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Translational activation maintains germline tissue homeostasis during adulthood

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Adult tissue maintenance is achieved through a tightly controlled equilibrium of 2 opposing cell fates: stem cell proliferation and differentiation. In recent years, the germ line emerged as a powerful *in vivo* model tissue to investigate the underlying gene expression mechanisms regulating this balance. Studies in numerous organisms highlighted the prevalence of post-transcriptional mRNA regulation, which relies on RNA-targeting factors that influence mRNA fates (e.g. decay or translational efficiency). Conserved translational repressors were identified that build negative feedback loops to ensure one or the other cell fate. However, to facilitate a fast and efficient transition between 2 opposing cell fates, translational repression *per se* appears not to be sufficient, suggesting the involvement of additional modes of gene expression regulation. Cytoplasmic poly(A) polymerases (cytoPAPs) represent a unique class of post-transcriptional mRNA regulators that modify mRNA 3' ends and positively influence cytoplasmic mRNA fates. We recently discovered that the 2 main cytoPAPs, GLD-2 and GLD-4, use distinct mechanisms to promote gene expression and that cytoPAP-mediated mRNA activation is important for regulating the size of the proliferative germ cell pool in the adult *Caenorhabditis elegans* gonad. Here, we comment on the different mechanisms of the 2 cytoPAPs as translational activators in germ cell development and focus on their biological roles in maintaining the balance between germline stem cell proliferation and differentiation in the *Caenorhabditis elegans* gonad.

Keywords: germ cells, RNA regulation, poly(A) polymerases, transit-amplifying cells, *C. elegans*

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Introduction

Maintenance of a healthy balance between proliferation and differentiation is an essential aspect during adult tissue homeostasis. Increased proliferation at the expense of differentiation leads to tissue overgrowth, and conversely, increased differentiation at the expense of proliferation leads to tissue shrinkage; both scenarios will eventually produce a non-functional tissue. The *Caenorhabditis elegans* (*C. elegans*) germ line is a powerful model system to study the molecular mechanisms underlying this balance of opposing cell fates and tissue maintenance.[1-3] In the adult gonad, germ cells form a syncytial tissue and are arranged in a distal-to-proximal organization that reflects subsequent developmental germ cell stages. Most distally and in close proximity to the germline niche (i.e. the somatic gonadal distal tip cell), proliferative germ cells are located that constitute the proliferative zone. At a defined distance from the distal tip, germ cells exit the mitotic cell cycle and further proximally start differentiation by entering prophase I of meiosis. This switch from proliferation-to-differentiation has been termed the mitosis-to-meiosis decision.[2,4]

Across species, gene expression regulation in germ cells occurs to a large extent at the post-transcriptional level. Hence, many conserved post-transcriptional RNA regulators have been identified to function in germ cell development.[1] Especially mRNA-associated translational repressors, *i.e.*, RNA-binding proteins and miRNA-containing protein complexes, were found to form self-enforcing negative feedback loops to maintain germ cell fate

decisions.[5] In *C. elegans*, although no clear involvement of miRNAs has been revealed to date, representatives of 2 translational repressor protein families influence the mitosis-to-meiosis decision: FBF-1 and FBF-2, 2 nearly identical PUF protein family members (commonly referred to as FBF), facilitate proliferation; the STAR protein family member, GLD-1, facilitates differentiation.[6,7] To prevent the switch from proliferation to differentiation, FBF limits among many differentiation-promoting mRNA targets GLD-1 protein synthesis to post-mitotic cells by selectively binding to *gld-1* mRNA in pre-meiotic cells.[7,8] Also in other germline stem cell model systems, translational repression emerged as a key mode of gene expression regulation.[5,9,10]

The poly(A) tail of mRNAs is a dynamic structure and changes of tail lengths in the cytoplasm are indicative of gene expression regulation.[11] Especially in developmental contexts, the length of the poly(A) tail correlates with mRNA stability and the amount of protein produced: long-tailed mRNAs are more stable and can attract a larger number of ribosomes per mRNA (termed polysomes); short-tailed mRNAs are more prone to degradation and attract less ribosomes.[12] Hence, translational repressors that recruit A-tail-shorting enzymes (*i.e.* deadenylases) generate mRNA species with trimmed poly(A) tails,[13] which upon translational de-repression probably require additional help to remain stable and efficiently engage in protein production. This help is likely provided in the form of cytoplasmic poly(A) polymerases (cytoPAPs). These conserved A-tailing enzymes are expected to counteract mRNA deadenylation, yet their mode of action and target mRNA repertoire remained unknown. Moreover, cytoPAPs lack common RNA-binding domains and, therefore, are hypothesized to recognize their mRNA targets via interactions with other RNA-binding proteins.[14,15] Recently, we investigated the underlying mRNA-regulatory mechanisms of the 2 distinct *C. elegans* cytoPAPs, GLD-2 and GLD-4, and how both represent 2 opposing forces in fine-tuning the mitosis-to-meiosis decision.[16,17]

GLD-4 promotes translational efficiency

GLD-4 is in its enzymatic domain evolutionarily most similar to that of non-canonical TRF4-type poly(A) polymerase family members.[18,19] Yet, in many organisms TRF4 proteins are nuclear enzymes that primarily target a variety of non-coding RNA substrates by adding short adenosine stretches (~10–20 nts) for exosome-mediated degradation.[20] By contrast, GLD-4 is predominantly located in the cytoplasm,[18] presumably targeting mRNAs for enhanced expression. GLD-4 shows poly(A) polymerase activity in a heterologous *in vivo* tethering system, but its endogenous activity on gene-specific A-tail length extension appears rather moderate at steady state.[17,18,21] Importantly, GLD-4 depends on its co-factor GLS-1 for efficient PAP activity in tethering assays, suggesting that the GLD-4/GLS-1 complex comprises the active cytoPAP.[18]

To reveal the impact of GLD-4 on global poly(A) tail metabolism, we recently measured A-tail lengths on bulk RNA and found that poly(A)-tails were only mildly reduced in the absence of GLD-4[16] or GLS-1 (unpublished results), arguing that GLD-4 cytoPAP may have an intrinsic low enzymatic activity that is similar to that of its nuclear counterparts.[22,23] A-tailing in the nucleus by TRF4 proteins leads to the degradation of the respective RNA substrates by the exosome.[24] In our transcriptome analysis of *gld-4*–compromised animals, we detected only minor changes in mRNA abundance,[16] arguing that GLD-4 has no major role in promoting cytoplasmic mRNA degradation. Taken together, this suggests that GLD-4–mediated polyadenylation in the cytoplasm might fulfill a different function.

GLD-4 function is linked to general translation (**Fig. 1A**). Our sucrose gradient analysis revealed a severe reduction of

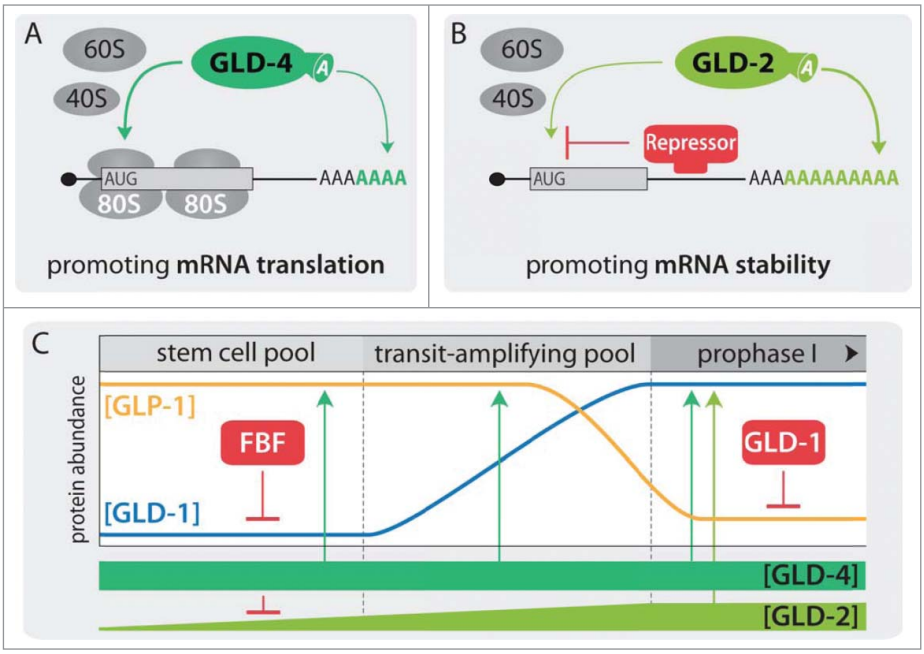

Figure 1. Working model of how GLD-2 and GLD-4 act as global and gene-specific translational activators in *C. elegans* germ cells. (**A**) GLD-4 promotes primarily polysome formation and oligoadenylates mRNAs. (**B**) GLD-2 primarily stabilizes translationally repressed mRNAs via polyadenylation. Ribosomal subunits (40S, 60S) and ribosomes (80S) are not drawn to scale. (**C**) In the mitosis-to-meiosis decision, translational repressors and activators maintain the balance between proliferation and differentiation. In GSCs, proliferation is promoted by the translational repressor FBF, which suppresses protein production of many differentiation-promoting genes, including GLD-1 and GLD-2. The equally expressed translational activator GLD-4 promotes expression of the proliferation-promoting gene, GLP-1/Notch. In cells committing toward differentiation, the translational repressor, GLD-1, blocks GLP-1 accumulation and both translational activators, GLD-4 and GLD-2, promote efficient GLD-1 protein synthesis.

poly-ribosomes in *gld-4*–compromised animals. Moreover, GLD-4 protein co-migrates with polysomes, suggesting a role in promoting efficient translation.[16] Although it still needs to be shown that GLD-4's polymerase activity is needed for modifying mRNAs before or during polysome formation, it is tempting to speculate that GLD-4–mediated polyadenylation may counteract the proposed gradual erosion of mRNA poly(A) tails in polysomes.[25] Also in favor of this idea is our finding that GLS-1, a strong potentiator of GLD-4 cytoPAP activity, co-migrates with polysomes in density gradient analyses and enhances polysome formation.[16] Alternatively, the GLD-4/GLS-1 complex might support bulk polysome formation in an enzyme-independent manner by a yet to be identified mechanism.

GLD-4 promotes proliferation

Many aspects of germline development require GLD-4 function.[18] However, a role in adult tissue homeostasis, regulating the mitosis-to-meiosis decision had remained uncharacterized. By following aging hermaphrodites, we found that GLD-4 maintains the normal size of the proliferative zone and prevents its progressive shrinkage.[17] The lab of Judith Kimble had recently provided convincing evidence that the proliferative zone contains 2 pools of cells with distinct properties; a distal pool of about 30–70 germline stem cells (GSCs) that primarily proliferate to self-renew, and a proximal pool of transit-amplifying cells with presumably limited proliferation potential that are primed for differentiation.[26,27] Interestingly, the extent of the proliferative zone that remains in *gld-4* mutants is similar to the distal GSC pool.[17] Hence, GLD-4 may function primarily in transit-amplifying cells to control their proliferative capacity. Alternatively, due to a global requirement of GLD-4 in maintaining the expression of broadly acting proliferative factors, both pools of cells may have shrunken in *gld-4* mutants.

Which proliferative factors might be targets of GLD-4–mediated translational activation? Initially, we suspected that FBF represents a suitable candidate. However, we found no convincing evidence that FBF expression depends on GLD-4 activity. By contrast, we discovered the Notch receptor-encoding mRNA *glp-1* as an important target of GLD-4 (**Fig. 1C**).[17] GLP-1 keeps germ cells undifferentiated and proliferative by sensing niche-produced Notch ligands.[28,29] GLP-1 protein expression is restricted to the distal part of the germ line,[30] and receptor function is essential for proliferation: too much GLP-1 activity prevents differentiation and causes the formation of a germline tumor; conversely, a loss of GLP-1 eliminates the proliferative zone and all germ cells differentiate.[31,32] To facilitate differentiation, *glp-1* mRNA is translationally repressed by GLD-1 and GLP-1 protein production is strongly reduced when cells enter meiotic prophase.[33] Interestingly, low levels of GLD-1 protein are already accumulating in transit-amplifying cells; therefore, translational activation of Notch may represent an active mechanism to delay premature repression. We found that GLD-4 associates with *glp-1* mRNA, contributes to its wild-type A-tail lengths, and enhances poly-ribosome formation of *glp-1* mRNA. Moreover, high GLP-1 protein levels require GLD-4 cytoPAP activity, arguing that GLD-4 enhances protein production of specific mRNAs. Although GLP-1 protein is less abundant in the germ line of *gld-4* mutants, its expression is not lost in aged animals.[17] Hence it is likely that GLP-1 expression is largely independent of GLD-4 in GSCs, but dependent in transit-amplifying cells. Moreover, it supports a differential requirement of GLP-1 activity in cells of the proliferative region.[34] Nonetheless, most likely other target mRNAs of GLD-4–mediated translational activation in transit-amplifying cells and/or GSCs exist.

Strikingly, also general differences of translational requirements between stem cells and early maturing cells are emerging. In the haematopoietic system, protein output differs between stem cells and restricted progenitors.[35] Although, it is not known whether GSCs behave in a similar manner, the protein synthesis rate in GSCs might be rather low and translation may need to be quickly up-regulated to meet higher protein synthesis demands in transit-amplifying cells while progressing toward differentiation. Consequently, the immediate stages leading up to post-mitotic differentiation in germ cells, for example pre-meiotic S phase, might require additional mechanisms that ensure efficient translation of key differentiation factors. Irrespective of a possible global nature of GLD-4–mediated enhanced translation in stem cell systems, GLD-4 cytoPAP is important for specific mRNAs to promote protein amounts that are required to execute a specific cell fate decision.

GLD-2 stabilizes translationally repressed mRNAs

C. elegans GLD-2 is the founding member of the GLD-2–type cytoPAP family, whose representatives are in many species central determinants of cytoplasmic poly(A) metabolism.[14,19] Its poly(A) polymerase capability was also demonstrated in a heterologous *in vivo* assay, by artificially tethering GLD-2 to an mRNA substrate.[36] A few gene-specific poly(A) tail measurements on putative GLD-2 targets also supported a role in A-tail extension of endogenous germ cell-specific mRNAs.[21,37-39] Recently, we analyzed the magnitude of GLD-2–mediated A-tailing by performing bulk poly(A) tail measurements, which provides a snapshot of the polyadenylation status of all mRNAs rather than that of individual mRNAs. With this assay, we detected a general shortening of tails in *gld-2*–compromised animals,[16] arguing that GLD-2 cytoPAP polyadenylates many germline mRNAs rather than a small subset of individual targets to control their expression.

Cytoplasmic polyadenylation is proposed to be important for translational activation of target mRNAs in many tissues across species.[11,40,41] To identify GLD-2 targets in *C. elegans* and reveal how GLD-2 promotes gene expression at the functional level in the worm, we analyzed the abundance and translation efficiency of mRNAs at the global scale, comparing *gld-2*–deficient animals to wild type in RNA deep sequencing and polysome profiling experiments. Our transcriptome analysis revealed that high, wild-type levels of many germline mRNAs depend on GLD-2. Interestingly, we could not detect a significant impact of

GLD-2 on the translatability of mRNAs in our polysome analysis.[16] This could be explained by our experimental set up, which was geared toward the detection of strong changes in mRNA translation efficiency; moderate or weak changes would have gone unnoticed. Alternatively, its initial role in mRNA stabilization may have overshadowed a subsequent role in promoting translation. A detection of the second GLD-2 role would have been masked in our data set due to the premature degradation of GLD-2 targets. Hence, we concluded that GLD-2 cytoPAP primarily stabilizes or promotes the initial expression of its target mRNAs (**Fig. 1B**). Therefore, we defined less abundant mRNAs as GLD-2 targets. Obviously, some mRNAs will also have been indirectly down-regulated as a consequence of GLD-2–dependent developmental changes in the mutant. Nonetheless, we think that our list of GLD-2–stabilized germline genes is highly enriched for direct targets and provides a valuable resource for studying GLD-2–regulated mRNAs.

GLD-2–mediated polyadenylation appears to be a major part of post-transcriptional gene expression networks in the germ line. Among all GLD-2–stabilized mRNAs, ~70% have been reported to be likely targets of several translational repressors: FBF-1, GLD-1, and OMA-1, a TIS11 zinc-finger protein family member.[8,16,42-44] This suggests that GLD-2–mediated mRNA stabilization is primarily important for mRNAs that are subjected to prior or continued translational repression. In this respect, it is important to note that mRNA deadenylation represents the first step of the major mRNA degradation pathway, and that numerous translational repressors, such as members of the PUF protein family, recruit deadenylases as part of their repressive activity.[13] Hence, GLD-2–mediated cytoplasmic polyadenylation appears to represent an effective counterforce to RNA decay-inducing poly(A) tail removal. Due to the large mRNA target overlap with various translational repressors, we propose that GLD-2 is a major mRNA stabilizer that protects many–if not all–translationally repressed genes in germ cells (**Fig. 1B**).

GLD-2 promotes differentiation

GLD-2 family members are important for late stages of female germ cell development in many organisms.[19] In *C. elegans*, also an earlier role during oogenesis had been revealed, in which GLD-2 promotes the expression of the FBF-target GLD-1 to facilitate differentiation in the mitosis-meiosis decision.[37,45] Based on the large overlap between GLD-2 and FBF targets,[16] we think that GLD-2 enhances the production of proteins from a broad range of differentiation-promoting mRNAs, most likely after their release from repression. In our recent work, we were able to show that GLD-2 protein production is actively repressed in the proliferative zone: GLD-2 protein abundance is very low in the distal half of the proliferative zone (i.e., GSCs), gradually increases further proximal in transit-amplifying cells, and reaches its peak expression in cells that have entered meiotic prophase.[14,17] We found that *gld-2* mRNA is targeted for translational repression by FBF in the proliferative zone. The 3' UTR of *gld-2* carries an active FBF-binding site and RNAi-mediated down regulation of FBF leads to an up regulation of GLD-2 protein in distal germ cells.[17] Although, it is at the moment unclear whether upregulated GLD-2 is actively promoting differentiation and how strongly other FBF targets, such as GLD-1, contribute to differentiation, the loss of FBF correlates with a complete loss of the proliferative cell fate.[7,8] This suggests that GLD-2 levels are kept low in proliferating cells by FBF to prevent premature entry into differentiation, and substantiates GLD-2's role as a major positive mRNA regulator that ensures a swift and efficient transition from proliferation to differentiation (**Fig. 1C**).

The relationship between GLD-2 and GLD-4

In the mitosis-to-meiosis decision, the 2 translational activators represent opposing forces. As described above, GLD-4 promotes the proliferative cell fate and loss of GLD-4 shrinks the proliferative zone.[17] By contrast, GLD-2 promotes the differentiation fate and loss of GLD-2 expands the proliferative zone.[45] Our analysis of adult *gld-2 gld-4* double mutants showed that the extent of the proliferative zone resembles wild-type size.[17] Although it is not clear at the moment whether the proliferative zone in the double mutant resembles a wild-type composition of GSCs and transit-amplifying cells, this result suggests that either single mutant defect reflects an imbalance between the 2 translational activators. How this apparent balance between GLD-4 and GLD-2 may be achieved at the functional level is currently unknown. One simple idea is that the mRNA target activation strength of both cytoPAPs sums up to be equal. Alternatively the 2 enzymes may be tightly connected and limit each other's activities, either directly as part of the same RNA-protein complexes or via indirect mechanisms, such as competing for similar co-factors. Nonetheless, our findings illustrate that counteracting translational activators of most likely equal strength, yet with different mechanistic properties, are involved in setting the boundary of proliferation and differentiation.

Translational activation vs. repression in germline homeostasis

GLD-2 and GLD-4 are part of an ever-expanding, highly redundant post-transcriptional RNA regulatory network that governs, downstream of niche signaling, the switch from mitosis to meiosis. But what is the contribution of translational activation and repression? A combined removal of translational repressors (*i.e.* FBF and GLD-1) leads to the loss of differentiation and a germline tumor forms.[7] By contrast, a combined removal of both translational activators (*i.e.*, GLD-4 and GLD-2) leads to differentiation onset in a superficially comparable manner to wild type.[17] This suggests that, in combination with GLP-1 signaling, translational repression may be sufficient to organize the mitosis-to-meiosis decision, and that cytoPAP-mediated translational activation by itself cannot initiate the switch to meiosis. However, this simplistic interpretation may be incorrect, given that translational activation may be mechanistically coupled to repression. Support for this idea comes also from the findings that many GLD-2 target mRNAs of early differentiation are FBF targets,[16] and that

FBF binds and stimulates GLD-2 cytoPAP activity.[46] Therefore, the effectiveness of translational activation may depend on prior repression and is hidden in its absence. Moreover, it remains possible that, next to GLD-2 and GLD-4, additional, yet to be discovered poly(A) polymerases or other translational activators may exist that function in the mitosis-to-meiosis decision.

Clearly, basic translation repression activity of GLD-1 is not enough to sustain the cell fate switch to meiotic prophase and commitment to meiosis relies on translational activation. This differential requirement of cytoPAPs for successive stages of differentiation is explained by a differential dose-dependent requirement of meiosis-promoting factors for initiation and maintenance. With respect to the mitosis-to-meiosis decision, basic levels of GLD-1 promote differentiation onset, but higher levels of GLD-1 are required to maintain meiotic commitment. This is achieved via redundant translational activation of *gld-1* mRNA,[18] whereby GLD-2 might primarily counteract FBF-mediated induced poly(A) shortening to stabilize mRNAs, and GLD-4 might predominantly promote ribosome engagement. Although the roles of GLD-2 and GLD-4 appear mechanistically distinct, the combination of both molecular mechanisms ensures the production of high protein levels in a short period of time.[18,37] In summary, we propose that translational repressors are paired with translational activators to establish and maintain a stable cell fate switch in a highly dynamic, yet homeostatic system.

Given the prevalence of translational repression across species, the importance of translational activation for germline stem cell development in other organisms is currently unknown. With the exception of a cytosolic form of canonical poly(A) polymerase in *Drosophila* which is important for early female germ cell stages,[47] little is known about the expression of cytoPAPs in other GSC lineages. However, as the known translational repressive networks utilizes deadenylases to silence gene expression in *Drosophila* GSCs,[48] it is quite conceivable that cytoplasmic poly (A) polymerases will emerge as conserved regulators of opposing cell fate decisions in GSCs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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