec 11;136(23):3881-7.