Role of TRA-1A/Gli transcription factor in the regulation of the *Hox* gene *lin-39/HoxD4* during *Caenorhabditis elegans* vulval development

PhD Thesis

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Introduction and aims

Identification of TRA-1A/Gli target genes in *Caenorhabditis elegans* vulval development

The Hedgehog-Glioma-associated (Hh-Gli) signaling pathway plays a crucial role in controlling embryonic patterning, cell growth, proliferation and cell fate determination during animal development. In adults, Hh-Gli signaling is involved in tissue maintenance and repair, as well as in stem cell behaviour in several instances, regulating organ homeostasis. Compromised Hh-Gli signaling has been implicated in a surprisingly unrelated and large number of human tumors, including among others glioblastoma multiforme (GBM), medulloblastoma, basal cell carcinoma (BCC), rhabdomyosarcoma, cancers of lungs, prostate, breast and pancreas.

Because most pathways are conserved across animal phylogeny, the molecular genetic study of human developmental pathways relies, in part, on the study obtained in model organisms. In nematode *Caenorhabditis elegans*, the Hh signaling pathway has undergone considerable divergence during evolution. As a result, obvious Hh receptor orthologs are absent whereas a large family of nematode Hh-related proteins are present. TRA-1A (sexual transformer) is the sole nematode representative of the Gli transcription factor family. The Gli-like transcription factor TRA-1A is the terminal regulator of the nematode sex determination cascade. In wild-type hermaphrodites, TRA-1A promotes female development in somatic tissues and abundant spermatogenesis in the germ line. Despite its known functions in all aspects of somatic sexual differentiation in this organism, there is no evidence to attest the obvious role of TRA-1 in vulval development. The vulva is the single hermaphrodite-specific organ, which is an elegant model for dissecting a gene regulatory network that directs postembryonic organogenesis.

The vulva of *Caenorhabditis elegans* hermaphrodites develops from subset [P(5, 6, 7).p] of six vulval precursor cells (VPCs) by the combined effect of RTK/Ras/MAPK, Wnt and LIN-12/Notch signaling, and of three redundant synMuv (synthetic Multivulva) pathways grouped into classes A, B and C. The P6.p cell generates eight descendants that form the center of the developing vulva, a lineage designated the primary (1°) cell fate, and the adjacent VPCs (P5.p and P7.p) generate seven descendants that form the outer part of the developing vulva, a lineage designated the secondary (2°) cell fate.

Using a combination of genetic and biochemical approaches (such as mutant and epistasis analysis, reverse genetics (RNA interference, RNAi), bioinformatics, electrophoretic mobility shift assay and gene expression techniques) my study aims to discover the regulatory role of *tra-1* in *C. elegans* vulval development and to investigate the genetic interaction between the terminal effector

of the sex determination pathway *tra-1* and other pathways. The *Hox* gene *lin-39* is the major point of convergence between different signaling pathways, thus it plays a central role in the vulval cell specialization. Hence I have focused on the investigation of molecular mechanisms by which the *tra-1* may regulate the activity of the key gene *lin-39* during vulval development.

Genetic interaction between autophagy genes and the TGF-β signaling pathway in controlling cell size in *Caenorhabditis elegans*

The regulation of cell growth and proliferation is fundamental for tissue homeostasis and normal development in divergent animal phyla, but the mechanisms that coordinate cell growth with cell cycle progression are poorly understood. It has generally been observed that cells grow to a certain size before they divide. Thus, cell growth is a prerequisite of cell proliferation and may be dysregulated in human malignancies. The regulation of cell size is intimately linked to nutrient and growth factor availability and requires a well-controlled balance between macromolecule synthesis and degradation. Autophagy is an evolutionarily conserved intracellular catabolic process, operating as a homeostatic mechanism in all eukaryotic cells. Autophagic degradation of subcellular constituents is a major route for turnover of cellular macromolecules and organelles, in particular proteins and mitochondria. The nutrient recycling and housekeeping functions of autophagy promote cell survival under environmental stresses (e.g. starvation). On the other hand, autophagy is also involved in cell death. Autophagic cell death or type II programmed cell death exhibits extensive autophagic degradation of Golgi apparatus, polyribosomes and endoplasmatic reticulum, which precedes nuclear destruction. In certain conditions autophagy may also mediate apoptosis or type I programmed cell death.

Both insulin-like growth factor-1 (insulin/IGF-1) and transforming growth factor-β (TGF-β) signaling pathways are known as major regulatory systems for cell growth, proliferation, and differentiation in various eukaryotic organisms. These cascades affect body length by controlling cell size.

Using *C. elegans* as model organism, another purpose of my study was to elucidate the functions of autophagy genes in the regulatory mechanism of cell growth, which is known to be controlled by the insulin/IGF-1 and TGF-β signaling pathways. First, I planned to monitore the effects of loss of function mutations in two autophagy genes *unc-51/Ulk-1* and *bec-1/beclin-1* on *C. elegans* body length (size). Second aim was to evaluate the effects of UNC-51 and BEC-1 deficiency on the long body size (Lon) phenotype of mutant strains defective in TGF-β signaling.

Materials and Methods

Nematode strains and alleles

Wild-type strain and parent of all mutant strains was *C. elegans* Bristol N2. The following strains were used in this study: CB2823 *tra-1(e1488)III; eDp6(III;f)*, CB2590 *tra-1(e1099)/dpy-18(e1096)III*, CB2810 *tra-1(e1575)/+ III*; *unc-42(e270) him-5(e1490) dpy-21(e428)V*, *CB3844 fem-3(e2006)IV*, MT688 *lin-12(n137)/unc-32(e189)III*; *him-5(e1490)V*), MT2375 *lin-12(n137)dpy-19(e1259)/lin-12(n676n909)unc-32(e189)III; him-5(e1467)V*, AH142 *zhIs4*[*lip-1::gfp + unc-119(+)*]; *unc-119(e2498)III*, NH646 *ayIs9*[*egl-17::gfp + dpy-20(+)*]*V*; *dpy-20(e1282ts)IV*, RA91 *qls76/hT2*[*qls48(tra-1::gfp)*]*(I;III)*, RA7 *rdEx1*[*tra-1::gfp + rol-6(su1006)*], AH30 *zhIs1*[*lin-39::gfp + unc-119(+)*]*IV*; *unc-119(e2498)III*, SU159 *ajm-1(ok160)X; jcEx44*, MT111 *lin-8(n111)II*, MT1808 *lin-38(n751)II*, MT1806 *lin-15A(n767)X*, MT8878 *dpl-1(n2994)II*, MT11147 *dpl-1(n3643)II*, MT10430 *lin-35(n745)I*, MT6034 *lin-36(n766)III, swEx520*[p*bec-1*::BEC-1::GFP + *rol-6(su1006)*], *unc-51(e369)*, *lon-2(e678)*, *him-8(e1489)* and *him-5(e1490)*.

Microscope imaging

For morphological analysis and to count the number of cells, optical recordings were performed on Olympus BX microscope. Images were obtained using brightfield illumination or fluorescence light with 60 or 100× objective lens. *C. elegans* strains transgenic for a fluorescent (GFP-fused) reporter were examined with U-M41025 filter. Worms were transferred to a thin (high-strength) agarose pad, which was formed over a standard glass microscope slide and 0.1 mM levamisole or 10 mM NaN_3 anesthetic solution was dropped onto the agarose pad.

RNA interference (RNAi)

To generate RNAi clones, total RNA was isolated from mix-staged wild-type nematode populations, and specific cDNA sequences were amplified by performing RT-PCR (Titan One-Tube RT-PCR System, Roche) reactions. Amplified cDNA fragments were purified (QIAquick gel extraction Kit, Promega), and cloned into pGEM-T Easy vector (Promega). Digested fragments were inserted into the pPD129.36 “feeding” vector (kindly provided by Andrew Z. Fire), and the resulted constructs were transformed into *E. coli* HT115(DE3). RNAi experiments were performed at 25 °C.

Promoter analysis

The alignment of the *lin-39* promoter sequences from *C. elegans* and *C. briggsae* were performed using the online software LALIGN (http://www.ch.embnet.org/software/LALIGN form.html). *Lin-39* complete genomic sequence and 10 kb upstream promoter region were downloaded from NCBI's GenBank database (http://www.ncbi.nlm.nih.gov/sites/entrez?db= nucleotide). Genomatix MatInspector program, Wormbase (www.wormbase.org) BLAST program and LALIGN were applied to probe the entire sequence of *lin-39* gene and promoter region for potential TRA-1A transcription factor-binding sites.

Electrophoretic mobility shift assay (EMSA)

For electrophoretic mobility shift assays, TRA-1A protein was generated by *in vitro* transcription and translation of full-length *tra-1* cDNA, pDZ118 (kindly provided by David Zarkower), using a T7-based coupled reticulocyte lysate system (Promega). After *in vitro* translation, ZnSO4 was added at 50 μM. For preparing DNA probes, single-stranded oligonucleotides were labeled by filling in the single-stranded termini with Klenow enzyme in the presence of 32Pα-dCTP according to standard procedures. Mutant *lin-39* oligonucleotides were used as negative control, while TRA-1A binding site from the *egl-1* promoter was used as positive control. EMSA experiments were essentially performed as described previously by Yi *et al*. (2000).

Gene expression analysis

To determine whether TRA-1 also accumulates in the vulval precursor cells (VPCs), or at least in some of them, we monitored the expression of a functional *tra-1::gfp* reporter. To test the influence of *tra*-1 on *lin-39* expression in the VPCs, I analyzed the vulval specific expression pattern of an integrated, rescuing *lin-39::gfp* reporter transgene. This construct contained the entire *lin-39* coding sequence and about 5 kb of the upstream promoter region. A so-called secondary (2°) vulval fate marker, *egl-17::gfp* was applied to examine vulval fate determination in *tra-1(-)* mutant animals. EGL-17 is known to be expressed strongly in descendants of VPCs that adopt 2° fate at the mid L4 larval stage. Using *tra-1::gfp*, *ajm-1::gfp* (Apical Junction Molecule) and *prk-1::gfp* [Pim (mammalian oncogene) Related Kinase] markers, cell number was assessed in autophagy-defective mutant animals. A *lip-1::gfp* (Lateral signal Induced Phosphatase) reporter transgene was used to test whether *tra-1* knockdown or over-activation influences LIN-12/Notch activity (*lip-1* is a direct downstream target of *lin-12*-mediated signaling).

Results

tra-1 controls vulval development in *C. elegans*

• Mutant analysis (dissecting and compound microscope studies) of *tra-1* deficient animals revealed that this gene is necessary for the development of a functional vulva in hermaphrodites. Reduction of function mutations of *tra-1* caused defects in vulval development: majority of the homozygous *tra-1(e1488)* mutant XX animals with a characteristic intersex phenotype were able to develop vulval structure exhibiting different abnormalities. The characteristic vulval phenotype of these intersexes is the protruding (Pvl) vulva (vulva hypertrophy / hyperplasia), the vulva of these animals was protruded from the plane of the ventral epidermis. Occasionally, these mutant nematodes developed more than one ventral protrusions. Thus, they were actually multivulva (Muv) animals. Consistent with the *tra-1(e1488)* phenotype, depletion of TRA-1 by RNAi treatment also influenced vulval development. *tra-1(RNAi)* animals displayed a Pvl phenotype, and they rarely also were able to develop more than one vulval protrusions. Moreover, knockdown of both *tra-1* transcripts *(tra-1a* and *tra-1b)* had more severe effects on vulval patterning than perturbing the larger *tra-1a* transcript alone.

• Loss-of-function (lf) mutations in *fem-3* (an upstream negative regulator of *tra-1*), which cause activation of *tra-1*, resulted in a Pvl phenotype in *him-5(e1490)* loss-of-function mutant or *him-8(RNAi)* genetic background.

• Using an e*gl-17::gfp* 2° cell fate marker, I determined that the most frequent vulval abnormality in these animals is the failure of P(5,7).p precursor cells to adopt 2° fates at the late L4 larval stage. Contrary to the wild-type developing vulva, which has a mirror-symmetric structure, in *tra-1* deficient animals the vulval primordium displayed an asymmetric pattern of cell division.

tra-1 influences LIN-12/Notch-mediated lateral signaling during vulval development

• The failure of *tra-1* deficient animals to specify 2° vulval fates raised the possibility that the sex determination cascade influences LIN-12/Notch-mediated lateral signaling during vulval patterning. I found that depletion of *tra-1* markedly enhance vulval induction in *lin-12(n137)* gain-of-function mutants. This indicates that *tra-1* interacts with *lin-12* gene (which encodes a Notch receptor) in the cell fate determination during vulval development. Consistent with this finding, loss-of-function mutation or RNAi-mediated knockdown of *fem-3* reduced the average number of pseudovulval protrusions in *lin-12 (n137gf)* background.

• In good accordance with the previous results, *lip-1::gfp* expression became increased in *tra-1* deficient mutants and significant decreased in *fem-3(RNAi)* animals as compared to wild-type.

TRA-1A controls the expression of *lin-39 Hox* gene

• I performed *in silico* analysis of the *lin-39* regulatory region for searching a putative TRA-1A binding site within this region. I have identified a consensus TRA-1A motif („TTTCNNNNTGGGTGGTC") 1 kilobase (kb) upstream of the ATG translational initiation site of *lin-39*, which is highly conserved between *C. elegans* and *C. briggsae.*

• To further confirm the binding specificity of TRA-1A with the *lin-39* promoter fragment containing the conserved TRA-1A binding site, I performed gel mobility shift assay (rEMSA), and found that *in vitro* translated full-length TRA-1A is able to bind to the wild-type *lin-39* promoter fragment. Although this association of TRA-1A to the *lin-39* fragment was weaker than to the positive control *egl-1* oligonucleotide, but the binding was specific, as the TRA-1A was unable to bind the mutant fragment of the *lin-39* promoter.

• To test the influence of *tra-1* on *lin-39* expression in the VPCs, I have analysed the vulva-specific expression pattern of an integrated, rescuing *lin-39::gfp* reporter transgene in wild-type males vs. *tra-1(-)* mutant XX animals. LIN-39 normally accumulates at basal levels in the Pn.p cells. In early L2 larval staged *tra-1(e1099)* animals the percentage of *lin-39::gfp*-expressing VPCs was significantly higher than in wild type worms at the same developmental stage. Consistently, Pn.p cells displayed excessive *lin-39::gfp* levels in tra-1 RNAi background as well.

***tra-1* is expressed in the vulval precursor cells and hypodermis**

• A translational fusion TRA-1::GFP reporter accumulated in VPCs prior to and at the time of vulval induction.

• This *tra-1::gfp* reporter gene was not only expressed in the VPCs and in their descendants but also was active in the region of the hypodermis, from which the inhibitory synMuv signals are known to be emitted.

***tra-1* interacts with the synMuv pathways**

• The *synMuv* genes are known to act in the hypodermis and repress *lin-39* during vulval development. Thus, epistasis analysis was performed to investigate whether *tra-1* interacts with the synMuv pathways to control vulval development. Double and triple mutant animals defective for *tra-1* and *synMuv A*, resp. *synMuv B* loss-of-function mutations were examined.

- Inactivation of *fem-3* suppressed vulval induction in *synMuv AB* double mutant hermaphrodites.
- In contrast, knockdown of *tra-1* significantly increased the average number of vulval protrusions in *synMuv AB* double mutants.
- The strong loss-of-function mutation *tra-1(e1099)*, which transforms XX animals into males, also promoted vulval induction in *synMuv AB* double mutant background, as compared with the corresponding *synMuv AB* mutant males. Inactivation of *tra-1* by strong loss-of-function allele resulted in weaker effects on vulval induction, than inhibiting *tra-1* by RNAi. This intriguing phenomenon may have resulted from the pleiotropic effect of *tra-1(e1099)*, which strongly masculinizes the ventral hypodermis, from which the vulval tissue develops in hermaphrodites.
- Although with a relatively moderate penetrance, *synMuv A; tra-1(RNAi)* and *synMuv A; tra-1(e1099)* animals exhibited a Muv phenotype, indicating that *tra-1* may act as a *synMuv B* gene (since animals with one or more mutations of the same class undergo wild-type vulval development, but animals with mutations of any two classes have a multivulva phenotype).
- Mutations in the *synMuv B* genes had no inductive effect on vulval formation in *tra-1(-)* mutant backgrounds, further suggesting that *tra-1* interacts with the synMuv B pathway or, alternatively, may be a *synMuv B* gene.

Genetic interaction between autophagy genes and TGF-β signaling

- Using *tra-1::gfp* and *prk-1::gfp* riporters to visualize intestinal cells, I demonstrated that mutational inactivation of two autophagy genes, *unc-51/Ulk-1* and *bec-1/beclin-1*, results in small body size without affecting cell number.
- I found twenty GFP-positive intestinal cells in wild-type animals, *unc-51(e369)* and *bec-1(ok691)* mutant adults. Thus, *unc-51* and *bec-1* do not affect cell lineage of *C. elegans*.
- The *unc-51(e369)* loss-of-function mutation reduced the size of intestinal and hypodermal cells in *C. elegans*, indicating that autophagy controls cell growth in this organism.
- The Sma (small body size) phenotype of *unc-51* and *bec-1* mutants were epistatic to the Lon (Long) phenotype of loss-of-function mutation of *lon-2* TGF-β signaling defective mutant. This epistatic relationship indicates that these autophagy genes act downstream of TGF-β signaling to control cell size in *C. elegans.*

Conclusions

The proper coordination of signals from different genetic pathways is crucial for cell fate specialization during animal development. The challenge is to understand the factors that comprise in this coordination. Vulval development in the nematode *Caenorhabditis elegans* is an excellent paradigm to study how cell fates are specified by different signaling pathways. The vulva (egg-laying structure) of nematode hermaphrodites is formed in the central body region by the progeny of three epidermal precursor cells P(5-7).p. Differential pattern of cell fate of vulval precursor cells (VPCs) is coordinated by the action of the evolutionarily conserved RTK/Ras/MAPK, Wnt and LIN-12/Notch signaling cascades (that activate vulval induction) and of three functionally redundant synMuv (synthetic Multivulva) pathways grouped into classes A, B and C (that generate an inhibitory signal in the hypodermal tissue surrounding the VPCs). Here I demonstrated that signaling via Gli (*Glioma-associated*) - like transcription factor TRA-1A (Transformer), the terminal regulator of the nematode sex determination cascade, is a newly discovered pathway influencing vulval development. To understand the roles of TRA-1A/Gli in vulval fate determination and its interactions with other signaling pathways, I performed a set of epistasis analysis, and revealed that *tra-1* influences LIN-12/Notch-mediated lateral signaling to specify secondary vulval fates. I also demonstrated that prior to and during vulval induction, TRA-1A accumulates in the VPCs where it controls the expression of the *Hox* gene *lin-39/HoxD4/Dfd*, the central regulator of vulval patterning. Consistently, TRA-1A is able to bind to the regulatory region of *lin-39 in vitro*. Thus, *lin-39* – which serves as a major integration site and relay in transmitting signals of RTK/Ras/MAPK, Wnt and SynMuv cascades – is a newly identified TRA-1A/Gli target gene.

Furthermore, I found that inactivation of *tra-1* promotes vulval induction in *synMuv A*, but not in *synMuv B* mutant background. This finding implies that TRA-1A may act as a synMuv B protein to repress *lin-39* expression. Many synMuv B proteins are involved in chromatin assembly, chromosome condensation, apoptosis and repression of cell proliferation. Identifying *tra-1* as a *synMuv B* gene, which regulates at least in part, the expression of specific *Hox* genes may help to understand how compromised Gli activity in humans leads to cancer.

Using *C. elegans* as model organism, I have applied genetic techniques to elucidate the functions of two autophagy genes (autophagy is a major catabolic process of eukaryotic cells) in controlling cell growth. I demonstrated that mutational inactivation of *unc-51/Ulk-1* and *bec-1/beclin-1* results in small body size without affecting cell number. Moreover, I defined that these autophagy genes affect cell size via acting downstream of the TGF-β signaling pathway, which is one of the major regulatory systems for cell growth, proliferation, and differentiation in various

eukaryotic organisms. The regulation of cell growth and proliferation is tightly integrated. This study suggests that autophagy genes are key modulators of cell size, and thus, their activity is critical for maintaining normal development and tissue homeostasis.

Publications related to the PhD thesis

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