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Analýza regulace komplexů cytoplazmatických poly(A) polymeráz

Analysis of regulation of cytoplasmic poly(A) polymerase complexes

Diploma Thesis

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Prohlášení

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V Praze, 29.08.2011 Jakub Novák

Statement of Academic Honesty

I hereby declare that I have written this thesis independently, with the use of listed literature. All used resources are listed in the list of reference. Neither this thesis, nor its significant part, was submitted for getting the other or the same academic degree.

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Abstract

The regulation of gene expression is achieved at many levels. Chromatin-based gene regulation has been the central focus of many decades of research; however, posttranscriptional control mechanisms are emerging as a fundamental complement to direct protein synthesis. This thesis is focused on a specific mechanism of posttranscriptional control - the translational regulation of mRNAs in the cell cytoplasm. This control is a consequence of the balance between translational repression and activation and hinges on the selective recognition of regulated mRNAs by RNA-binding proteins and their ability to recruit RNA modifying proteins. In this thesis, *Caenorhabditis elegans* germline was used to study translational control of the germ cell-enriched gene, *gld-2*. Mutants of known RNA-binding proteins of the PUF and CPB protein families were analyzed by performing Western blots, using anti-GLD-2 antibodies. Yeast 3-Hybrid system was used to identify the *cis*-regulatory sites in the *gld-2* mRNA conferring translational regulation by members of PUF and CPB protein families. Potential autoregulatory loop of *gld-2* gene expression was also investigated. This thesis shows that FBF proteins positively regulate expression of *gld-2* and bind to a conserved sequence in the 3´UTR of its mRNA. Mutations of *gld-2* negatively affect protein levels of GLD-2, suggesting the presence of autoregulatory loop. However, this effect was not that strong, probably because of redundant effect of other cytoplasmic poly(A) polymerase GLD-4.

Abstrakt

Regulace genové exprese je dosahováno mnoha způsoby. Výzkum se dlouhá léta zaměřoval především na regulaci genové exprese na úrovni chromatinu, nicméně posttranskripční regulace se na cestě k syntéze proteinů ukazuje být jejím nezbytným doplňkem. Tato práce se zaměřuje na studium jednoho specifického mechanismu posttranskripční kontroly - regulace translace mRNA v buněčné cytoplazmě. Tato regulace je důsledkem rovnováhy mezi aktivací a represí translace a závisí na selektivním rozpoznání cílových mRNA RNA-vazebnými proteiny a jejich schopností navazovat proteiny modifikující RNA. V této práci byla zárodečná linie *Caenorhabditis elegans* využita ke studiu translační regulace mRNA genu hojně exprimovaného v zárodečné linii, *gld-2*. Mutanti známých RNA-vazebných proteinů z rodin proteinů PUF a CPB byli analyzováni pomocí Western blotů za použití protilátek proti GLD-2. K identifikaci *cis*-regulačních míst na *gld-2* mRNA, zodpovídajících za regulaci translace PUF a CPB proteiny, bylo využito kvasinkového 3-hybridového systému. Také byl zkoumán potenciální autoregulační systém exprese genu *gld-2*. Tato práce ukazuje, že proteiny FBF pozitivně regulují expresi genu *gld-2* a váží se na jednu konzervovanou sekvenci v nepřekládaném regionu na 3´ konci jeho mRNA. Mutace v genu *gld-2* negativně ovlivnily hladinu proteinu GLD-2, což naznačuje existenci autoregulační smyčky. To, že tento účinek nebyl ještě výraznější, lze vysvětlit redundantním účinkem další cytoplazmatické poly(A) polymerázy GLD-4.

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List of Abbreviations

BAC	Bacterial Artificial Chromosome
Brat	Brain Tumor protein
CCCH	C-x8-C-x5-C-x3-H type zinc finger domain
ccr-4	CCR (yeast CCR4/NOT complex component) homolog
ccf-1	yeast CCR4 associated Factor family
CDK9	cyclin-dependent kinase 9 protein
cpb-1/-2/-3	CPEB polyA binding family
CPE	Cytoplasmic Polyadenylation Element
CPEB	cytoplasmic polyadenylation element-binding protein
CPSF	cleavage/polyadenylation specificity factor
CstF	cleavage stimulation factor
daf-12	abnormal DAuer Formation
daz	Deleted in AZoospermia
dpy	DumPY: shorter than wild-type
DSL family	Delta, Serrate, Lag-2 family
DTC	Distal Tip Cell
eIF	eukaryotic Initiation Factor
EMS	ethyl methane sulfonate
FBE	FBF-binding elements
fbf-1/-2	Fem-3 mRNA Binding Factor-1/-2
fem-3	FEMinization of XX and XO animals
fog-1	Feminization Of Germline
gld-1/-2/-3	defective in Germ Line Development
glp-1	abnormal Germ Line Proliferation
hrg	*hiiragi*
hb	*hunchback*
HRP	horseradish peroxidase
KH domain	K Homology domain
lag-2	Lin-12 And Glp-1 phenotype
let-7	LEThal
lin-4/-14/-28/-41/-42	abnormal cell LINeage
mex-1/-3	Muscle EXcess
miRNA	microRNA
nos-3	NanOS related
OD	optical density
oma-1/-2	Oocyte MAturation defective
ORF	open reading frame
PAA	polyacrylamide
PABP	poly(A)-binding protein
PAGE	polyacrylamide gel electrophoresis
paip-1	PolyAdenylate-binding protein-Interacting Protein
PAP	poly(A) polymerase
par-1/-2/-3	abnormal embryonic PARtitioning of cytoplasm
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
pos-1	POsterior Segregation

pie-1	Pharynx and Intestine in Excess
PK	protease K
PTR	PostTranscriptional Regulation
puf	Pumilio and FBF [fem-3 binding factor]
RING	Really Interesting New Gene finger domain
RNAi	RNA interference
rnp-8	RNP (RRM RNA binding domain) containing
RRM	RNA-recognition motif
SDS	Sodium Dodecyl Sulfate
sel-8	Suppressor/Enhancer of Lin-12
skn-1	SKiNhead
SOC	Super Optimal broth with Catabolite repression
TBE	Tris/Borate/EDTA
TCA	trichloroacetic acid
TEMED	N, N, N´, N´ - Tetramethylethylen diamine
unc	UNCoordinated
UTR	UnTranslated Region
WB	Western blot(ting)

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1. *Caenorhabditis elegans*

1.1 *C. elegans* as a model organism

Caenorhabditis elegans is non-parasitic, in soil living nematode feeding on microbes. It was introduced as an effective experimental model system by Sydney Brenner during the 1960´s.

In 1998 the genome of *C. elegans*, with its size of 102 Mbp stored in six chromosomes, was the first one sequenced of a multicellular organism. [1] *C. elegans* is also the only multicellular organism, which is fully sequenced and assembled without gaps. [2] This, together with relatively easy chemical, radiation or transposon mutagenesis, hermaphrodite/male reproduction system, easy storage of frozen worm strains and supported by many easily visible motility or morphology mutants, make *C. elegans* powerful model for genetic experiments. Self-fertilization allows propagation of homozygotes and mating with males enables creating different mutant combinations. [3, 4]

From a total of 959 somatic cells in hermaphrodite and 1031 in male adults around 650 are the same in both sexes. Despite this relative simplicity (see fig.1) it shows many different types of behaviour - locomotive, sensoric (taste, smell, osmotic pressure, touch), survival (foraging, feeding, defecation and formation of dauer larvae in case of unfavorable conditions), reproductive behaviour (mating, egg laying) and also complex behaviour such as social behaviour and memory is present. The effects of different substances on the neuronal system was performed in *C. elegans*, too. [5] It is a frequently used model organism for neuronal development and aging studies.

This nematode has five pairs of autosomal chromosomes and one pair of sex chromosomes - XX in hermaphrodites and XO in males. Males are usually very rare, around 0.1 % in the population. Their incidence raises up till 50 % when hermaphrodites are mated.

The genome of *C. elegans* contains around 24 000 protein coding genes, including 4474 alternatively-spliced forms (WormBase data release WS221). The average gene covers a genomic region of about 3 kbp and contains 6.4 coding exons, which are usually small (with a median of 123 bases) and separated by small introns (with a median of 47 bases).[6] This nematode has probably the best described interactome of all model organisms.

A very useful and not very labor-intensive method used mainly for larger genetic screens is RNA interference (RNAi). In 1998, Fire and his colleagues described the effect of

double stranded RNA (dsRNA) injection, which led to degradation of its target mRNA in *C. elegans*.[7]

RNAi could be performed in three major ways: First, microinjection into a young adult followed by scoring for phenotypes in the progeny. Second, RNAi by soaking in solution with high concentration of dsRNA, which is used for bigger amounts of worms. Lately, RNAi feeding, which is done by feeding worms on agarose plates with a special bacterial strains producing dsRNA from plasmid. Every method has its cons and pros. They differ in efficiency, labor intensivity and cost of the whole procedure. It is a wide topic and needs to be carefully designed for each particular experiment. [7]

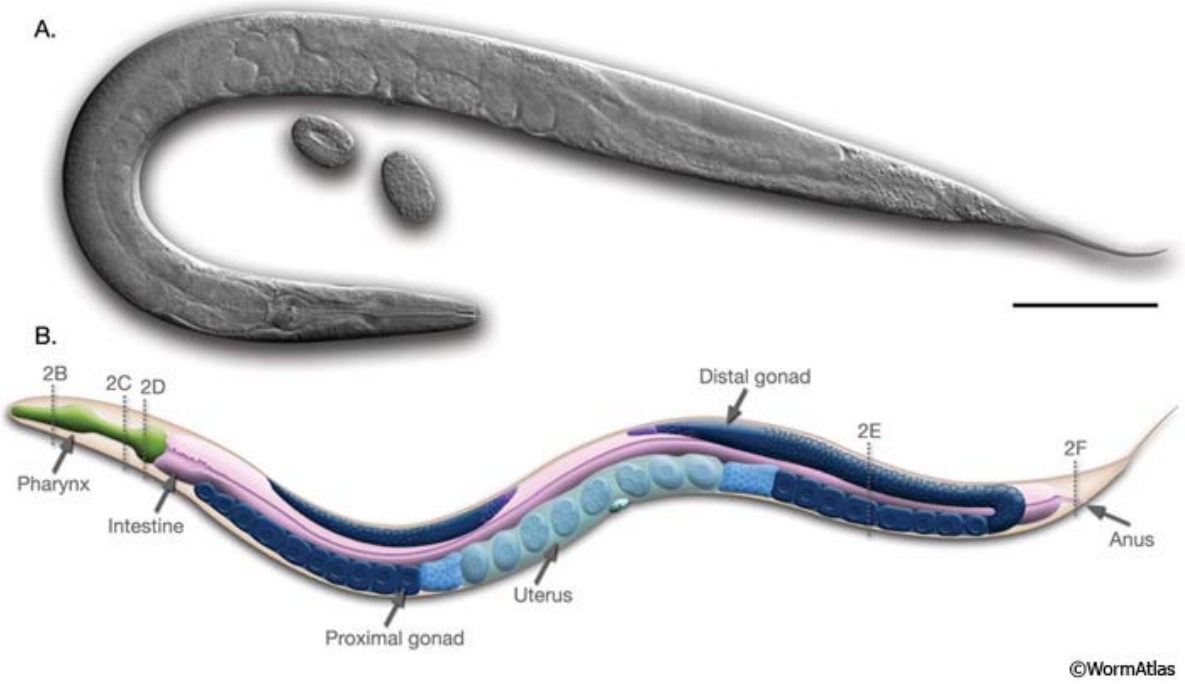

Fig.1: *C. elegans* anatomy - DIC image (A) and schematic illustration (B) of adult hermaphrodite. Scale bar 0.1 mm. Source: http://www.wormatlas.org/

1.2 Development and life cycle

Generation time of *C. elegans* takes three days (at room temperature), embryogenesis itself lasts for about 14 hours. Although the development (depicted in fig.2) of *C. elegans* is mostly autonomous and mosaic, fate of some types of cells depends also on cell-cell interactions[8].

On the beginning of embryonal development five asymmetric divisions give rise to six founder cells AB, E, MS, C, D and P_4. The first division results in AB, an anterior blastomer and a germline progenitor blastomer P_1, which further divides into EMS and P_2. EMS divides in E (the sole founder of the intestine) and MS (the founder of the somatic gonad, muscle, and

the majority of the pharynx, neurons and gland cells) blastomeres and P_2 into P_3 and C (founder of muscle, hypodermis and neurons). Division of P_3 gives rise to P_4 (precursor of germ-line) and D (muscle).[9]

It takes about half an hour until the fertilized zygote develops a chitinous shell and a vitelline membrane. In three hours after fertilization gastrulation proceeds and at that time eggs are deposited outside the mother. Embryogenesis continues first by proliferation and organogenesis. Proliferation ceases about seven hours after fertilization and during the next seven hours morphogenesis occurs - then L1 hatches from the egg.

Larval development comprises four larval stages, designated as L1 to L4, and takes about 50 hours. Transitions between these stages are punctuated by molts. At the second molt, in case of unfavourable environmental conditions, the worm will undergo dauer arrest.

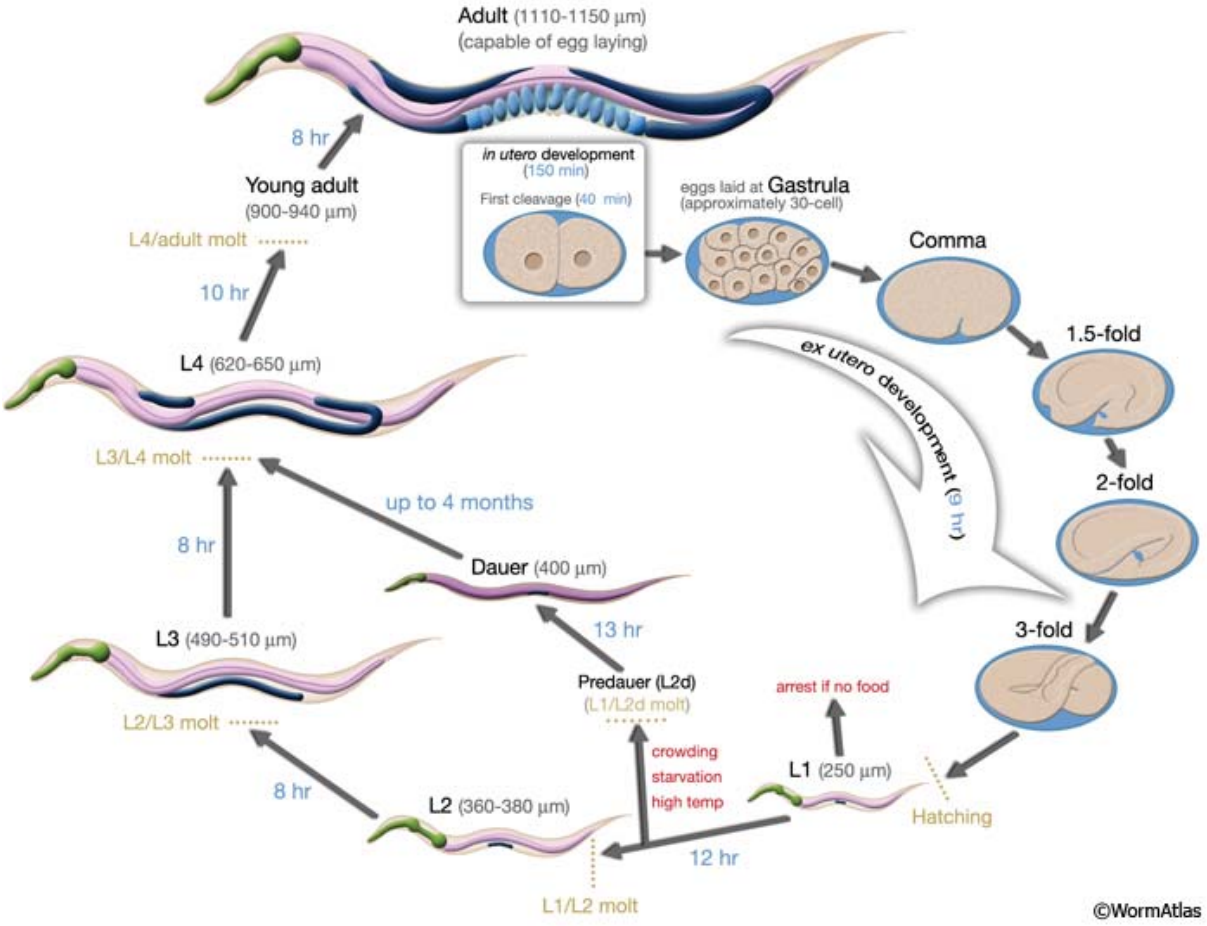

Fig.2: Depiction of *C. elegans* life cycle. Source: http://www.wormatlas.org/

Dauer larvae do not feed and are very resistant to chemicals. Animals survive for up to three months in the Dauer stage. If environmental conditions are getting better and food is available, dauer larvae enter L4 and continue development normally.

Postembryonic divisions are proceeding in almost invariant patterns, resulting in a fixed number of cells - 959 somatic cells in hermaphrodites and 1031 somatic cells in males. The only exception from these patterns presents germ line proliferation.

Under normal conditions (adequate amount of food, temperature 20°C) both sexes live about 17 days after becoming adults.[10]

1. 3 Germ line of *C. elegans* (as a model for stem cell biology)

Germline development proceeds entirely postembryonically in a lineage dependent manner. It is the only tissue that produces cells for the whole life of worm[11]. Two germlines are present in the hermaphrodites whereas one is present in the male. In each case the germline co-develops with a support of somatic tissue, the gonad.

The gonad of *C. elegans* is formed as a blind-ended tube. Hermaphrodites have a two-armed gonad, males possess only a single arm. On the closed - distal - end of the tube the distal tip cell (DTC) is found, which is followed by proliferating germ cells and more proximally by cells entering meiosis. The DTC is a somatic gonadal cell whose depletion leads to defects in proliferation of germ cells. Its mislocation causes ectopic proliferation. Therefore it acts like a germ cell niche. [12]. Germ cells in the most distal part of the gonad are behaving in the way the stem cells do - they are proliferating, but are also able to give rise to more specialized, differentiating cells[13] - either oocytes or sperm.

The germline is derived from embryonic germline blastomeres (P_0 - P_4, see above). These blastomeres are specified mainly by the presence of so called P granules, which are cytoplasmic non-membrane organelles. P granules are rich for RNAs and also RNA-regulating proteins. Another typical characteristics of germline blastomeres is transcriptional silencing caused by PIE-1, a CCCH-type zinc finger protein[14]. Basically, PIE-1 prevents totipotent germline blastomeres to respond to signals causing somatic fate as transformation was shown when this protein is mutated - in that case, one additional posterior blastomere adopts an MS-like fate[15]. Initially PIE-1 is localized on both centrosomes of the mitotic spindle during each germline blastomere division. After that it disappears from somatic daughter cell, but is kept in the germline daughter cell[16]. It acts at the step of transcriptional elongation by inhibiting CDK9 - it behaves like its “pseudosubstrate” and CDK9 is then not further able to phosphorylate C-terminal domain of RNA pol II[17].

The development and function of the germ line is tightly controlled in *C. elegans*. The underlying genetic pathways are highly redundant, further highlighting the importance of its proper control.

One of the most important signaling pathway responsible for germ line development is the GLP-1/Notch pathway[12]. This pathway is known across the phyla to control proliferation and differentiation in animals. It is assumed that germ cells, which are close to DTC, receive a strong signal and are therefore behaving as stem cells.

The Notch signaling pathway has been dissected into its main components. Shortly described, the DTC produces LAG-2, a DSL (Delta, Serrate, Lag-2) family ligand. Germ cells express GLP-1, the Notch family receptor. After LAG-2 binding, GLP-1 is cleaved and the C-terminal fragment enters the nucleus where it forms a complex with LAG-1 and SEL-8/LAG-3 proteins. This complex regulates the transcription of its target genes[18].

Generally said, germ cells under the influence of active GLP-1/Notch pathway are proliferative. Unfortunately, just a few direct targets of GLP-1/Notch pathway are known. GLP-1 protein level is highest in the mitotic region, which resides at the most distal end of the germ line. On the contrary *glp-1* mRNA level is more or less uniform throughout germ line - this suggests some kind of post-transcriptional control. Not surprisingly, proteins acting downstream of GLP-1 are also mostly RNA-regulators - i.e. FBF-1/-2, GLD-1, GLD-2 or GLD-3[19].

1.4 P granules

P granules function as a storage of many RNA-regulating proteins, allowing them to be targeted to specific cell type - presumptive germ line. They are worm version of so-called "germ granules" presented across the species. P granules are non-membrane, enclosed protein-rich cytoplasmic bodies responsible for promoting of germline development[20]. These granules are maternally loaded and passed on from one germline blastomere to the next during embryogenesis, i.e. from P_1 to P_4. Subcellulary, they are localized close to the nucleus.

Although uniformly localized during the one-cell stage, P-granules become asymetrically localized under the influence of cortically localized polarity (PAR) proteins. PAR-2 is supposed to regulate the cortical association of P granules together with PAR-1 protein. PAR-2 is also responsible for promoting anterior localization of the PAR-3, on the other hand PAR-2 remains restricted to the posterior cortex as a result of *par-3* gene activity. This localization also depends on microfilaments[21]. PAR-2 is a RING protein - this domain is responsible for PAR-2 stabilization on the posterior cortex[22].

Basically, all known proteins present in P granules contain some putative RNA-binding motifs[23]. The only components presented for the whole time are members of the

PGL (RGG motif) family and the VASA-related DEAD box helicase motif family of GLH proteins - these are most likely responsible for the P granules formation and stability[20, 24]. The other protein components are mostly proteins acting as translational regulators. These include Sm proteins, involved in RNA splicing, CCCH zinc finger proteins involved in RNA processing such as PIE-1, MEX-1, POS-1 and OMA-1/-2, the KH-domain proteins MEX-3 and GLD-1, and the GLD-2/GLD-3 non-canonical cytoplasmic poly(A)-polymerase[14].

It was shown by Brangwyne and colleagues[25] that P granules behave as liquid-like droplets. They can fuse, drip, they also dissolve and condense rapidly. Their localization is determined by variations in the condensation point in the cell caused by polarity proteins. This leads then in increased posterior condensation of P granules and their enrichment.

Furthermore P granules contain maternally loaded mRNAs of *pos-1*, *mex-1*, *par-3*, *skn-1*, *nos-2* and *gld-1* genes. All of these mRNAs are developmentally regulated[23]. The way P granules act could be deduced from experiments done by Pitt and his colleagues[26]. They found that P granules are tightly associated with nuclear pores and even when they are detached from the nucleus they are still associated with pore-like structures. So it is possible that mRNAs transported out of the nucleus may pass through and be retained in P granules. Then they could be targeted by posttranscriptional regulators presented there for further regulation, deciding on the fate of the mRNA[14].

2. Posttranscriptional regulation (PTR)

The development of every organism requires an orchestrated expression of chosen sets of genes, in exact and perfectly regulated spatial and temporal expression patterns specific for each tissue. Posttranscriptional regulation is particularly important in establishment of morphological patterns, body axes, stem cell maintenance and many other examples.

Regulation of developmental processes is very often based on mechanisms as gradients (i.e. of morphogens) or molecular switches ("yes or no" decisions). Complicated and highly redundant interaction networks and combinatorial regulatory processes are very common. Everything has one aim - precise and coordinated gene expression.

Posttranscriptional control is way much faster than control on the transcriptional level and thereby enables to react rapidly and dynamically to external inputs.

Probably two best explored cell types utilizing posttranscriptional regulation are neuronal and germ cells. Translational control in neuronal cells, is involved in controlling synaptic plasticity, memory formation and other activities[27],[28]. Germline and early embryonic development relies heavily on translational control.

2.1 General mechanisms of translational repression vs. activation

As was indicated above, RNA-regulating proteins are abundant in the germ line. There are more reasons for posttranscriptional control, probably the most important is that it enables embryo to regulate the spatial and temporal gene expression mainly during late germline and early embryo development. When entering meiosis, chromosomes are becoming condensed and transcription becomes progressively inactive. Hence in certain developmental contexts (i.e. late oogenesis/spermatogenesis, fertilization, meiotic divisions, and early embryogenesis) it is necessary to regulate the amount of protein produced from a given mRNA pool[29]. To this end either localized or stored mRNA is used.

mRNA has two major features for translation - 5´cap and 3´poly-A-tail. Both structures are added co-transcriptionally in the nucleus and work synergistically. Each structure is targeted to control the translational output of an mRNA. This form of posttranscriptional gene regulation (PTR) in the cytoplasm is termed translational control.

2.2 Regulation of poly(A) tail length - important aspect of PTR

Translationally unregulated mRNAs are readily translated and the amount of protein synthesized is a consequence of mRNA stability. In contrast to unregulated RNAs, specific RNAs undergo translational repression. Naturally, these need to be reactivated later. One prevalent mechanism how to achieve that is through poly(A) tail length control, because translational repression is coincidental with deadenylation and poly(A) tail shortening. On the contrary, translational activation is coincidental with readenylation and poly(A) tail lengthening.

During maturation, mRNA is polyadenylated in the nucleus. Unregulated mRNAs form translational initiation complex and are readily translated. After every round of translation, poly(A) tail becomes shorter - it occurs during pre-elongation phase. Since it works also as a protection of the coding sequence against exonucleases, the length of poly(A) tail determines mRNA´s lifespan [30].

When translationally regulated mRNA is transported to the cytoplasm, poly(A)-tail is shortened - mRNA is then stable but barely translated. Readenylation by cytoplasmic poly(A)-polymerases allows binding more molecules of poly(A)-binding protein (PABP) and increased ribosomal translation [31].

2.3 Overview of translational regulators involved in poly(A) tail metabolism

Both proteins and nucleic acids (microRNAs [miRNAs] respectively) function as translational regulators. The most important classes of these regulators involved in poly(A) tail length control are PUF proteins (more described in chapter 5 of this introduction), CPEB proteins, cytoplasmic poly(A) polymerases (chapter 4) and microRNAs.

CPEB (cytoplasmic polyadenylation element-binding) proteins are evolutionary conserved RNA-binding proteins. They posses a highly conserved zinc-finger domain and RNA-recognition motif (RRM). They are involved in polyadenylation and - in indirect way - translational activation and repression, by masking mRNAs with the help of maskin and eIF4E and thus preventing their translation [32]. In *C. elegans* four CPEB homologs could be found - *cpb-1, cpb-2*, *cpb-3* and *fog-1*. These show similarity to other CPEB homologs mainly in carboxy-terminal region, where the functional domains are contained: two distinctive RRM motifs and consecutive C4 and C2H2 zinc fingers. Products of *cpb-1* and *fog-1* genes control two different steps in spermatogenesis - progression through meiosis (CPB-1) and specification of the sperm fate (FOG-1) [33]. CPB-3 is involved in multiple processes in

germline. Together with GLD-3 it promotes meiotic entry, participates in the sperm/ocyte switch and in contrast to other three members of CPEBs in worms, CPB-3 is important for oocyte production[34].

MicroRNAs (miRNAs) are known translational repressors - this function could be executed either in deadenylation independent[35], [36] or dependent[37] manner. miRNAs are short (about 22 nucleotides long) noncoding RNAs[38]. Their function was first revealed in *C. elegans*, by discovery of two *lin-4* transcripts (one 22 nucleotides and other 61 nucleotides long) interacting with *lin-14* 3´UTR in RNA - RNA manner and, as a result, causing translational repression of *lin-14* mRNA[39]. Another breakthrough was discovery of *let-7* gene, whose transcript, 21-nucleotides long RNA, is targeting mRNAs of multiple heterochronic genes *lin-14*, *lin-28*, *lin-41*, *lin-42* and *daf-12*[40] - it was found conserved among metazoans, from nematodes to vertebrates. It was not found in *Saccharomyces cerevisiae*, *Escherichia coli* or *Arabidopsis* and some cnidarians and poriferans[41]. MicroRNAs are quiet abundant, they present about 1 % of human, worm and fly genes. They are involved in control of many different processes as cell proliferation, cell death, metabolism, neuronal patterning and other, both in animals and plants[42].

2.4 3´UTR-mediated poly(A) tail length regulation

Specific RNAs, which undergo some kind of posttranscriptional regulation are sharing a common feature - their mRNAs encode this information in their sequence. Those *cis*-regulatory elements, important for PTR are often found in 3´UTR. This region often contains PTR control elements for i.e. cytoplasmic deadenylation and readenylation, regulation of ribosomal subunit joining or translational repression by miRNAs. Latter is usually executed also by means of de-/re-adenylation. Other 3´UTR-binding proteins and small RNAs could influence mRNA stability, its localization or translational efficiency. During development, different 3´UTR isoforms are expressed and its length shortens with age[43].

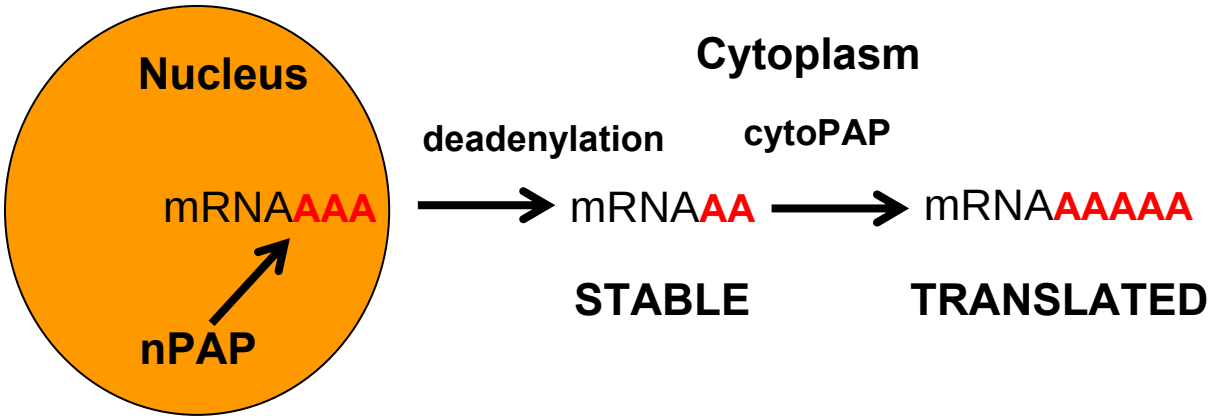

Fig. 3: Schematic illustration of mRNA polyadenylation events

3. Polyadenylation and poly(A) polymerases

3.1 General characterization

Most eukaryotic mRNAs are processed, on 3´end, by endonucleolytic cleavage event, eventually also exonucleolytic digestion[44]. Afterwards a tract of adenosines is added to the 3´end of pre-mRNA[45]. This is one of the essential steps in mRNA maturation, which influences its metabolism and turnover in the cell by acting in many ways. It enables its transfer to cytoplasm[46], confers its stability and improves translation.

3.2 Nuclear polyadenylation

In the nucleus, about 20 - 250 adenosine nucleotides are added by canonical nuclear PAP[44]. Both for cleavage and polyadenylation, consensus sequence of polyadenylation signal - AAUAAA - is required. Any change in this sequence, except of one - AUUAAA, which has similar effects - strongly reduces the accuracy and efficiency of cleavage and polyadenylation *in vitro* in nuclear extracts from HeLa cells [47]. About eight nucleotides downstream of the polyadenylation signal are enough for polyadenylation (without any sequence preference). On the contrary, cleavage requires another downstream, usually U-/UG-rich sequences. The consensus hexanucleotide lies about 15 - 20 nucleotides upstream of polyadenylation site. Its presence is essential for addition of first ten nucleotides, then the mechanism of the reaction changes and further continues until there are about 250 nucleotides added[44].

The polyadenylation hexanucleotide is necessary, for nuclear polyadenylation and it is bound by a protein called cleavage/polyadenylation specificity factor (CPSF). Formation of ternary complex, formed when the cleavage stimulation factor (CstF) is bound strongly enhances CPSF - hexanucleotide interaction. Formation of this complex is step which could by very easily controlled and thereby 3´end processing could be regulated.

3.3 Cytoplasmic polyadenylation

Poly(A) tails of most of the mRNAs entering cytoplasm are continuously shortened and mRNAs are consequently decapped and degraded. But specific mRNAs are kept “intact”, yet are translationally inactive.

In developing oocytes, many maternally derived mRNAs are stored in translationally inactive state - they have short poly(A) tails (20 to 40 nucleotides) and therefore are not

translated nor degraded - they are “masked”. Before translation could start, upon oocyte maturation or after fertilization, their poly(A) tail needs to be elongated - to a length of about 80 or 250 adenosine residues[48].

The phenomenon of cytoplasmic poly(A) tail lengthening is known since 1980s where it was found to happen in different organisms - in clams first[49] and then also in other organisms.

Long poly(A) tails attract efficiently poly(A)-binding proteins (PABPs). Each molecule needs at least 12 adenosine residues to bind and when bound it covers around 25 adenosines. PABPs have also another feature - they bind in multiple fashion - basically, the longer the poly(A) tail is, the more PAB protein molecules are bound[50]. PABP bound to a poly(A) tail interacts with eIF4G and through eIF4G - eIF4E - 5´cap interactions causes circularization of the mRNA[31] and presumably also stabilizes the eIF4E - eIF4G interaction[32].

In the cytoplasm, *cis*-acting sequences necessary for polyadenylation differ from the one in the nucleus. Cytoplasmic polyadenylation element (CPE) is U-rich sequence, i.e. UUUUUAU or UUUUAAU. As was shown in *Xenopus* by Stebbins-Boaz and colleagues[51], polyadenylation hexanucleotide and CPE are necessary, but if (and in which developmental stage) polyadenylation occurs is dependent also on other sequences presented in 3´UTR.

CPEs are bound by Cytoplasmic Polyadenylation Element Binding (CPEB) proteins. Their characteristic features are two RNA-recognition motifs (RRMs) and an RNA-interacting zinc-binding C-H domain.

As was shown on *cyclin B1* mRNA, CPE in combination with CPEB are able to repress (in immature oocytes) and activate translation (after oocyte maturation starts)[52]. The exact mechanisms is not clear yet - they include indirect effect of masking of 5´end, but more important is the effect of protein called Maskin, which is recruited by CPEB to particular RNA and then bind the translational initiation factor eIF4E - this binding repress formation of complete translational initiation complex[53].

C. elegans genome, however, does not encode any homolog of Maskin, whereas it encodes subunits of CPSF - so it is disputable if CPB-3, CPEB homolog in worms, can function as a repressor in this way[34].

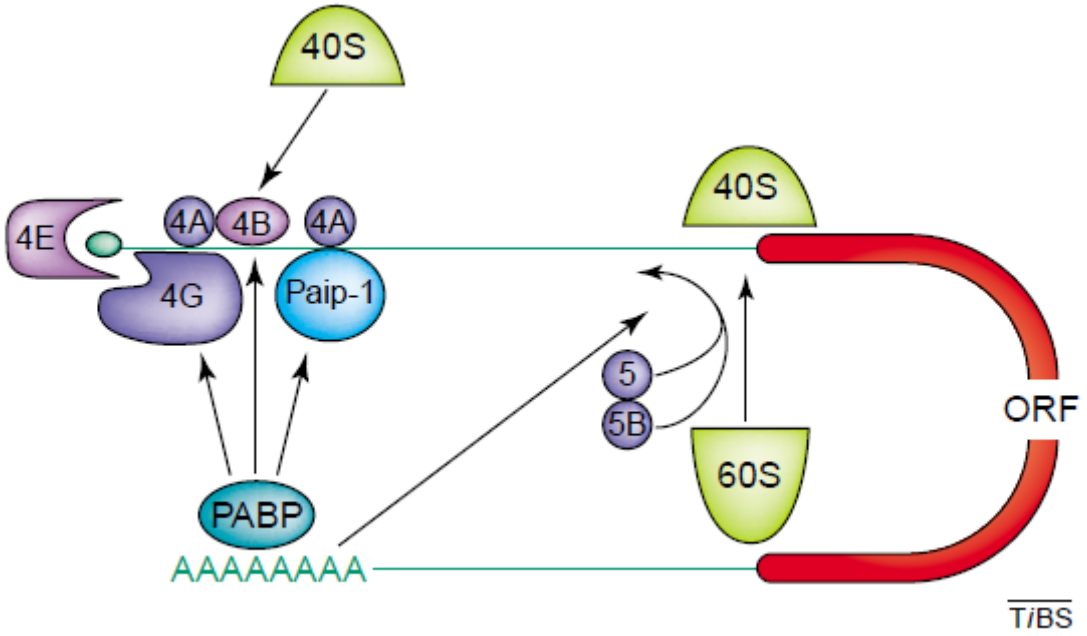

Fig.3: Regulation of translational initiation by poly(A) tail - PABP interacts with eIF4G (causes circularization of mRNA via eIF4G - eIF4E - 5´cap interactions), Paip-1 (interacts with eIF4A and eventually stimulates translation by unknown mechanism) and eIF4B (enhances PABP´s poly(A) binding). **PABP** - poly(A) binding protein; **4A/B/E/G** - eIF4A/B/E/G; **5/5B** - eIF5/eIF5B, **Paip-1** - polyadenylate-binding protein-interacting protein 1. Source: Wilkie, G. S. et al., 2003[31].

During oocyte maturation, CPEB is phosphorylated. That probably weakens the interaction between Maskin and eIF4E and increases the binding affinity of CPEB to CPSF[54]. CPSF then binds the hexanucleotide sequence and recruits the cytoplasmic poly(A) polymerase XGLD-2, which promotes polyadenylation[53]. Another interesting possibility is that polyadenylation and translational derepression are coincidental, meaning that PABP which binds to poly(A) tail is interacting with eIF4G and that it stabilizes eIF4E - eIF4G interaction[32].

4. GLD-2 (*germ line defective-2*)

4.1 Characterization, functions, interactions

In 1998 a gene was described, which was required for normal progression through meiotic prophase and promoted entry into meiosis- this gene was called germline-defective-2 (*gld-2*) [55] and its function was redundant with previously described gene *gld-1*[56]. Epistatic analysis showed, that both genes are redundant, acting in two different genetic pathways downstream of *glp-1*[55]. Further research revealed a complex and tightly regulated protein network controlling germ line development (reviewed i.e. in Kimble, J. & Crittenden, S.L. 2007[19]).

GLD-2 was first identified in *C. elegans* as the first metazoan cytoplasmic poly(A) polymerase[57] (PAP). It belongs to the group 2 of DNA polymerase beta-like superfamily of nucleotidyltransferases. GLD-2, like other PAPs, harbors catalytic and central domains - but shares little outside sequence similarity toDNA beta-like polymerases. *gld-2* gene encodes the catalytic subunit of a noncanonical cytoplasmic PAP.

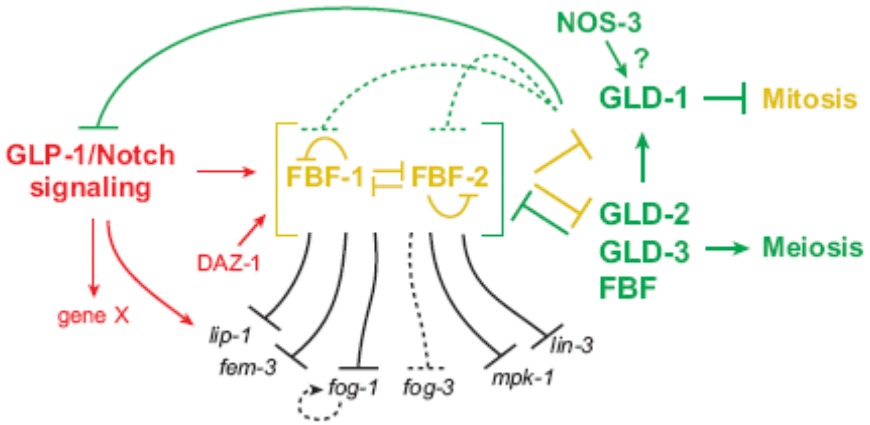

Fig.5: Illustration of complex network controlling the mitosis/meiosis decision in the adult germ line. From: Kimble, J. & Crittenden, S.L. 2007

What is interesting is that it lacks any predictable RNA-binding domain and has only little enzymatic activity on its own. Therefore, it has to form complexes with RNA-binding proteins. One of them, first described, is with GLD-3, which contains five KH-domains and belongs to Bicaudal-C family of RNA-binding proteins[58]. Second known binding partner of GLD-2 is RNP-8 with RNA recognition motif (RRM) [59]. It was shown that complexes of GLD-2/RNP-8 and GLD-2/GLD-3 exist separately, the first one promoting spermatogenesis

and the latter oogenesis[60]. Although GLD-3 and RNP-8 act antagonistically in spermatogenesis/oogenesis decisions, they act synergistically to ensure normal oogenesis[59]. Specific mechanisms in which these complexes act are not yet known and await for determination of targets of each of those complexes.

In *Drosophila*, *Wispy*, the homolog of *gld-2*, encodes the cytoplasmic poly(A) polymerase called WISP. It is required for polyadenylation of *bicoid*, *Toll*, and *torso* mRNAs upon egg activation. Its depletion blocks oogenic development and the transition from egg to embryo[61]. In addition, three other cytoplasmic poly(A) polymerases could be found in *Drosophila*. A canonical one, encoded by gene *hiiragi* (*hrg*)[62] and acting in the earliest steps of oogenesis, together with Orb, the *Drosophila* CPEB protein[63]. Second cytoplasmic poly(A) polymerase is Wispy, which is also interacting with with Orb. It acts later in oogenesis than canonical polymerase - during late oogenesis and early embryogenesis. Its function is required at the final stage of oogenesis for metaphase of meiosis I arrest and for progression beyond this stage[64]. Third cytoplasmic PAP is the WISPY paralog expressed in testis was found - GLD2. Its absence causes defects in development of post-meiotic spermatids (i. e. problems with chromatin reorganization, protamins incorporation) and leads to sterility due to the absence of mature sperm[65].

Recently was shown, that GLD-2 is not the only noncanonical poly(A) polymerase in *C. elegans* important for germline development - in 2009 GLD-4 was discovered[66]. Both polymerases are presented in P granules and form fail-safe system ensuring progression in early meiosis.

4.2 Expression of GLD-2

The *gld-2* gene encodes multiple mRNAs - germ line specific transcript has size about 4.7-kb. Northern analysis showed it is abundant in embryos, L4 stage and adults. In situ hybridization showed that it was abundant in the meiotic pachytene region and in oogenesis, but decreased during spermatogenesis.

gld-2 mRNA is also the first known non-mammalian target of cytidine to uridine RNA-editing event. This change is germ line-specific and occurs on cytidine at the position 10791. It is supposed to cause proline-to-leucine (P400L) change in GLD-2 protein, outside the conserved nucleotidyltransferase domain. No polymerase activity change was observed, but it is possible that maybe folding rate of the protein or its interaction with binding partners could be affected[67].

GLD-2 was found to be predominantly cytoplasmic. Its protein level was detectable in the mitotic region and increases more proximally - it was abundant during pachytene and oogenesis, but decreased during spermatogenesis in both sexes, and was undetectable in mature sperm[57].

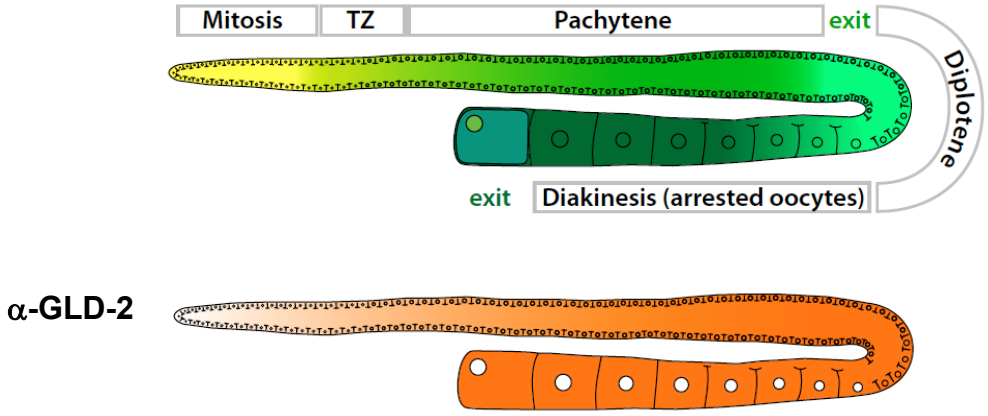

Fig.4: Schematic illustration - *C. elegans* germline (up), expression of GLD-2 in the wild-type germ line (bottom). Credits: Judith Kimble lab

4.3 *gld-2* mutants

For proper analysis of gene function, analysis of functional and structural mutants is of great importance.

Two *gld-2* mutations were identified in previous studies, both carrying lesions in common exons: null mutant, *gld-2(q497)*, has premature stop codon, and *gld-2(h292)* is a missense mutant (E875K)[55, 57]. *gld-2(q497)* lacks practically all functionally relevant parts. *gld-2(h292)* is structural mutant, which has aberrant GLD-3 binding site. Three other mutations - predicted nonsense mutant *gld-2(q535)* and two missense mutations *gld-2(q540*), *gld-2(dx32)* were further analyzed in this work.

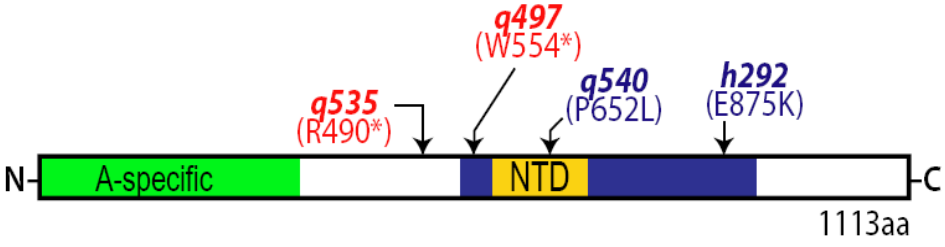

Fig.5: Schematic illustration - GLD-2 with highlighted nonsense (red) and missense (blue) mutations. Adapted from: Wang et al., 2002

5. PUF (Pumilio and FBF [*fem-3* binding factor]) protein family

5.1 PUF protein family

PUF proteins are named after *D. melanogaster* Pumilio and *C. elegans* FBF proteins, first two members of the family, analyzed in detail. The main features of all PUF protein family members, which are conserved in eukaryotic organisms, is that they are all 3′UTR regulatory proteins and - in most cases - act as translational repressors[68]. In a few incidences, one PUF protein, FBF in *C. elegans*, was found to act as a repressor and activator, which may depend on its binding partner[69].

Repressor function of FBF involves regulation of poly(A) tail length, its shortening respectively, and eventual mRNA decay or translational repression[70]. In yeast, two PUF proteins, Puf4p and Mpt5p cooperate in a repression of the target mRNA HO and for full repression, mRNA has to be bound by both of them. They provide sequence specificity to Ccr4p deadenylation activity. Puf4p and Mpt5p bind Pop2p, a component of the cytoplasmic Ccr4p-Pop2p-Not deadenylation complex, and eventually recruits the active deadenylase complex to target RNA[71]. Although Puf4p definitely works in deadenylation-dependent manner[71], it is not that clear for Mpt5p, where some other mechanisms of translational repression may exist[72].

The outcome of PUF protein - mRNA interaction depends, in some cases, on the presence of additional cofactors. Well described example of this is the regulation of posterior embryonic pattern development in *Drosophila*. *hunchback* (*hb*) mRNA is bound by Pumilio. Nanos interacts with Pumilio and recruits Brat (Brain Tumor)[73, 74].

PUF proteins, although usually translational repressor, were also shown to be able act as activators. In the mitotic region of *C. elegans*, FBF binds to *gld-1* mRNA, recruits CCF-1/Pop2p deadenylase complex and thereby promotes deadenylation. The opposite action of FBF is executed in cooperation with GLD-2, but the exact mechanism is still not yet clear. It could either form ternary complex with GLD-2/GLD-3, “tag” the *gld-1* mRNA for activation by GLD-2/GLD-3 complex or repress the repressor of *gld-1* [69].

A different mechanism of translational activation through a PUF protein is observed between the human PUF protein Pumilio-2 and DAZ (Deleted in AZoospermia)[75]. DAZ-Like (DAZL) proteins, which are members of the DAZ protein family possess the ability to translationally activate target mRNAs, at the level of translation initiation, most probably through the direct recruitment of PABPs[76].

PUF proteins are involved in many developmental processes - in both *Drosophila*[77] and *C. elegans*[78] they control germ line maintenance, sex determination[79], stem cell maintenance[80] and others.

While hugely conserved, individual species differ by the number of PUF proteins - one in *Drosophila*, 11 in *C. elegans*[68] and 26 in *Arabidopsis thaliana*[81]. In general, vertebrates seem to have a much smaller number - in humans and mice two members are encoded in the genome[68].

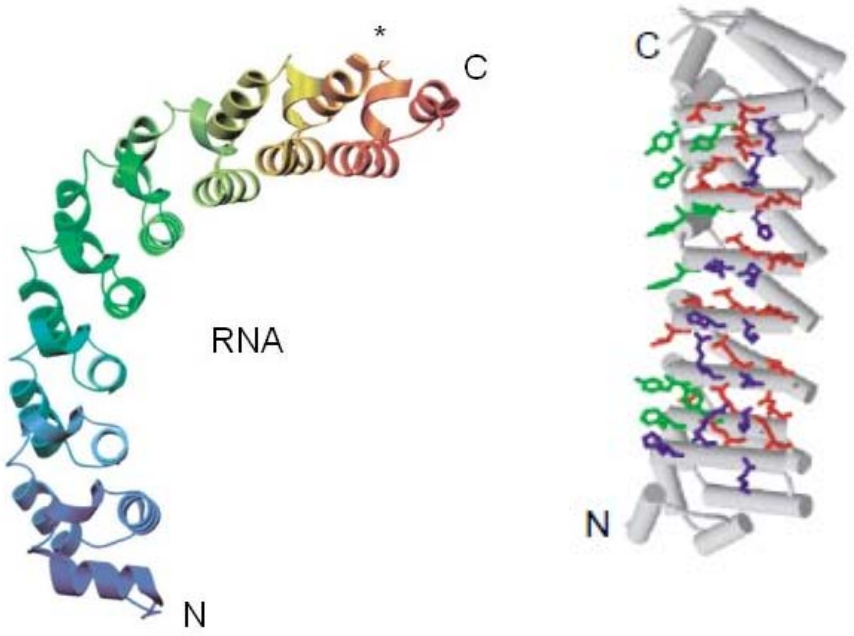

Fig.6: Structure of *Drosophila* Pumilio - Protein structure is formed by eight tandem repeats (each illustrated with different colour). RNA binds to the inner, concave part of the helix. (left) Asterisk indicates Nanos binding site, RNA binds to the inner part of the crescent. (right) Same structure rotated 90 degrees with RNA binding site facing to the reader. From: Wickens, M. et al., 2002; originally Wang, X. et al., 2001[82]

The most typical feature of PUF proteins is so called Pumilio homology domain (PUM-HD) [83] or Puf domain[79] is formed by eight tandem copies of so called PUM repeats - 36 amino acids long motifs. Repeats form a single structural motif and together, they form a crescent-shaped right-handed helix. The outer, convex and acidic side of this helix binds to regulatory proteins such as Nanos, BRAT or a cytoplasmic polyadenylation element binding protein. The inner, concave and highly basic part of the helix is responsible for sequence specific RNA-binding[82].

Each repeat binds to a different RNA base. A single base is bound by three amino acid side chains at conserved positions. Alternations of the side chains cause a change in sequence specificity of the target RNA sequence[84], which is defined as a Puf binding element (PBE).

All known PBEs share common core of a tetranucleotide sequence UGUR (R for A or G). The flanking nucleotides are specific for each individual PUF protein[68]. The position of a PUF binding element is usually in the 3´UTR of an mRNA. Its proximity to the ORF, may also have a partial effect on the translational repression efficiency[85]. The same work also showed ability of FBF protein from *C. elegans* to function - although less efficiently - in different species. This finding further underlines conservation of PUF protein interactions and functions.

5.2 FBF proteins

Hermaphrodite worms produce both types of gametes. From an initial production of spermatocytes they have to switch to oocytes production. This switch is evoked by the suppression of *fem-3* activity (FEMinization of XX and XO animals). Loss of *fem-3* activity prevents sperm production. *fem-3* gain-of-function mutants produce instead of oocytes a vast excess of sperm[86], consequently its repression is essential for the sperm/oocyte switch. Interestingly, *fem-3* mRNA is controlled through its 3´UTR[87]. This control is regulated post-transcriptionally and alterations of poly(A) tail length are involved[88].

Later, a protein was identified, that binds to the 3´UTR of *fem*-3 mRNA and it is responsible for *fem-3* translational repression. This protein is a founding member of the PUF protein family, which was termed FBF (for "fem-3 mRNA binding factor")[79].

Two genes, *fbf-1* and *fbf-2*, encode two almost identical proteins (they share 91 % of amino acid sequence) and represent a functional redundant product of a recent gene duplication[79]. FBF-1 and FBF-2 are also largely functionally redundant, in mRNA binding and are therefore referred to as a single activity of FBF. Single mutants are without any serious defects, but in double mutant worm are defective in oocyte production and mitotic activity[78].

Besides its role in sex determination, FBF activity turned out to be essential for germ line stem cells control: their maintenance and self-renewal. However it is not required for early larval germ line proliferation[78]. Thus FBF promotes both mitosis[89] and oocyte production. On the contrary, GLD-1, a member of the STAR/Quaking/GSG family[90], functions as its antagonist. It inhibits germ cell proliferation[91] and although it is essential for oogenesis[56] it also has a non-essential function in spermatogenesis[56, 91].

FBF seems to act as a molecular switch controlling *gld-1* expression. First, translationally represses *gld-1* mRNA, most likely by interacting with the CCF-1/Pop2p

deadenylase[69]. But later activates it, presumably, via GLD-2 poly(A) polymerase binding and thereby positively affects *gld-1* mRNA polyadenylation.

FBF prevents premature meiotic entry in two ways - first is a control of *gld-1* expression and second is inhibition of proteins involved in synaptonemal complex formation. FBF works as a proper timer of meiotic entry[92].

The FBF RNA-binding consensus, is similar to other PUF proteins, in its core region, but still distinct in the flanking sequences. The minimal required sequence is UGUnnnAU. The *in vitro* further defined FBF-1 binding site is UGURHHAU (R is either A or G and H is A, C or U). Flanking sequences are also important and affect the strength of binding [93].

Merritt and Seydoux then derived a so called "repressive FBE" consensus - CNUGUVNHAU (N is any base and V is A, G or C)[92].

5.3 Other PUF proteins in *C. elegans*

In *C. elegans*, eleven PUF proteins are found. Systematically, they could be divided into two clusters - PUF-8 and PUF-9 belong to the so called "Pumilio cluster" (together with *Drosophila* Pumilio or several vertebrate PUFs) and to "*C. elegans* cluster", where are, besides FBFs, also PUF-3/-4/-5/-6/-7/-10 and PUF-11[68].

PUF-3 is expressed in ovary and functionally included in cell timing, spindle positioning, formation of the polar bodies and pronuclei (http://www.wormbase.org/db/gene/gene?name=WBGene00004239;details=1, last updated 17.07. 2004). PUF-4 was not characterized yet in detail (http://www.wormbase.org/).

PUF-5 shares 48% amino acid identity to PUF-6 and PUF-7. PUF-6 and PUF-7 are almost identical - they have 98% nucleic acid and amino acid identity. These three proteins redundantly control translation of *glp-1* mRNA and some other maternal mRNAs during specific stage of oogenesis (i.e. PUF-5 was found to be expressed during mid and late oogenesis). Their depletion leads to severe defects in oocyte formation and embryonic cell division[94].

PUF-8 protein is highly enriched in germline mitotic region and functions - redundantly with KH domain protein MEX-3 - germline stem cell (GSC) maintenance by promoting mitosis[95]. PUF-8 also blocks dedifferentiation of primary spermatocytes back into the mitotic cells and tumor formation[96].

PUF-9 promotes adult hypodermal state by repression of *hbl-1* (HunchBack Like) via its 3´UTR.. HBL-1 is a temporal regulator and *hunchback* ortholog in *C. elegans*. It promotes

continued cell division and prevent differentiation. Genetic data suggest, that *puf-9* and *let-7* together promote larval-to-adult transition by repression of *hbl-1* and other targets[97].

puf-10 also has high similarity to *puf-6/-7*. It has about 79% nucleotide identity to them. *puf-10* contains in frame stop codon between third and fourth PUF repeat[94].

6. Aim of this work

FBF proteins act in preventing entry into meiosis. GLD-2, on the contrary acts as its antagonist. As shown in Fig.7, both proteins have opposite expression patterns - GLD-2 is barely detectable in the distal (mitotic) zone but increases more proximally in regions where the germ cells undergo meiosis. FBF is mostly expressed in the distal part and its expression decreases more proximally.

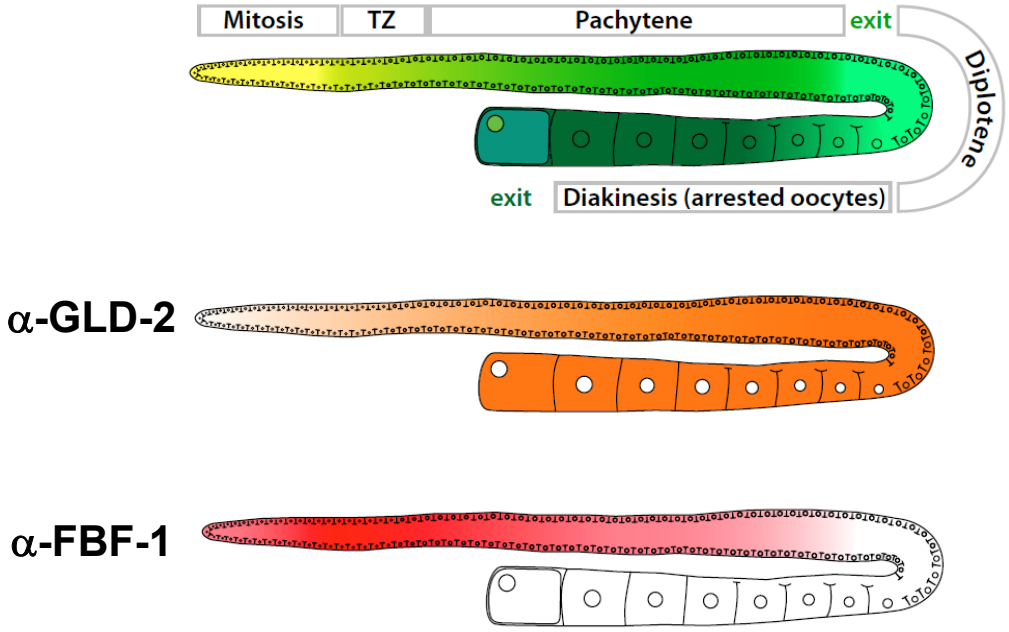

Fig.7: Schematic illustration - (up) *C. elegans* germline. (middle) Expression of GLD-2 in the wild-type germ line. (bottom) Expression of FBF-1 in the wild-type germ line. Credits: Christian Eckmann

These results suggest possible interaction between two antagonists. This interaction would show one of the ways how to finely balance gene expression of their targets and - possibly - of each other. It is even more interesting when one think about ambivalent role of FBF in posttranscriptional regulation. The aims of this work are clear - to analyze if this interaction exists and how it works.

Because FBF acts as a repressor of some meiosis-promoting genes (i.e. *gld-3* or *gld-1*) to which *gld-2* belongs too and expression patterns are opposite, two main questions arise - does FBF binds to *gld-2* mRNA? If yes, does FBF act as a repressor, activator or doesn´t have any role in *gld-2* regulation at all? The aim of this work is to find answers to these two questions.

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1. Genotyping

1.1 Sample preparation

Individually picked worm was put into 6.5 µl protease K (PK) buffer (supported with 1 µl of protease K prewarmed for 5 minutes at 55 °C per 100 µl of buffer). Afterwards, PCR tube was put for 10 - 15 minutes in the - 80°C freezer. Then, the tube was transferred in the PCR cycler and went through the program as followed:

PK treatment

*step*1 65 °C, 1 hour, 15 minutes

*step*2 95 °C, 15 minutes

*step*3 20 °C, forever

For sequencing of regions about 1 kbp long (or more), nested - PCR was used. PCR mix was added to 4 µl of PKed solution (due to the evaporation). PCR mix was prepared as followed:

PCR setup (primers diluted 1:10):	**setup for 1nd round PCR**	**setup for 2nd round PCR**
PKed template	5.0 µl	0.5 µl (1st round PCR)
fwd primer	1.0 µl (10 µM)	1.0 µl (10 µM)
rev primer	1.0 µl (10 µM)	1.0 µl (10 µM)
10x PCR Buffer	2.5 µl	2.5 µl
10mM dNTPs	0.5 µl	0.5 µl
HiFi polymerase	0.05 µl	0.05 µl
H2O	14.95 µl	20.45 µl
Total	25 µl	25 µl

PCR program

*step*1 95 °C, 4 min

*step*2 95 °C, 1 min

*step*3 55 °C, 50 seconds

*step*4 72 °C, 30 seconds

*step*5 goto *step2*, repeat 34 more times

*step*6 72 °C, 7 min

*step*7 20 °C, forever

Enzymatic clean up of PCR product

SAP (shrimp alkaline phosphatase) 1U / µl	0.30 µl
ExoI (exonuclease I) 20 U / µl	0.08 µl
H_2O	1.62 µl

To 2 µl of this mix add 8 µl of diluted template (4 µl 2nd round PCR product + 4 µl of H_2O) and run the following program:

*step*1 37 °C, 15 min

*step*2 72 °C, 15 min

1.2 Sample submission

All the samples were submitted to DNA Sequencing Facility at the MPI-CBG in Dresden. Total volume of the submitted sample was 5 µl - general rules are:

PCR DNA:

10 -25 ng of template per 1000 bases

5 pmol of primer*

H2O to a final volume of 5 µl

Plasmid DNA:

35 - 75 ng of template per 1000 bases to a maximum of 1 µg

5 pmol of primer*

H2O to a final volume of 5 µl

BAC or other large DNA templates:

1 - 2 µg template

10 - 15 pmol primer*

Usually, 2 µl of 10 µmol primers were used in each sample.

2. Western blotting

2.1 Introduction

Western blotting (or immunoblotting) is very useful method for protein detection in various mixtures (in this case worm and yeast lysates). In the first step, all the proteins in the mixture are resolved by denaturating SDS gel electrophoresis, then transferred to the nitrocellulose membrane and stained.

First, membrane is washed in buffer and blocked by milk to minimise unspecific binding by antibodies. Afterwards, primary antibody is added, membrane is washed again with the buffer and then secondary antibody against the primary is added. This secondary antibody is coupled with an enzyme - depending on the substrate used during exposure - either horse-radish peroxidase or alcalic phosphatase.

2.2 Western blot protocols

2.2.1 Worm lysates

1. Single-pick 40 worms into 1,7ml eppendorf tube with 5 µl of M9 buffer
2. Freeze the sample at - 80 °C
3. Add 20 µl of hot (95 °C) SDS sample buffer and boil for 5 - 10 minutes
4. Spin and sonicate in a water bath at 70 °C for 10 minutes
5. Spin, boil for 5 minutes before loading
6. Spin, full-speed for 3 - 5 minutes, use supernatant for loading on the gel

2.2.2 Yeast lysates

1. Grow cells in 5 ml of the medium, until OD_{600} equals 1
2. Harvest 4 OD units (4000 rpm, 5 - 8 minutes)
3. Wash in 1 ml of 20% TCA (samples could be freezed at this point)
4. Resuspend in 200 µl at room temperature
5. Add 200 µl of glass beads
6. Vortex 8-times for 30 seconds or leave for 10 minutes in beads-shaker
7. Save supernatant and repeat steps 4 - 7 for washing, use 200 µl 5% TCA, vortex 45 seconds each (two times) or let shake for 5 minutes
8. Combine supernatants

9. Spin at 3000 rpm for 10 minutes (room temperature)
10. Resuspend pellet in 200 µl of SDS sample buffer
11. Add 100 µl of 1M Tris - base
12. Boil for 3 minutes, spin again, save supernatant in fresh tube, load 10 to 15 µl

2.2.3 SDS-PAGE

Separation gel:	**8% PAA**	**9% PAA**
40% acrylamide	2 ml	2.25 ml
1M Tris - pH 8,8	3,75 ml	3,75 ml
H_2O	4,05 ml	3,80 ml
10% SDS	100 µl	100 µl
10% APS	80 µl	80 µl
TEMED	20 µl	20 µl

Total volume is 10 ml, which is enough for two gels. Poor the mixture fast between the glasses (1mm thick) and layer with iso-propanol. Let it polymerize.

Wash isopropanol with water and poor stacking gel:

Stacking gel (10 ml):

40% acrylamide	0,63 ml
1M Tris pH - 6,8	0,67 ml
H_2O	4,05 ml
10% SDS	100 µl
10% APS	50 µl
TEMED	20 µl

Solution without 10% APS and TEMED pre-prepared and stored in refrigerator. Before use, add 15 µl of TEMED and 40 µl of APS. Poor fast and insert a comb, let polymerize. Load samples and run through the gel with about 25mA per one gel and max. 80V per gel for about 1 hour.

2.2.4 Blotting and probing protocol

1. Soak nitrocellulose (NC) membrane in distilled water

2. Soak well in 1x blotting buffer:

2 Whatman filter papers

2 scotchpads

PAGE gel

membrane filter (a) or (b)

3. On bench, prepare the "sandwich" as follows (bottom → top (1 → 4)):

(1) Whatman paper (2) gel (3) membrane (use glass rod to remove air bubbles) (4) Whatman paper

4. Move sandwich on a scotchpad (black side) and cover with other scotchpad (white side)

5. Transfer in 1x blotting buffer by cooling and very gently stiring the buffer solution, parameters: 400mA, 1hrs/60kDa, (check V, ~100)

6. Stain with Ponceau marker bands unless stained Marker has transfered well

(Membrane could be eventually stored in 1 x PBS with 0.05% sodium azide at 4 °C).

7. Rinse the membrane with 1x PBST for 1 - 2 minutes

8. Block the membrane in 1x PBST, 5% non fat dry milk solution for at least 10 minutes (better longer ~20 - 30 minutes)

9. Add primary antibody, dissolved in 1x PBST, 0.5% non fat dry milk solution for 1 - 2hrs (or at 4 °C overnight)

10. Remove the antibody solution (could be used again two or three times), rinse once generously and wash membrane 3x with 1x PBST.

11. Add secondary antibody in 1x PBST 0.5% non fat dry milk solution for one hour usually (when the weaker signal is expected leave it there for two hours)

12. Remove the secondary antibody (don´t use it again) and wash membrane 4x with 1x PBST.

13. Get rid of excess fluid - membrane should never bee dry - and put on foil. Add 1ml of ECL solution (0.5ml solA and 0.5ml solB) per 8 x 6 cm membrane for horse-radish peroxidase-coupled secondary antibody

14. Incubate @ RT for 1 - 5 min.

15. Remove excess solution

16. Close foil and press tight (wick off excess solution)

17. Incubate with X-ray film from 10 seconds to 1 hour (start with 10 seconds and 1 minute exposures first).

2.2.4 Stripping protocol

1. Prepare the stripping solution - add 1 ml of 100% acetic acid and 1 ml of 10% SDS in 100 ml of H_20
2. Put the membrane in stripping solution and leave for 30 minutes at room temperature, shake couple of times
3. Afterwards, wash well with PBS, add about 80 µl of Tris pH 8,8. Repeat three times for about 10 minutes
4. Block the membrane with milk

3. Yeast three-hybrid (Y3H) assay

3.1 Introduction

The importance of mRNA posttranscriptional regulation was already mentioned in the chapter "Introduction". To analyze this type of regulation is thereby necessary to have efficient tool for RNA - protein interaction analysis. As Bernstein and colleagues mentioned in their work from 2002 [98] the only known system used to identification of new RNA binding proteins of biological significance was yeast three-hybrid system.

Design of this experiment (also illustrated in fig.8) was as follows - first hybrid molecule consists of DNA-binding protein, LexA, which is fused to bacteriophage MS2 coat protein. LexA recognizes 17-nucleotide long binding site, placed upstream of reporter gene - *lacZ*. Second hybrid molecule consists of two MS2 coat protein binding sites, which form stem-loop structure, linked to the tested RNA sequence. Third hybrid molecule consists of analyzed RNA-binding protein (PUF-5/-6/-8, FOG-1, FBF proteins and CPB-1/-2/-3) and Gal4 activation domain. In case that there is interaction between RNA-binding protein and RNA of our interest, Gal4 initializes expression of β-galactosidase. Yeast colonies producing this enzyme will turn blue after addition of X-gal substrate. First hybrid molecule is present in the genome of *S. cerevisiae* strain YBZ-1, RNA construct is introduced in pIIIA/MS2-2 plasmid vector (described in fig-9) [98] and third hybrid is introduced in pACT2 or pACT4 plasmid vectors.

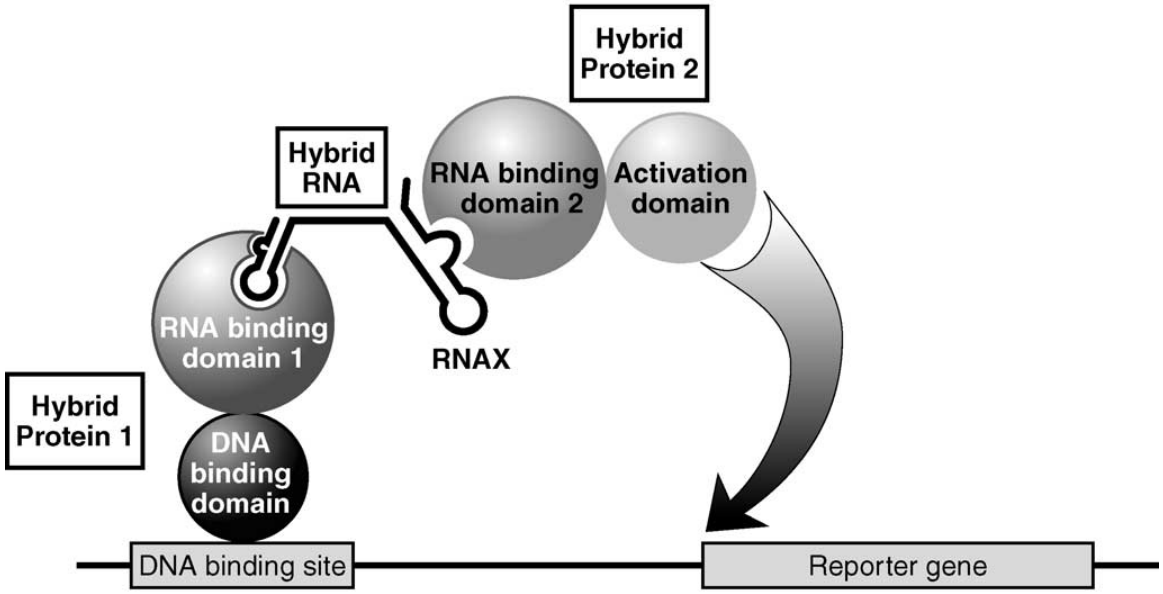

Fig.8: General strategy of yeast three-hybrid assay experiment - DNA binding site - *LexA* operator; DNA-binding domain - LexA; RNA-binding domain 1 - MS2 protein; hybrid RNA - two *MS2*-binding sites with RNA of our interest; RNA-binding domain 2 - protein, whose interaction is analyzed; activation domain - Gal4 activation domain; reporter gene - *lacZ*. From: [98]

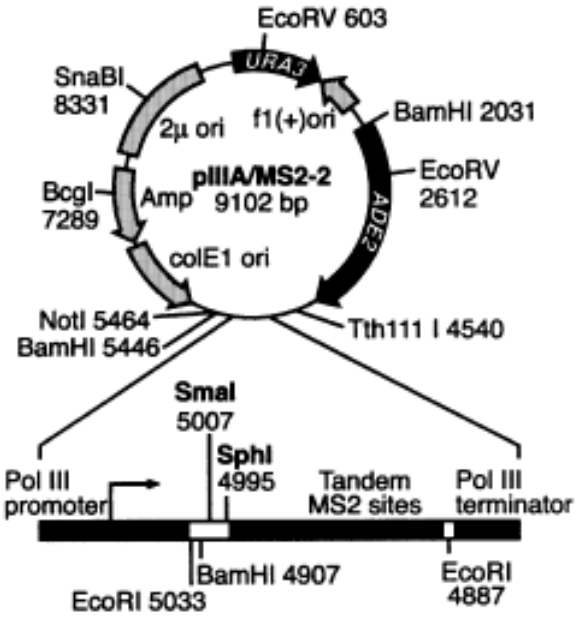

Fig.9: Plasmid vector used to express RNA - pIIIA/MS2-2 - restriction sites for SmaI and SphI are unique are were used for insertion of the desired fragment. From: [98]

3.2 Protocols

3.2.1 Preparation of RNA constructs

Cloning - oligonucleotides

CE2319(gld2-FBE1bdsF)

ccggAATCATCTATCAGATATGTGATGATCTCTACAATTTTTgcatg

CE2320(gld2-FBE1bdsR)

cAAAAATTGTAGAGATCATCACATATCTGATAGATGATT

CE2321(gld2-FBE2bdsF)

ccggCCTTCTCTCCACCCACATTTCCCTGTATTTTAACACGAAAAgcatg

CE2322(gld2-FBE2bdsR)

cTTTTCGTGTTAAAATACAGGGAAATGTGGGTGGAGAGAAGG

CE2323(gld2-FBE3bdsF)

ccggTAAAATACAAATTATATGTACAAACTTTCGAAAAgcatg

CE2324(gld2-FBE3bdsR)

cTTTTCGAAAGTTTGTACATATAATTTGTATTTTA

CE2325(gld2-FBE4bdsF)

ccggAACTTTCCACAATTCCTGTTCTGTAATTTTCTCTCgcatg

CE2326(gld2-FBE4bdsR)

cGAGAGAAAATTACAGAACAGGAATTGTGGAAAGTT

Cloning - PCR fragments

a) **CE2329(gld2-FBEsbdsSma)** tgctCCCGGGatcatttgttcctcgaaaatc

CE2330(gld2-FBEsbdsSph) gtgtGCATGCaacgtgtacctcgttttgaac

gld-2 3'UTR in plasmid was used as a template.

b) **CE2329(gld2-FBEsbdsSma)** tgctCCCGGGatcatttgttcctcgaaaatc

CE2331(gld2-FBE1bdsSph) gacgGCATGCcatcgtattctgaaaattcgcaa

gld-2 3'UTR in plasmid was used as a template.

c) **CE2332(gld2-FBE1bdsSma)** attgCCCGGGtttcggttcatcagaactttcc

CE2333(gld2-FBE1bdsSph)	tttcGCATGCaccaatcacacagaaactcacaac

Fosmid ZC308 was used as a template.

Double restriction digest of vector

restriction buffer	4 µl
Sph I	0.25 µl
Xma I	0.25 µl
vector pIIIA/MS2-2	2 µg
BSA	0.4 µl
dH2O	up to 40 µl

1. Let for 1 hour at 37 °C
2. Separate on the agarose gel (1,5%)
3. Purify the DNA with PROMEGA kit

Undigested vector used as a control.

Vector dephosphorylation

eluted vector	
10x SAP buffer	4 µl
SAP enzyme (2 U/ µl)	2 µl
total volume	40 µl

1. Incubate at 37 °C for 1 hour
2. Heatkill the enzyme activity at 65 - 72 °C for 25 minutes
3. Gel-purify and elute in 40 µl of H2O
4. Check 1 - 2 µl on the agarose gel

Oligo-phosphorylation with polynucleotide kinase (PNK)

H_2O	15,5 µl
Oligo fwd	3 µl
Oligo rev	3 µl

BSA (10 µg / ml)	0,5 µl
10 mM ATPs	3 µl
10x PNK Buffer	3 µl
T4 PNK (5 U / µl)	2 µl

Incubate for 1 hour at 37 °C and heatkill at 65 °C for 25 minutes.

Double digestion of PCR fragments

restriction buffer	2 µl
Sph I	0,25 µl
Xma I	0,25 µl
vector pIIIA/MS2-2	1 µg
BSA	0,2 µl
dH2O	up to 20 µl

1. Incubate for 1 h at 37 °C
2. Separate on the agarose gel (1,5%)
3. Purify the DNA with PROMEGA kit

Dilution and ligation (in multiple concentrations)

H_20	10 µl - (insert) – (vector)
vector 50 ng / µl	1-2 µl
insert (3 x more molar than vector)	x µl
2 x ligation buffer	10 µl
T4 DNA ligase (Roche)	1 µl

<u>Quickchange protocol</u>

T_m should be more or equal to 78°C. T_m calculation:

$T_m = 81.5 + 0.41(\%GC) – 675 / N - \%mismatch$

where: N = primer length (25 - 45 bp)
% number are whole numbers

GC pair content should be more than 40 %; FPLC or PAGE purified primers are preferred.

Primer mismatches should be in the middle of the primer.

PCR setup:

DNA (5 ng / µl)	2 µl
primer 1 (125 ng / µl)	1 µl
primer 2 (125 ng / µl)	1 µl
10x PCR buffer	5 µl
10mM dNTPs	1 µl
Pfu Turbo Polymerase	1 µl
H2O	39 µl

DNA template shoud vary in three different concentration

PCR program:

*step*1 95 °C, 1min
*step*2 95 °C, 50 seconds
*step*3 60 °C, 50 seconds
*step*4 68 °C, 1 kb = 2 min
*step*5 goto STEP2, repeat 17 more times
*step*6 68 °C, 7 min
*step*7 END

1. Add 1 µl DpnI (restriction enzyme)
2. Incubate at 37 °C for 60 - 90 minutes
3. Transform 2 - 5 µl (or even more) electrocompetent XL1 blue cells

3.2.2 Transformation of electrocompetent *E. coli*

1. Prepare on ice - cuvettes for electroporation, 1 ml of water, eppendorf tubes and XL - 1 cells
2. Pre-warm SOC medium on 37 °C
3. Put 900 µl of cold water into the tube with XL - 1 cells
4. Take 100 µl of solution per each sample

5. Add 1 µl (but could be much more, even 15 - 30 µl) of ligation product (and empty vector as a negative control)
6. Let it stay on ice for a while (15 minutes)
7. Transfer it into the cuvettes and electroporate them - voltage 2,5 V
8. Add, immediately, 800 µl of SOC medium
9. Let them grow for 1 hour at 37 °C
10. Plate 100 µl on one plate, harvest the rest, leave about 200 µl and plate the rest on another plate
11. Incubate overnight at 37 °C

3.2.3 Transformation of yeast

1. Inoculate single colony in 5 - 20 ml of YPD, grow at 30 °C overnight
2. Dilute the culture to 5 x 10^6 / ml in 50 ml YPD - add (8.3 / OD_{600}) ml of culture
3. Grow at 30 °C, till OD_{600} is between 0.7 to 1
4. Harvest cells, at 4000 rpm for 5 minutes
5. Discard entire supernatant (remove all residual water)
6. Resuspend cell pellet in 1 ml of 0.1M LiAc, transfer to eppendorf tube and pelet them (full speed for about 15 seconds), remove supernatant
7. Resuspend cells (100 µl pellet) in 400 µl of 0.1M LiAc (total desired volume is 500 µl)
8. Dispense 50 µl for one transformation, harvest cells (8000 rpm for 15 seconds) and discard supernatant
9. Add 326 µl of transformation mix to cell pellet and 300 ng of each plasmid diluted in 34 ml of water
10. Rack cell vigorously, vortex
11. Incubate at 30 °C for 25 minutes, then shift to 42 °C for another 25 minutes
12. Harvest cells (8000 rpm, 15 seconds), gently resuspend in 1 ml H_2O, plate 150 µl / 500 µl on desired plates with selective medium
13. Incubate for 2 - 4 days at 30 °C

Transformation mix (for one reaction)

PEG (50% w/v)	240 µl
1M LiAc	36 µl
salmon sperm DNA (2 mg/ml)	50 µl

ssDNA should be boiled before use.

3.2.4 β - galactosidase filter assay

1. Pick one colony of transformed yeast out of the plate with selective medium with the pipette tip
2. Put the nitrocellulose filter on new plate with selective medium
3. Streak colony onto the nitrocellulose membrane and let it grow overnight
4. Submerge filter in liquid N_2 for 5 minutes
5. Let it thaw at room temperature
6. Prepare two Whatman filter circles in the lid of Petri dish and pour 1,5 ml of Z - buffer + 30 μl of X-gal
7. Put the nitrocellulose membrane on the Whatman filters, cover the dish and seal
8. Turn it over, put in 37°C incubator and check it every half an hour for the color change

4. Chemicals & materials

4.1 List of solutions and chemicals

Acrylamide - Bis

Serva, Lot # P100205

5x blotting buffer

15.14 g/L Tris base and 72.06 g/L glycine

10x DNA loading dye

0.25 % bromophenol blue, 0.25 % xylene cyanol, 50 % glycerol.

HRP - Juice Components A

p.j.k, Lot # 09239A

HRP - Juice Components B

p.j.k, Lot # 09239B

LB medium:

1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, adjusted to pH 7 and autoclaved.

1M LiAc

1 M lithium acetate adjusted to pH 7,5 with diluted acetic acid, filtered.

2 - Mercaptoethanol

Serva, Lot # 100367

M9 Buffer

22mM KH_2PO_4, 22mM Na_2HPO_4, 85mM NaCl, 1mM $MgSO_4$

5% milk

5% (w/v) powdered, non-fat, dry milk in PBS-T

50% PEG 3350 (polyethylene glycol)

Prepared with sterile H_2O, autoclaved.

PBS

137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4

PBS-T

0.05% Tween-20 in PBS

Salmon sperm DNA

10x SDS-PAGE running buffer

30.2 g/l Tris buffer grade, 142.0 g/l glycin, 10.0 g/l SDS.

2x SDS sample buffer

2 ml Tris pH 6.8 (1 M), 4.6 ml glycerol (50%), 1.6 ml SDS (10%), 0.4 ml bromophenol blue (0.5%), 0.4 ml β-mercaptoethanol.

SOC medium

20 g/l bacto tryptone, 5 g/l yeast extract, 20 mM glucose, 0.5 g/l NaCl, 2.5 ml/l KCl (1 M), adjusted to pH 7 and autoclaved

Stacking gel ready mix

213.5 ml ddH2O, 25 ml 40% Acrylamide/Bis (37.5:1), 31.5 ml 1M Tris pH 6.8 and 2.5 ml 10% SDS were mixed and stored at 4°C. For 2 stacking gels 5.45 ml were used, 15 µl TEMED and 40 µl APS (10%) were added.

5x TBE

89mM Tris (54 g/l), 89mM boric acid (27.5 g/l), 2mM EDTA (2.93 g/l).

20% TCA (trichloroacetic acid)

N, N, N´, N´ - Tetramethylethylen diamine (TEMED), SIGMA Life Sciences

X-Gal

5-bromo-4-chloro-3-indolyl-β-D-galactoside; 66 µl mixed with 5 ml of Z - buffer

Z - buffer

60mM Na_2HPO_4, 40mM NaH_2PO_4, 10mM KCl, 1mM $MgSO_4$

4.2 Cells

***E. coli* HT115** feeding bacteria: F-, mcrA, mcrB, IN (rrnD-rrnE)1, lambda -, rnc14::Tn10 (DE3 lysogen: lacUV5 promoter -T7 polymerase) (IPTG-inducible T7 polymerase) (RNase III minus). The Tn10 transposon interrupting the rnc14 gene carries a tetracycline resistance gene.

***E. coli* OP50**: uracil auxotroph, standard feeding bacteria for nematode propagation.

***E. coli* XL-1** blue electro-competent cells: endA1 gyrA96(nalR) thi-1 recA1 relA1 lac glnV44 F'[::Tn10 proAB+ lacIq delta (lacZ)M15] hsdR17(rK- mK+).

***S. cerevisiae* YBZ-1**: genotype MATa, ura3-52, leu2-3, 112, his3-200, trp1-1, ade2, LYS2two colons(LexAop)-HIS3, ura3two colons(lexA-op)-lacZ, LexA-MS2 MS2 coat (N55K)

4.3 Worm strains

Worms were grown in the fridge at 20 °C (sometimes also 16 °C) and handled according to standard procedures[99].

EV108: *gld-1(q485) / ccIs4251 unc-15(e73) I; fbf-1(ok91) fbf-2(q738) / [mIs14 dpy-10(e128)] II*

EV244: *gld-2(dx32) / hT2g(I;III)*

EV271: *gld-2(q540) I / hT2g(I;III)*

JK1976: *gld-2(q535) unc-13(e51) / dpy-5(e61) unc-87(e843) I*

JK2497: *gld-2(h292) I / hT2g(I;III); fem-1(hc17ts) IV*

JK2589: *nos-3(q650)*

JK2879: *gld-2(q497) gld-1(q485) I / hT2g(I;III)*

JK3008: *gld-3(q730) / mIn1[mIs14 dpy-10(e128)] II*

JK3026: *gld-2(q497) / hT2[qls48](I;III)*

JK3128: *gld-1(q485)/ccIs4251 unc-15(e73) I; fbf-1(ok91) fbf-2(q704) / mIn1[mIs14 dpy-10(e128)] II*

JK3182: *gld-3(q730) nos-3(q650) / mIn1[mIs14 dpy-10(e128)] II*

JK3542: *fbf-2(q704) fbf-1(ok91) gld-3(q730) nos-3(q650) / mIn1[mIs14 dpy-10(e128)] II*

The wild-type strain was bristol N2

4.4 Antibodies

Antibody	Dilution
mouse anti-GLD-2	1:2000
rabbit anti-GLD-2	1:2000
mouse anti-CPB-3 (173)	1:1000
mouse anti-tubulin	1:60 000
rabbit anti-PAB-2	1:4000
rabbit anti-GLD-4	1:5000
mouse anti-HA	1:4000

References - Eckmann laboratory

4.5 Materials

Amersham Hyperfilm™ ECL, GE Healthcare

Centrifuge 5415D, eppendorf

Electrophoresis Power Supply EPS 301 and EPS 601, Amersham Biosciences

GFP - scope MZFLIII, Leica

magnetic stirring hotplate MR 3001, Heidolph

Megafuge 1.0 R centrifuge, Thermo Scientific

Nytran SPC 0.45 µm Nylon Transfer Membrane, Whatman™, Item # 10416216

PCR cycler MJ research PTC 200, BIO - RAD

Precise™ Protein Gels (Tris - HEPES - SDS) Precast Polyacrylamide Mini Gels (4 - 20% gradient gels), PIERCE

Protran BA85 Nitrocellulose Transfer Membrane 300 mm x 3 m 0.45 µm pore wire, Whatman™, Lot # D119354

shaker Unimax 1016, Heidolph

sonicator Sonorex Super RK102M, Bandelin

table shaker Unitwist RT

Thermomixer comfort 5355, eppendorf

Thermomixer compact, eppendorf

UltraPure™ Agarose, Invitrogen, Cat # 15510-027

Vortex Genie - 2, Scientific Industries

Wizard® Plus SV Minipreps DNA Purification Systems, Promega, Cat # A1460

Wizard® SV Gel and PCR Clean-Up System, Promega, Cat # A1460

X-OMAT 2000 Processor, Kodak

yeast medium CSM-HIS-LEU-URA, BIO 101® Systems, Cat # 4531-212

Results

1. Molecular lesions in different *gld-2* mutants

1.1 Confirmation of *gld-2(h292)* and *gld-2(q497)*
1.2 Identification of two novel mutations - *gld-2(q535)* and *gld-2(q540)*
1.3 *gld-2(dx32)* mutation

2. Western blotting - protein levels in *gld-2* mutants

2.1 Final Western blots
2.2 GLD-2 protein levels
2.3 CPB-3 protein levels
2.4 PAB-2 protein levels

3. Western blotting - protein levels in different genetic backgrounds

3.1 Final Western blots
3.2 GLD-2 protein levels
3.3 CPB-3 protein levels
3.4 PAB-2 protein levels

4. Yeast three-hybrid (Y3H) assay results

4.1 β - galactosidase assay results
4.2 Control of expression of tested proteins

1. Molecular lesions in different gld-2 mutants

1.1 Confirmation of *gld-2(h292)* and *gld-2(q497)*

Molecular lesions in two *gld-2* mutations - *gld-2(h292)* and *gld-2(q497)* - were previously identified [57] and confirmed in my experiments. *gld-2(h292)* is a missense mutation. Guanosine-to-adenosine transition causes change of aspartic acid for lysine (E875K) and affects the interaction of GLD-2 with GLD-3. *gld-2(q497)* is a nonsense mutation. Guanine-to-adenine transition causes a change of amino acid tryptophan into the premature stop codon (W554*). *gld-2(q497)* is considered to be null mutant.

1.2 Identification of two novel mutations - *gld-2(q535)* and *gld-2(q540)*

I have identified the molecular lesions in two *gld-2* mutants - *gld-2(q535)* and *gld-2(q540)*. *gld-2(q535)* is a nonsense mutation, located upstream of *gld-2(q497)*. Cytosine-to-thymidine transition causes a change of amino acid arginine into the premature stop codon (R490*) - see figures 10 and 11. *gld-2(q540)* is a missense mutation positioned in a nucleotidyl transferase domain. Cytosine-to-thymidine transition causes a change of proline to lysine (P652L) - see figures 12 and 13.

1.3 *gld-2(dx32)* mutation

Finally, *gld-2(dx32)* is a novel mutant, previously unidentified in the lab. I sequenced all the exons of *gld-2* and some of the intronic regions, but did not find the molecular lesion of this mutant. This mutation could be either in the promoter region, in one of the intronic sequences, 5´UTR or 3´UTR, which were not sequenced, possibly modulating splicing outcome.

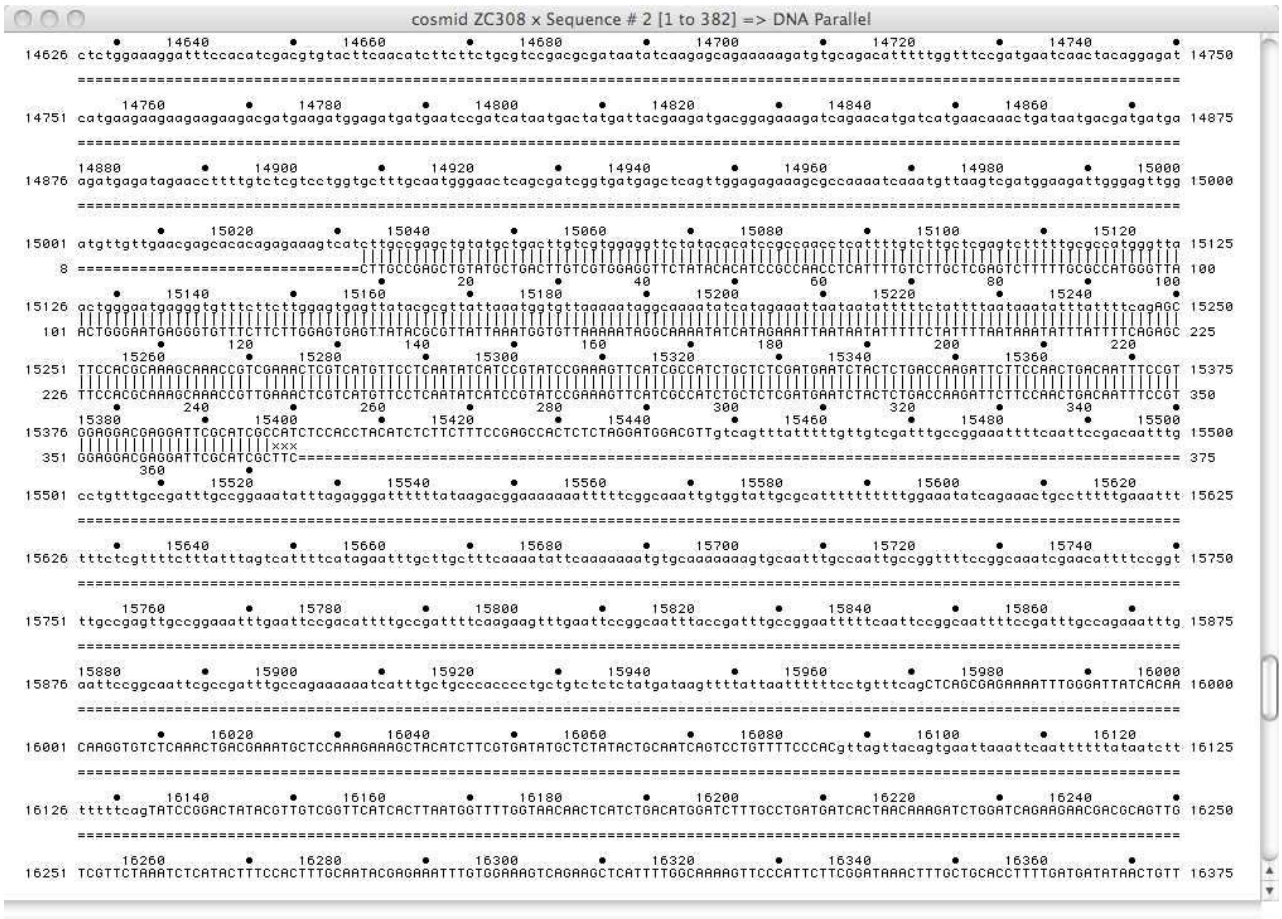

Fig.10: Comparison of wild-type sequence and that of the JK1976 strain, carrying the *gld-2(q535)* mutation. The lesion was found at the position 15271 of the ZC308 cosmid.

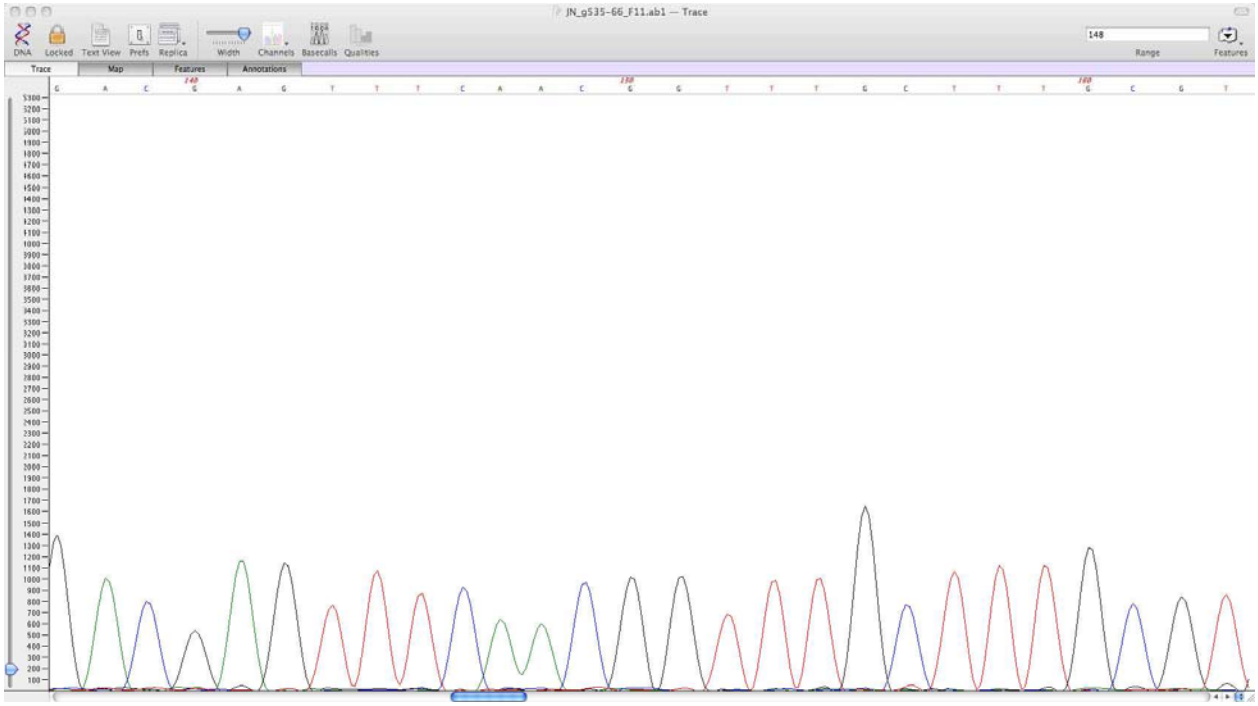

Fig.11: Sequencing diagram of the JK1976 strain, carrying the *gld-2(q535)* mutation. The diagram shows that the sequencing result is highly confident.

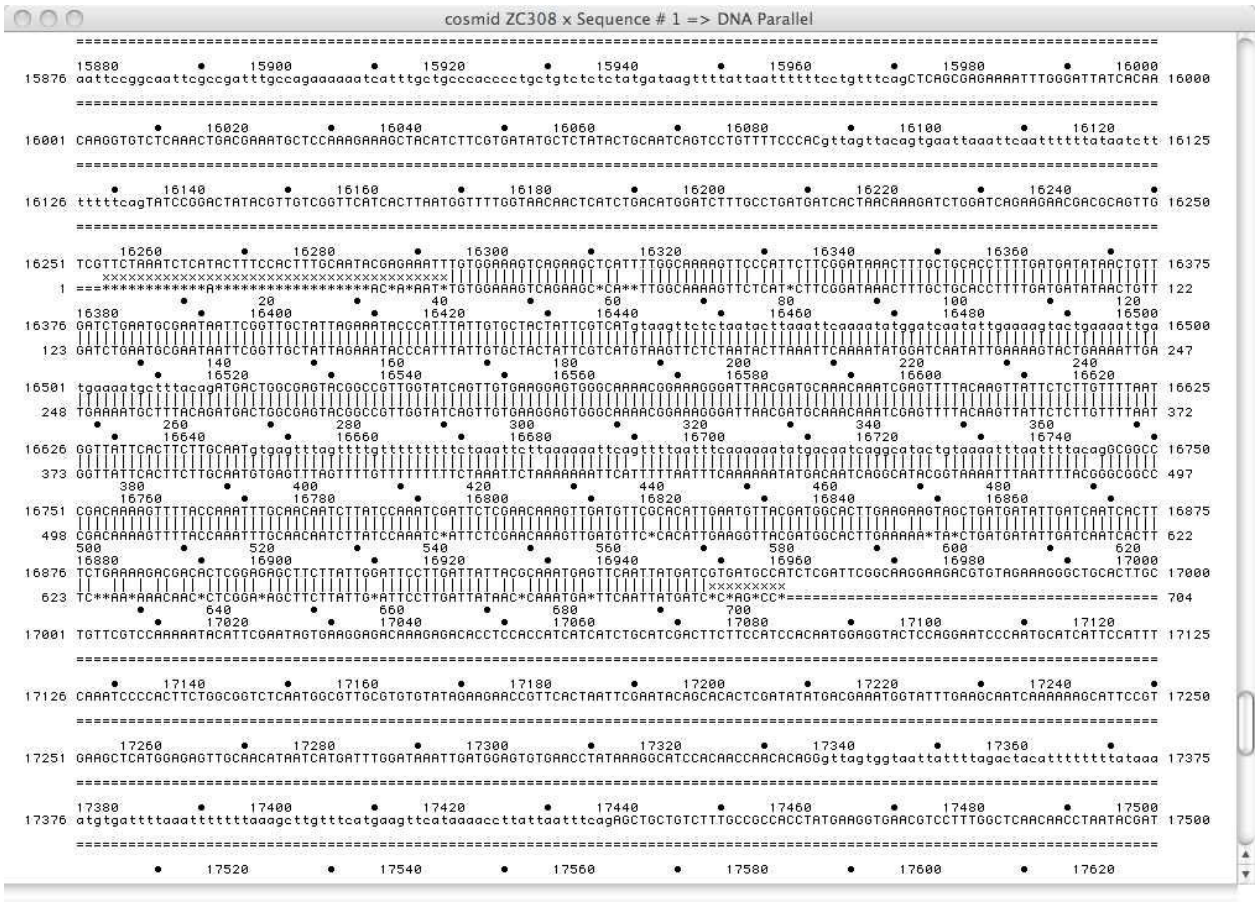

Fig.12: Comparison of the wild-type sequence and the sequence of the EV271 strain, carrying the *gld-2(q540)* mutation. The lesion was found at the position 16329 of the ZC308 cosmid.

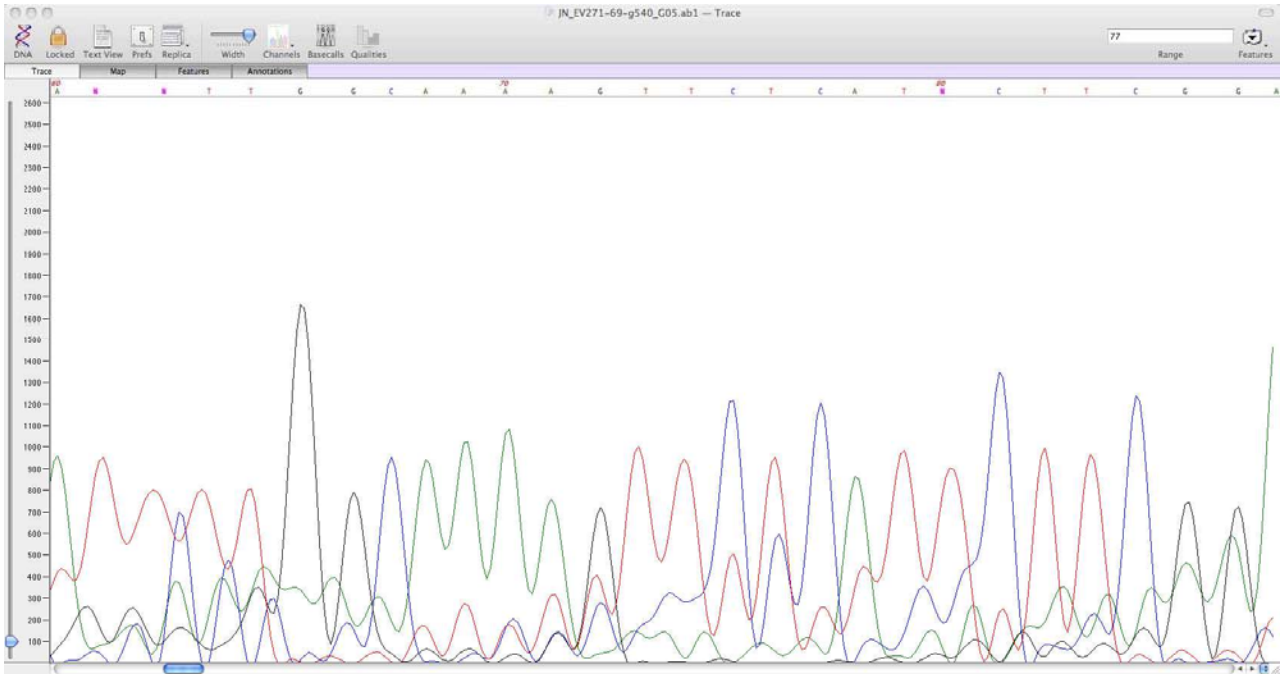

Fig.13: Sequencing diagram of the EV271 strain, carrying the *gld-2(q540)* mutation. Diagram shows high reliability of the sequencing result.

2. Western blotting - protein levels in gld-2 mutants

All Western blots were performed as described in “Materials & Methods“ in the chapter “Western blotting”. Usually the membrane was probed for GLD-2 (molecular weight about 130 kDa) together with CPB-3 (molecular weight about 100 kDa). Without stripping, after washing in PBS-T, membrane was probed with antibodies against tubulin (molecular weight about 55 kDa) and PAB-2 (molecular weight about 70 kDa.

Scans of Western blots were processed by Adobe Photoshop CS4 and Adobe Illustrator CS4.

Western blot scans were analyzed by ImageJ 1.44p (http://imagej.nih.gov/ij) as follows - image was inverted and brightness was measured. Background values were subtracted from resulting numbers. Afterwards, loading of each mutant was measured by dividing brightness of tubulin band by the brightness of N2 band. Values for particular proteins in each mutant were divided by the value for the wild-type (N2) and finally divided by the loading ratio. Resulting values were put in the graphs, with the N2 having a default value of 1.

2.1 Western blots

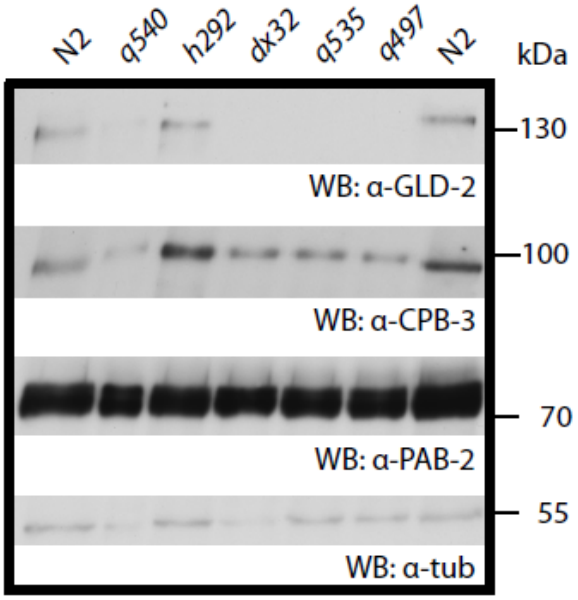

Fig.14: #1 - Western blot from protein lysates from 40 individually picked worms - *gld-2* mutant strains and wild-type strain (N2). In *gld-2* mutants *q497*, *q535* and *dx32* there was no GLD-2 expression detected. Mutants *h292* and *q540* show negatively affected expression of GLD-2 when compared with N2 and loading control. *h292* mutant shows higher expression of CPB-3 protein.

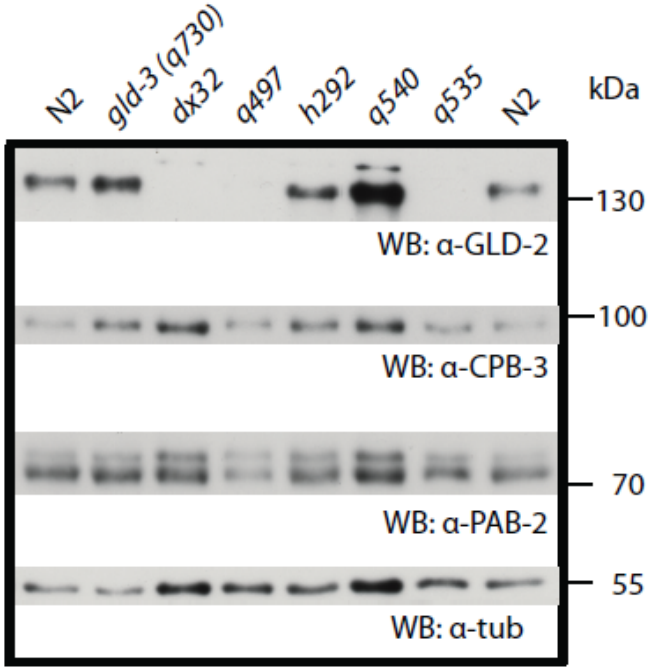

Fig.15: #2 Western blot from protein lysates from 40 individually picked worms - wild-type strain (N2), *gld-2* mutants and *gld-3(q730)* mutant. In *gld-2* mutants *q497*, *q535* and *dx32* there was no GLD-2 expression detected (the upper band in *dx32*mutant is background band. Mutants *h292* and *q540* show different expression of GLD-2 when compared with N2, but further analysis and comparison of loading control (tubulin) is needed. *gld-3(q730)* mutant shows highly elevated expression of GLD-2. The effects of *gld-2* mutants on expression of CPB-3 protein need to be analyzed in relation to the loading control (tubulin).

2.2 GLD-2 protein levels

As shown in figures 17 and 18, previously published results, the characterization of *q497* and *h292* mutants were confirmed in my experiments. Together with *q497*, also *q535* and *dx32* were shown to behave as *gld-2* null mutants. *q540* showed reduced expression of GLD-2, compared to wild-type, by about 5 - 10 %, but not as much as in the case of *h292*, whose expression was reduced by about 23 %. GLD-2 expression in *gld-3(q730)* was elevated by more than 100 %, maybe because more *gld-2* mRNAs are available for binding by FBFs. These results suggest that neither a structural mutation affecting interaction between GLD-2 and GLD-3, nor a mutation in the polyadenylation domain, severely affect GLD-2 protein levels. Possibly, GLD-2 could be substituted by action of GLD-4 poly(A) polymerase, forming “fail-safe“ system.

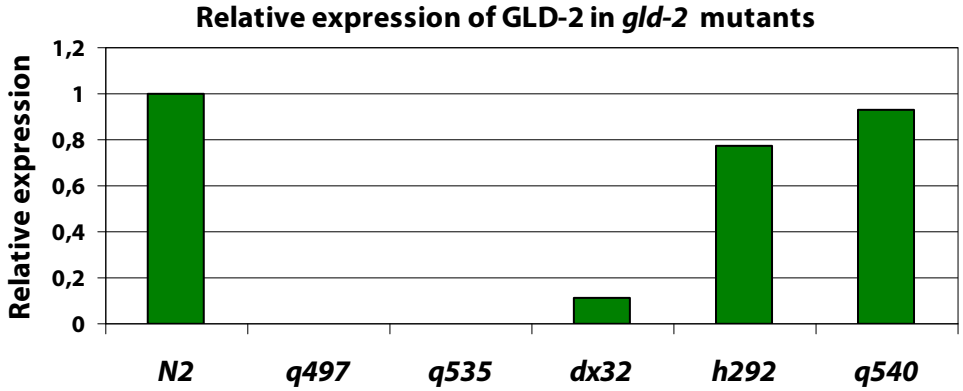

Fig.16: WB #1 - ImageJ analysis of relative expression of GLD-2 in *gld-2* mutants and wild-type strain (N2). In *gld-2* mutants *q497*, *q535* and *dx32* there was no GLD-2 expression detected (weak signal from *dx32* is considered irrelevant when compared with fig.14). Mutants *h292* and *q540* show negatively (but not severely) affected expression of GLD-2 in comparison with N2 worms.

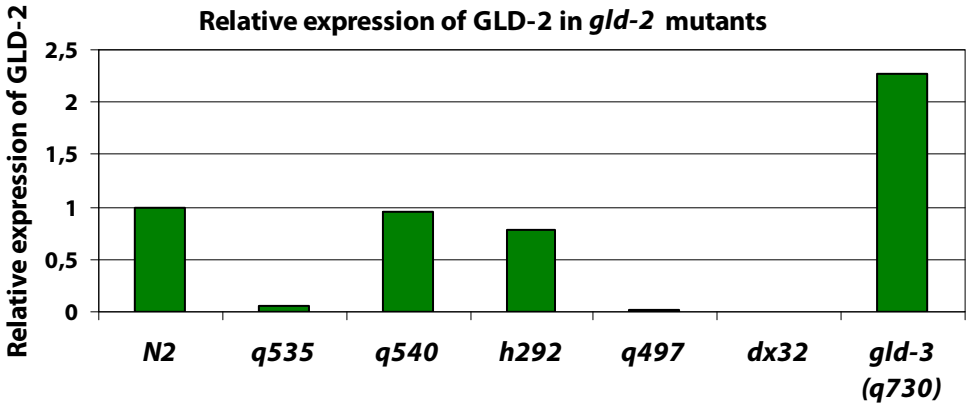

Fig.17: WB #2 - ImageJ analysis of relative expression of GLD-2 in *gld-2* mutants and wild-type strain (N2). In *gld-2* mutants *q497*, *q535* and *dx32* there was practically no GLD-2 expression (weak signal is in *q497*, *q535* is considered irrelevant when compared with fig.15). Mutants *h292* and *q540* negatively (but not severely) affected expression of GLD-2 in comparison with N2 worms. *gld-3(q730)* mutant shows highly elevated expression of GLD-2 in comparison with wild-type worms.

2.3 CPB-3 protein levels

In both *q497* and *q535* null mutants, CPB-3 expression was reduced - especially in the *q497* mutant. CPB-3 expression in the strain carrying the *dx32* mutation was elevated to more than 250 % of wild-type expression. In *h292* mutants, CPB-3 levels were more or less elevated, but two opposite results were shown for *q540* mutants. The *gld-3* mutant has shown highly - more than five times - elevated CPB-3 expression.

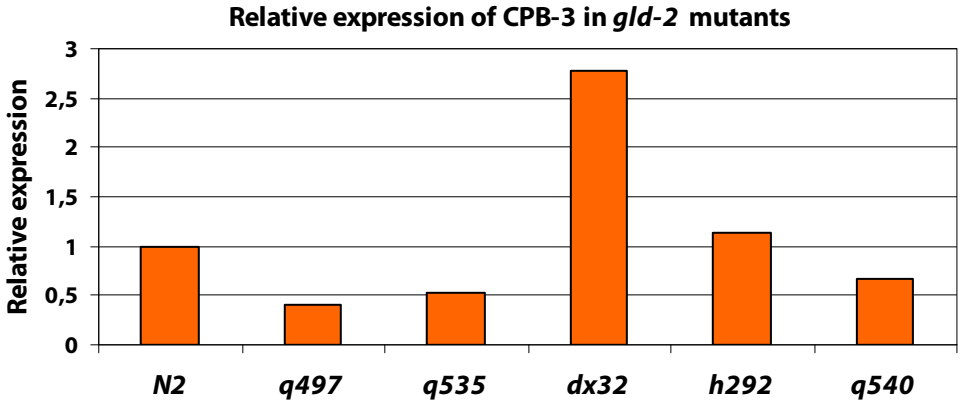

Fig.18: WB #1 - ImageJ analysis of relative expression of CPB-3 in *gld-2* mutants and wild-type strain (N2). In comparison with wild-type worms, *gld-2* mutants *q497*, *q535* showed negatively affected expression of CPB-3 protein. Surprisingly, the *dx32* mutant showed highly elevated expression of CPB-3. Mutant *h292* had similar CPB-3 levels as N2 and *q540* had reduced expression about less than half of N2.

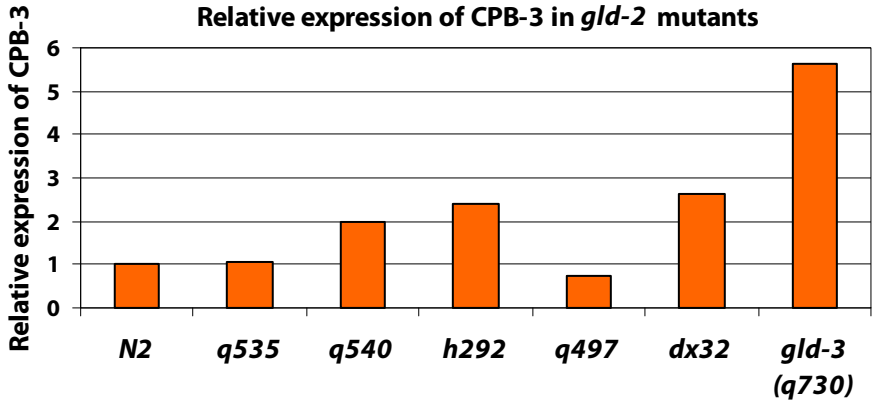

Fig.19: WB #2 - ImageJ analysis of relative expression of CPB-3 in *gld-2* mutants and wild-type strain (N2). In comparison with wild-type worms, *gld-2* mutants *q497*, *q535* showed slightly lower or similar expression of CPB-3 protein. Surprisingly, the *dx32* mutant showed highly elevated expression of CPB-3, and similar results were obtained in *h292* and *q540* mutants. The *gld-3(q730)* mutant shows highly elevated expression of CPB-3 in comparison with wild-type worms.

2.4 PAB-2 protein levels

Because of very high signal from PAB-2 bands in all samples, it is hard to determine the real ratios of expression. PAB-2 levels in *q497* and *q535* mutants are either similar or lower than in wild-type worms. The *h292* mutant shows lower expression of PAB-2, results for *dx32* and *q540* are unreliable. *gld-3* mutant shows elevated expression of PAB-2.

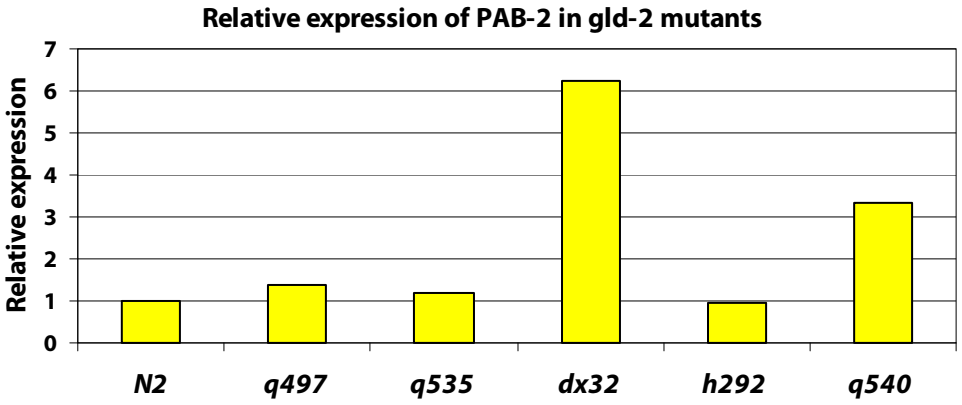

Fig.20: WB #1 - ImageJ analysis of relative expression of PAB-2 in *gld-2* mutants and wild-type strain (N2). PAB-2 levels in *gld-2* mutants *q497*, *q535* and *h292* were not much affected and were similar to wild-type worms. Surprisingly, the *dx32* mutant showed highly elevated expression of PAB-2; asimilar result - but not that high - was found in the *q540* mutant.

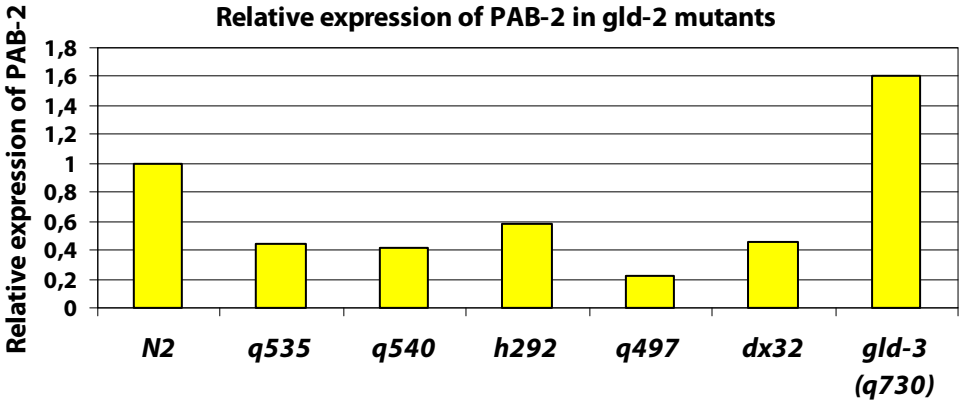

Fig.21: WB #2 - ImageJ analysis of relative expression of PAB-2 in *gld-2* mutants and wild-type strain (N2). In comparison with wild-type worms, *gld-2* mutants *q497*, *q535*, *dx32*, *h292* and *q540* had lower expression (by at least 40 %) of CPB-3 protein. The *gld-3(q730)* mutant showed expression of PAB-2 elevated by about 60 % in comparison with wild-type worms.

3. Western blotting - protein levels in different genetic backgrounds

The methods used in this chapter are similar to those used in the previous one - the only difference is that in the case of probing with antibodies against GLD-4 (which has molecular weight similar to GLD-2 - about 130 kDa), the membrane was first stripped.

3.1 Western blots

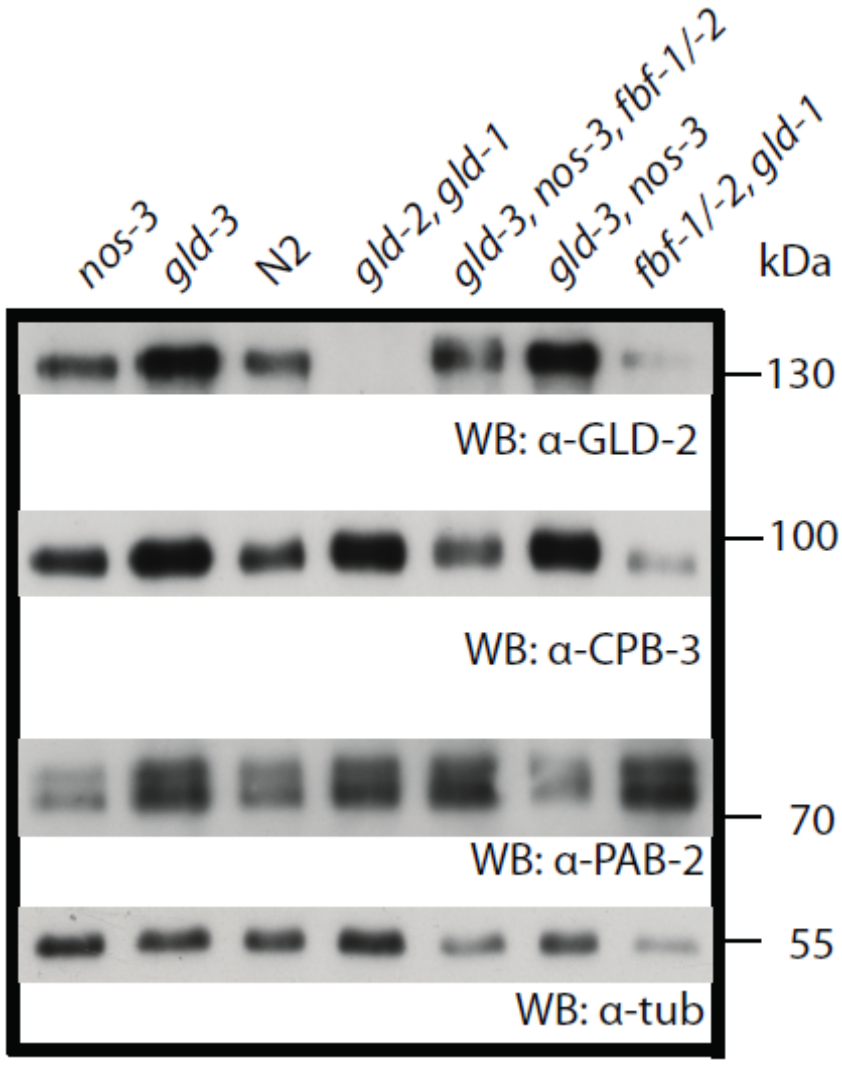

Fig.22: #1 - Western blot of mutants with tumorous and other genetic backgrounds and wild-type strain (N2). Mutants with tumorous germlines - *gld-1, fbf-1.2*, then *gld-3, nos-3, fbf-1.2* and finally tumor control *gld-3, nos-3* and non - tumorous single mutants *gld-3* and *nos-3* were examined. As a negative control (no GLD-2 expressed), *gld-1, gld-2* double mutant was used and as a positive control N2 worms were used. Tubulin levels were used as loading controls.

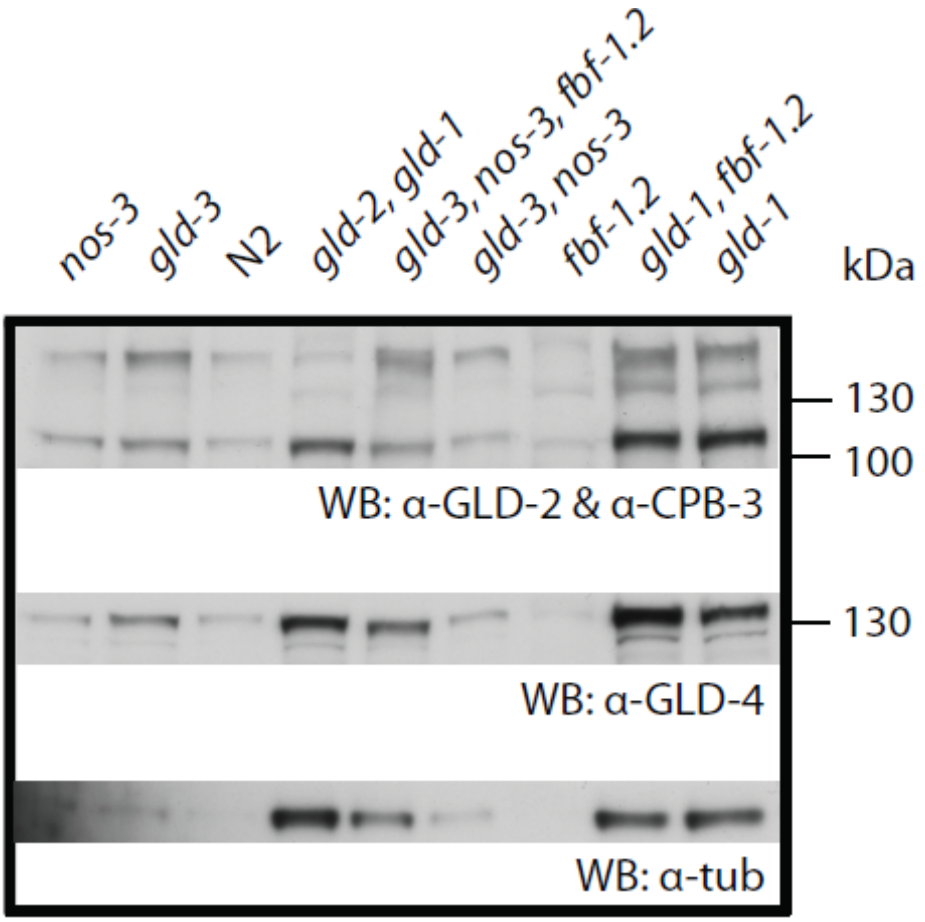

Fig.23: #2 - Western blot of mutants with tumorous and other genetic backgrounds and wild-type strain (N2). Mutants with tumorous germlines - *gld-1, fbf-1.2*, then *gld-3, nos-3, fbf-1.2* and finally tumor control *gld-3, nos-3* and non - tumorous single mutants *gld-1*, *gld-3* and *nos-3* were examined. As a negative control (no GLD-2 expressed), *gld-1*, *gld-2* double mutant was used and as a positive control N2 worms were used. Tubulin levels were used as loading controls. In this blot, the*fbf-1.2* double mutant was also used, but could not be analyzed due to insufficient loading volume.

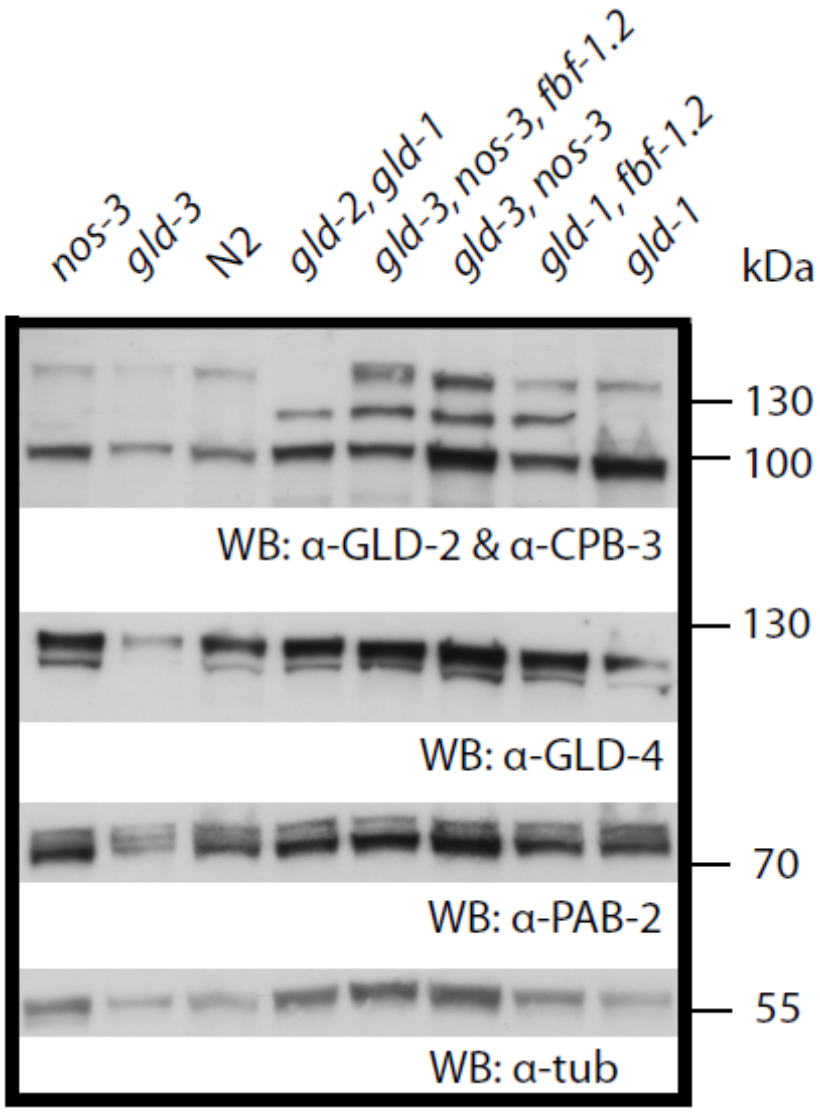

Fig.24: #3 - Western blot of mutants with tumorous and other genetic backgrounds and wild-type strain (N2). Mutants with tumorous germlines - *gld-1, fbf-1.2*, then *gld-3, nos-3, fbf-1.2* and finally tumor control *gld-3, nos-3* and non - tumorous single mutants *gld-1*, *gld-3* and *nos-3* were examined. As a negative control (no GLD-2 expressed), *gld-1, gld-2* double mutant was used and as a positive control N2 worms were used. Tubulin levels were used as loading controls. This blot was probed with polyclonal rabbit antibodies against GLD-2.

3.2 GLD-2 protein levels

The *gld-1, fbf-1.2* mutant showed similar levels of GLD-2 expression as wild-type worms, the same result was shown for the *nos-3* mutant. On the contrary *gld-3, nos-3* double mutant showed elevated levels of GLD-2, an effect which was slightly reduced in *gld-3, nos-3, fbf-1.2* triple mutants. GLD-2 expression more than doubles in the *gld-3* single mutant, as was also shown in one of the Western blots of *gld-2* mutants (fig.15). The *gld-1* mutant showed the opposite behavior and the *fbf-1.2* mutant was not able to be analyzed, due to extremely low signal from tubulin (see figure 21). A mutation in *fbf* genes caused lower expression of GLD-2 protein levels in tumorous germlines than in *gld-3, nos-3* tumors.

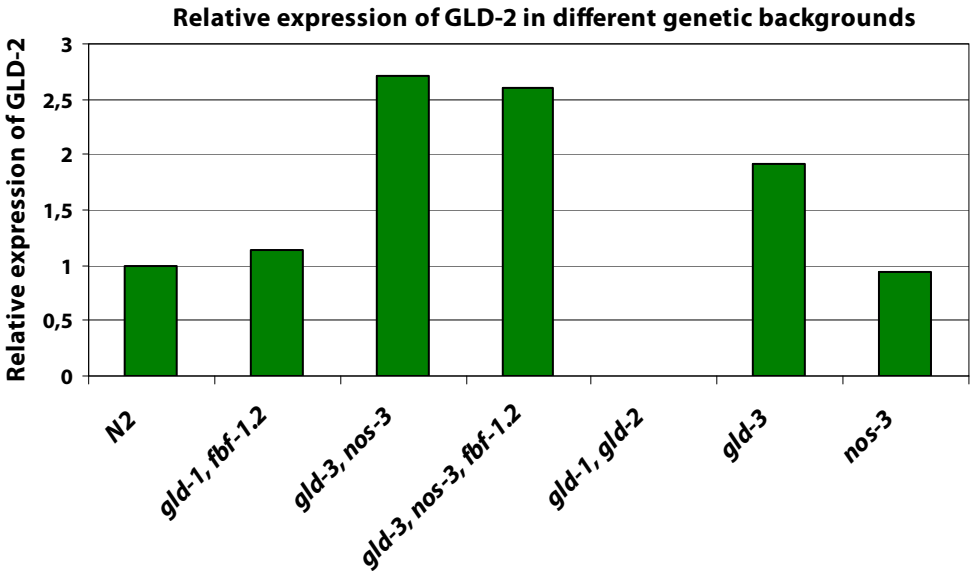

Fig.25: WB #1 - ImageJ analysis of relative expression of GLD-2 in tumorous and other genetic backgrounds and wild-type strain (N2). *gld-1, fbf-1.2* and *nos-3* mutants showed similar expression of GLD-2 as the wild-type worms. The *gld-3, nos-3* double mutant and *gld-3, nos-3, fbf-1.2* quadruple mutant showed elevated expression of GLD-2. This effect was lower when *fbf-1.2* were mutated. *gld-3* showed, as in previous cases elevated expression of GLD-2. The *gld-1, gld-2* double mutant was used as a negative control and no GLD-2 was detected.

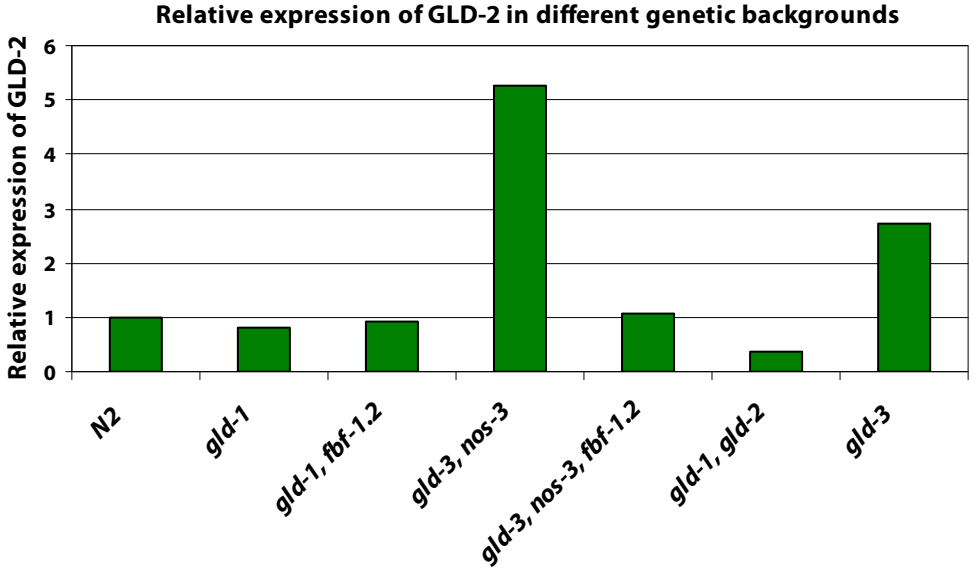

Fig.26: WB #2 - ImageJ analysis of relative expression of GLD-2 in tumorous and other genetic backgrounds and wild-type strain (N2). The *gld-1* single mutant, *gld-1, fbf-1.2* and the *gld-3, nos-3, fbf-1.2* quadruple mutant all showed a similar level of expression of GLD-2 as the wild-type worms. The *gld-3, nos-3* double mutant showed elevated expression of GLD-

2. The same effect was found, again, in the *gld-3* single mutant. *gld-1, gld-2* double mutant was used as a negative control and only some background signal was detected.

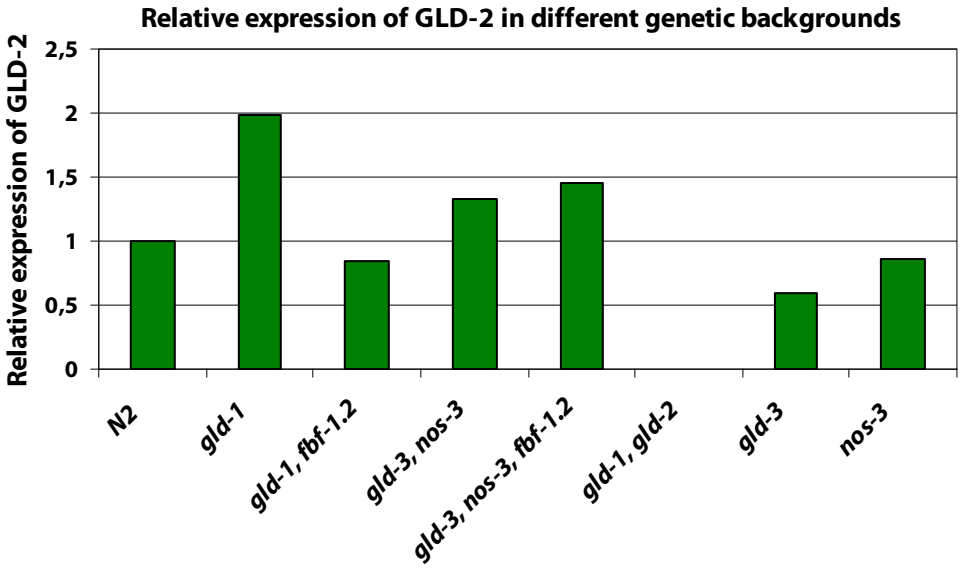

Fig.27: WB #3 - ImageJ analysis of relative expression of GLD-2 in tumorous and other genetic backgrounds and wild-type strain (N2). The *gld-1* single mutant, *gld-1, fbf-1.2* and the *gld-3, nos-3, fbf-1.2* quadruple mutant showed a similar level of expression of GLD-2 as the wild-type worms. The *gld-3, nos-3* double mutant showed elevated expression of GLD-2. The same effect was found in the *gld-3* single mutant. The *gld-1, gld-2* double mutant was used as a negative control and only some background signal was detected.

3.3 CPB-3 protein levels

The *Gld-1* mutant showed highly elevated levels of CPB-3. But this effect is reduced in *gld-1, fbf-1.2* triple mutants (WB #2 is the only exception - but there is also big difference in the amount of loaded samples between this mutant and wild-type worms and this could cause errors in measurement). Mutation of *fbf-1.2* also caused a the reduction in CPB-3 expression in *gld-3, nos-3* mutants. Double mutants showed enrichment of CPB-3 protein in WB #1 and WB #2, when compared to wild-type, but quadruple mutants showed significant decreases in all cases. Like GLD-2 protein, also CPB-3 protein expression was elevated in *gld-3* mutants. On the other hand, *nos-3* mutants showed a slight decrease, when compared to wild-type.

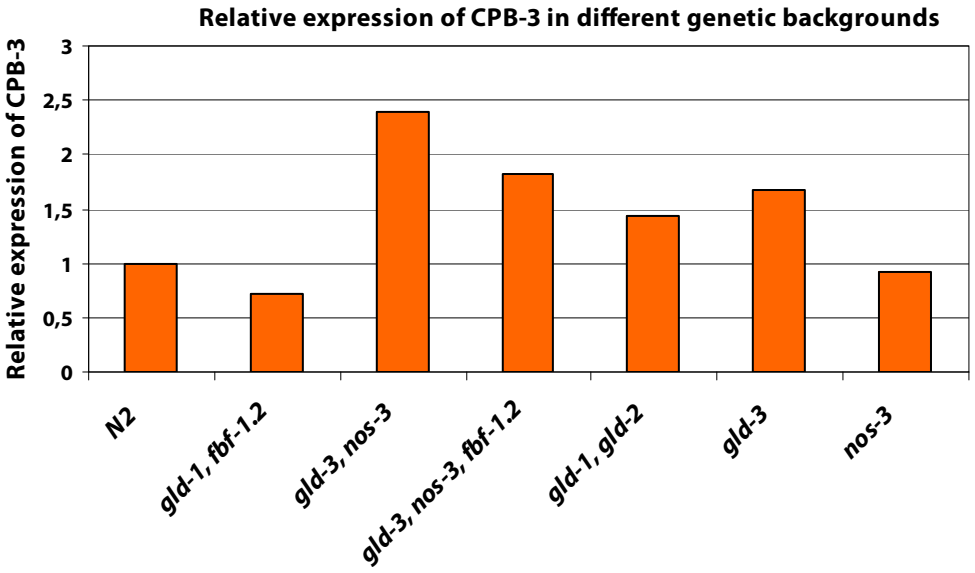

Fig.28: WB #1 - Relative expression of CPB-3 in tumorous and other genetic backgrounds and wild-type strain (N2). *gld-1, fbf-1.2* showed lower CPB-3 expression than in N2 worms, *gld-3, nos-3* double and *gld-3, nos-3, fbf-1.2* quadruple mutants showed elevated expression of CPB-3, but this effect was lower when *fbf-1.2* were mutated. The *gld-1, gld-2* double mutant and *gld-3* showed higher expression of CPB-3 than in wild-type worms, the *nos-3* single mutant showed similar expression like in N2 worms.

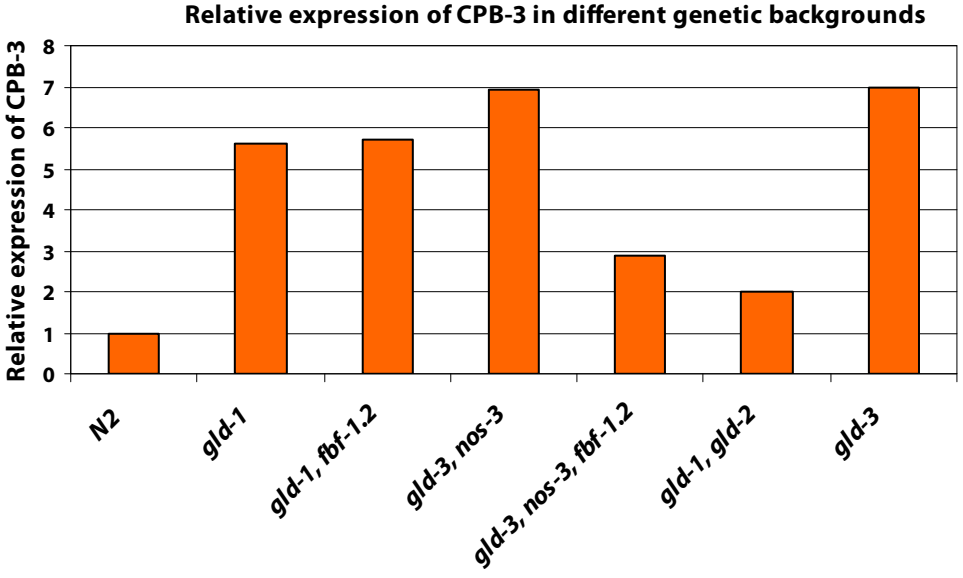

Fig.29: WB #2 - Relative expression of CPB-3 in tumorous and other genetic backgrounds and wild-type strain (N2). The *gld-1* single and triple mutant *gld-1, fbf-1.2* both show much higher expression of CPB-3 than N2 worms. The highest expression (about seven times) of CPB-3 was found in the *gld-3* single mutant and the *gld-3, nos-3* double mutant. In the *gld-3, nos-3, fbf-1.2* quadruple mutant - although still higher than wild-type - CPB-3 expression was strongly reduced, in comparison with the *gld-3, nos-3* mutant. The *gld-*

1, gld-2 double mutant showed higher - twice as much - expression of CPB-3 than in wild-type worms.

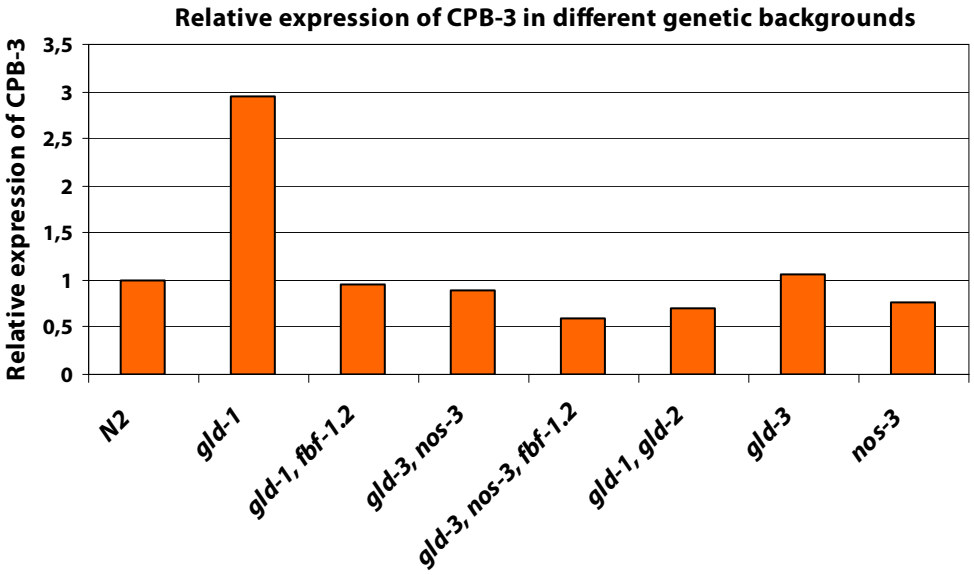

Fig.30: WB #3 - Relative expression of CPB-3 in tumorous and other genetic backgrounds and wild-type strain (N2). The *gld-1* single mutant showed three times higher expression of CPB-3 than the wild-type worms. In the *gld-3* mutant, only a slight increase in CPB-3 expression was shown. The other mutants showed similar or lower expression as in wild-type. There was also a slight difference between the *gld-3, nos-3* double mutant and the *gld-3, nos-3, fbf-1.2* quadruple mutant - the latter had lower expression of CPB-3.

3.4 PAB-2 protein levels

Because PAB-2 was not probed for in Chapter 3-WB #2 and in other cases where it was probed for gave extremely high signals, scan analysis of western blots did not show any concrete trend - the only exception is in the *nos-3* mutant which shows a reduction of PAB-2 expression to one half of the wild-type level.

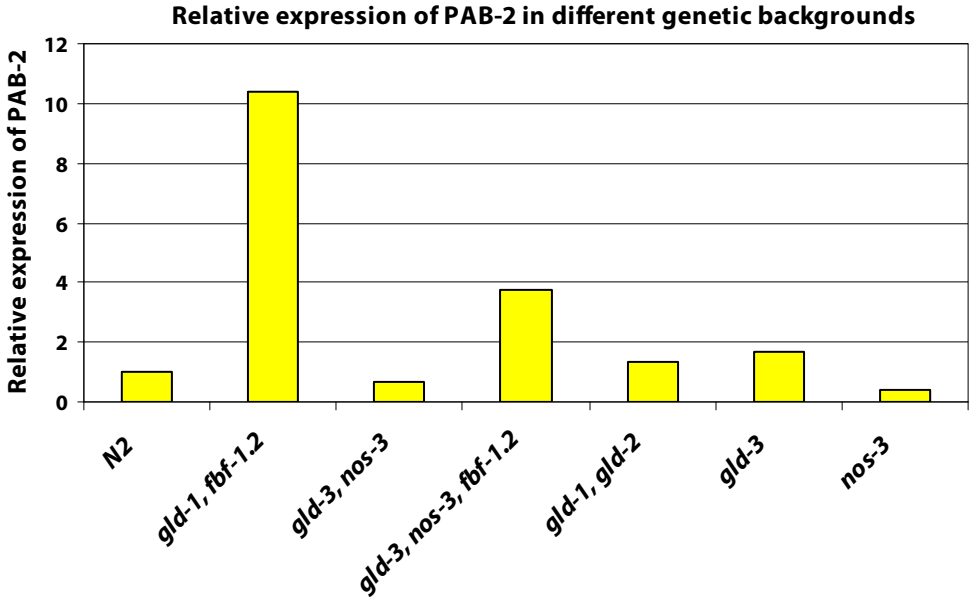

Fig.31: WB #1 - Relative expression of PAB-2 in tumorous and other genetic backgrounds and wild-type strain (N2). The *gld-1, fbf-1.2* triple mutant showed strongly increased expression of PAB-2 compared to wild-type worms. In the *gld-3* mutant, only a slight increase in PAB-2 expression was shown. The other mutants showed similar or slightlyhigher or lower expression as compared to wild-type. There was also a slight difference between the *gld-3, nos-3* double mutant and the *gld-3, nos-3, fbf-1.2* quadruple mutant - the latter had higher expression of PAB-2.

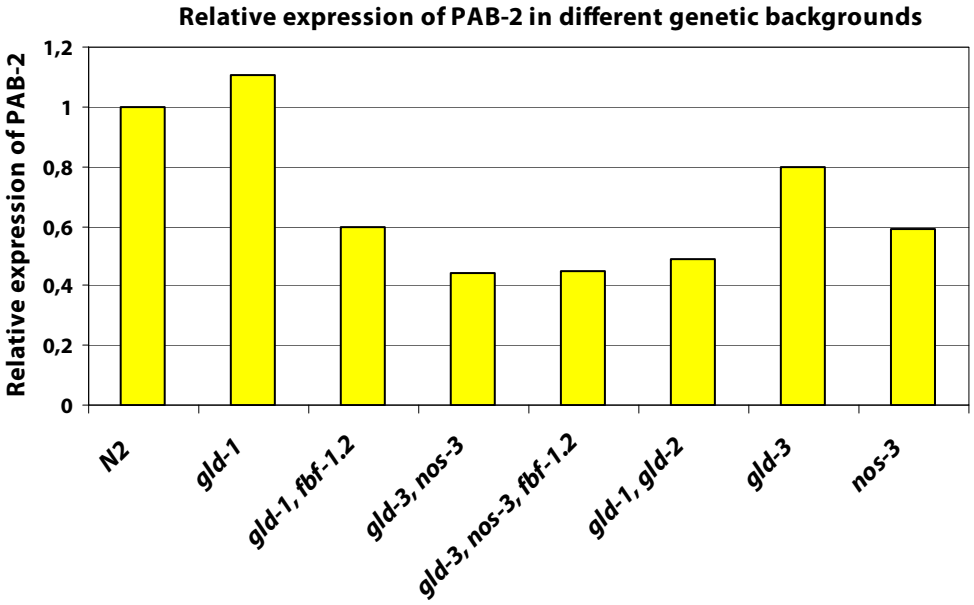

Fig.32: WB #3 - Relative expression of PAB-2 in tumorous and other genetic backgrounds and wild-type strain (N2). The *gld-1* single mutant showed slightly higher expression of PAB-2 than the wild-type worms. The other mutants showed similar or lower

expression as in wild-type. There was barely any difference between the *gld-3, nos-3* double mutant and the *gld-3, nos-3, fbf-1.2* quadruple mutant.

3.5 GLD-4 protein levels

Again, as in the case of PAB-2, I got the inconclusive results, partly because of underloaded wild-type sample in WB #2 and partly because of high signal from GLD-4 antibody staining. Both blots show opposite behavior - however one observation that can be made is that, in *gld-1* and *gld-3, nos-3* mutants, an additional mutation in *fbf-1.2* in three out of four cases elevated the expression of GLD-4 in comparison with *gld-1* and *gld-3, nos-3* mutants by themselves.

Fig.33: WB #2 - Relative expression of GLD-4 in tumorous and other genetic backgrounds and wild-type strain (N2). The *gld-1* single and triple mutant *gld-1, fbf-1.2* both show much higher expression of GLD-4 than N2 worms; when *fbf-1.2* were mutated the expression was four times higher than in the single mutant of *gld-1* (which is actually not tumorous). Higher expression of GLD-4 was found in all other mutants, with the *gld-3, nos-3* double mutant having higher expression than the *gld-3, nos-3, fbf-1.2* quadruple mutant. A strong signal was observed in the *gld-3* single mutant.

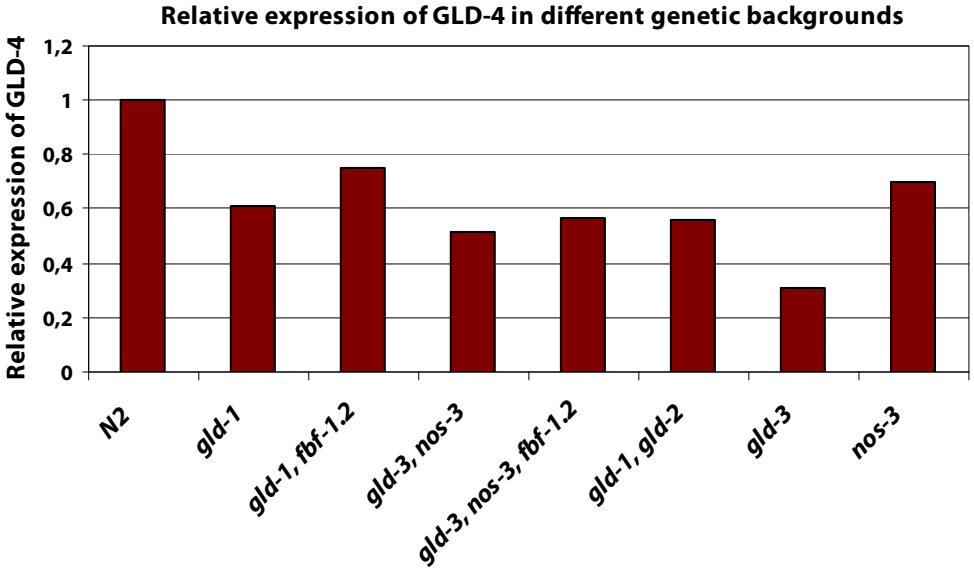

Fig.34: WB #3 - Relative expression of GLD-4 in tumorous and other genetic backgrounds and wild-type strain (N2). All the mutants showed lower expression, in contrast to the previousblot (fig.33) making the results inconclusive.

4. Yeast three-hybrid (Y3H) assay results

Five predicted sequences (presumptive FBF-binding elements) (see fig.35 and 36) and their combinations (FBE1+2*gld-2*, FBE1+2+3*gld-2*, FBE5+end*gld-2*) were tested for binding of FBF-1, FBF-2, PUF-5, PUF-6, PUF-8 and cytoplasmic polyadenylation element binding (CPEB) proteins - CPB-1, CPB-2, CPB-3 and FOG-1 (*Feminization Of Germline*). FBF-2 does not interact with the *tra-2* site, so this sequence was used as a negative control; as a positive control, a double *fem-3* site was used, which is known to interact with FBF proteins. In later experiments, another positive control - a FBE4-*gld-2* site bound by FBF-2 - was used.

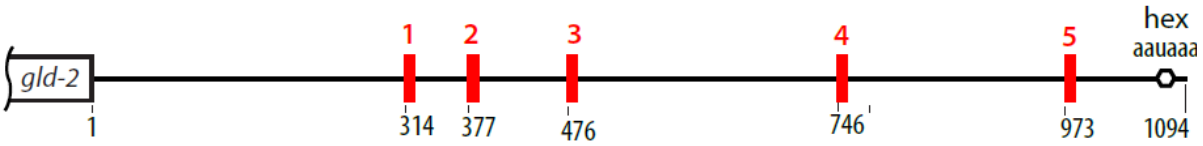

Fig.35: Schematic illustration of 3´UTR from *gld-2* mRNA and localization of individual presumptive FBF-binding elements (FBE1-5*gld-2*)

```
FBE1      c c g g A A U C A U C U A U C A G A U A U G U G A U G A U C U C U A C A A U U U U U U A g c a u g c
FBE2      c c g g C C A C A U U U C C C U G U A U U U U A A C A C G A A A A G U U C A A A A C G g c a u g c
FBE3      c c g g A A A A U A C A A A U U A U A U G U A C A A A C U U U C G A A A g c a u g c
FBE3bds   c c g g A A U A C A A A U U A U A U G U A C A A A C U U U C G A A A g c a u g c
FBE3mut   c c g g A A U A C A A A U U A U A a c U A C A A A C U U U C G A A A g c a u g c
FBE4      U U U U A U G A U U G U A C C A U A A G U A C C U C C C
FBE4mut   U U U U A U G A U a c U A C C A U A A G U A C C U C C C
FBE5      c c g g A A C U U U C C A C A A U U C C U G U U C U G U A A U U U U C U C U C C U C U C C g c a u g c
FBE5mut   c c g g A A C U U U C C A C A A U U C C U G U U C a c U A C A A A C U U U C G A A A g c a u g c
```

Fig.36: Sequences of individual presumptive FBF-binding elements (FBE1-5*gld-2*)

4.1 β - galactosidase assay results

As is shown in fig.37 - 46 and summarized in fig.47 (with respective controls shown in fig.48), the only found interaction was between FBE4*gld-2* and FBF proteins. That this interaction was sequence-specific was confirmed by the mutation of the consensus binding sequence by Quickchange, in which first trinucleotide “UGU” was changed in “ACU”. This change abolished interaction between FBE4*gld-2* and its binding partners (see fig.41).

The interaction between FBF-2 and *fem-3* mRNA was used as a positive control, later also the FBF-2 and FBE4*gld-2* interaction was used; as negative controls FBF-2 with *gld-1* mRNA and FBF-2 with *tra-2* mRNA were used, which are known to not interact with each other.

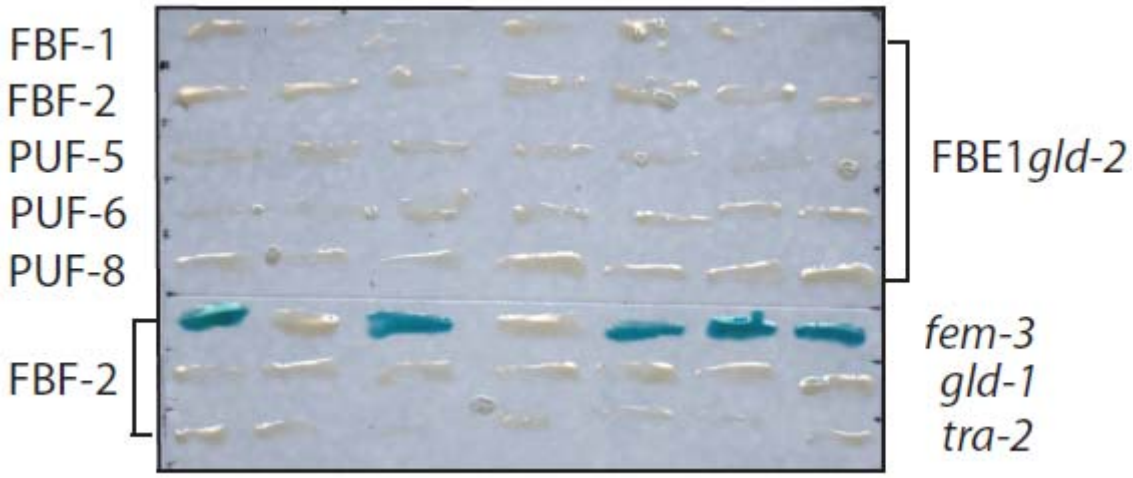

Fig.37: β - galactosidase assay results of interaction analysis of FBE1*gld-2* presumptive binding site and some of the PUF proteins. No interaction was found for this site. As a positive control the confirmed interaction between FBF-2 and *fem-3* mRNA was used; as negative controls FBF-2 with *gld-1* and *tra-2* mRNAs were used, which are known to not interact with each other. All controls worked as expected.

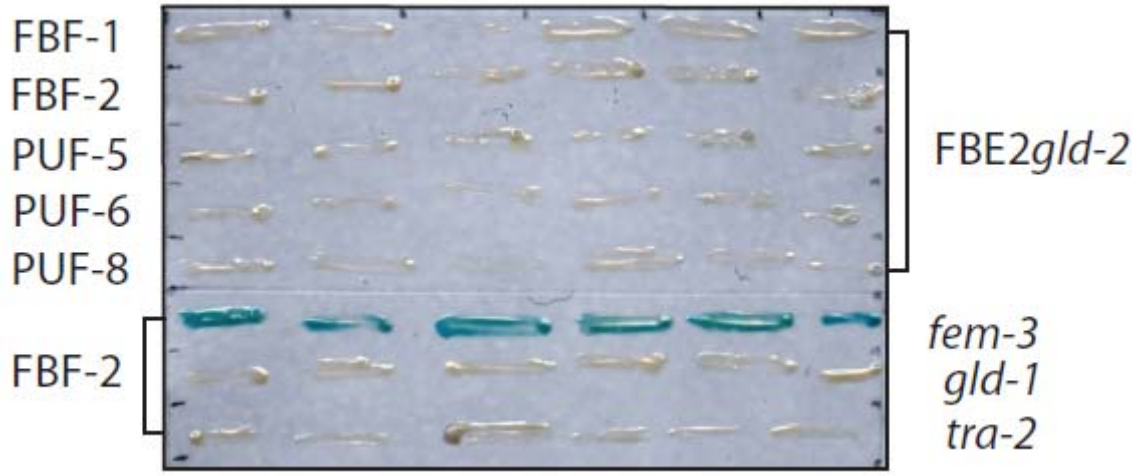

Fig.38: β - galactosidase assay results of interaction analysis of FBE2*gld-2* presumptive binding site and some of the PUF proteins. No interaction was found for this site. As a positive control the confirmed interaction between FBF-2 and *fem-3* mRNA was used; as negative controls FBF-2 with *gld-1* and *tra-2* mRNAs were used, which are known to not interact witch each other. All controls worked as expected.

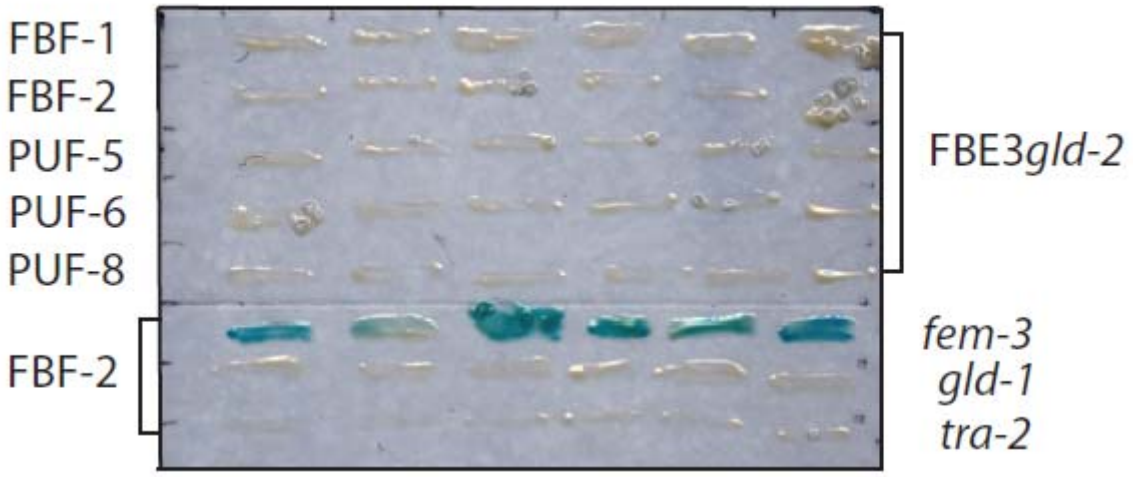

Fig.39: β - galactosidase assay results of interaction analysis of FBE3*gld-2* presumptive binding site and some of the PUF proteins. No interaction was found for this site. As a positive control the confirmed interaction between FBF-2 and *fem-3* mRNA was used; as negative controls FBF-2 with *gld-1* and *tra-2* mRNAs were used, which are known to not interact with each other. All controls worked as expected.

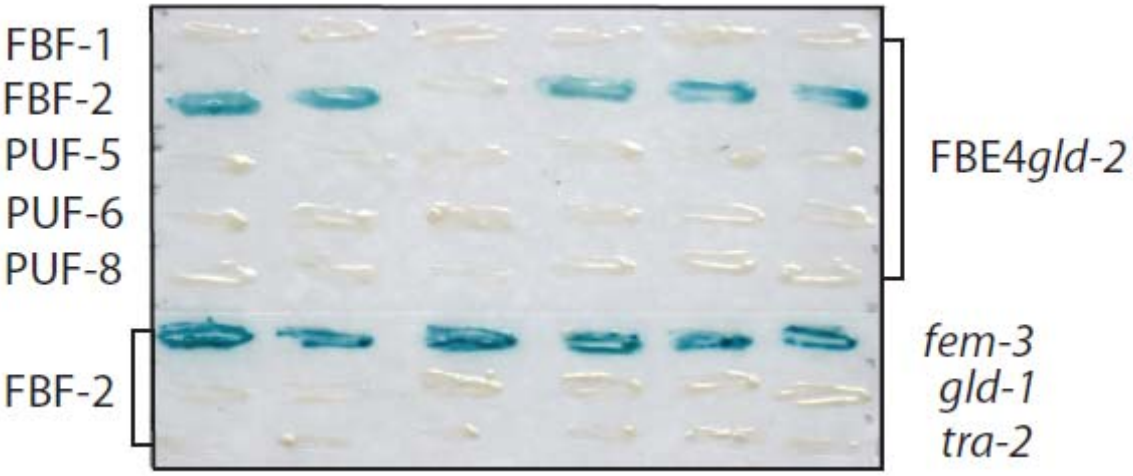

Fig.40: β - galactosidase assay results of interaction analysis of FBE4*gld-2* presumptive binding site and some of the PUF proteins, where FBF-2 was found as an interacting partner. Interaction between this site and FBF-2 was found. As a positive control the confirmed interaction between FBF-2 and *fem-3* mRNA was used; as negative controls FBF-2 with *gld-1* and *tra-2* mRNAs were used, which are known to not interact with each other. All controls worked as expected.

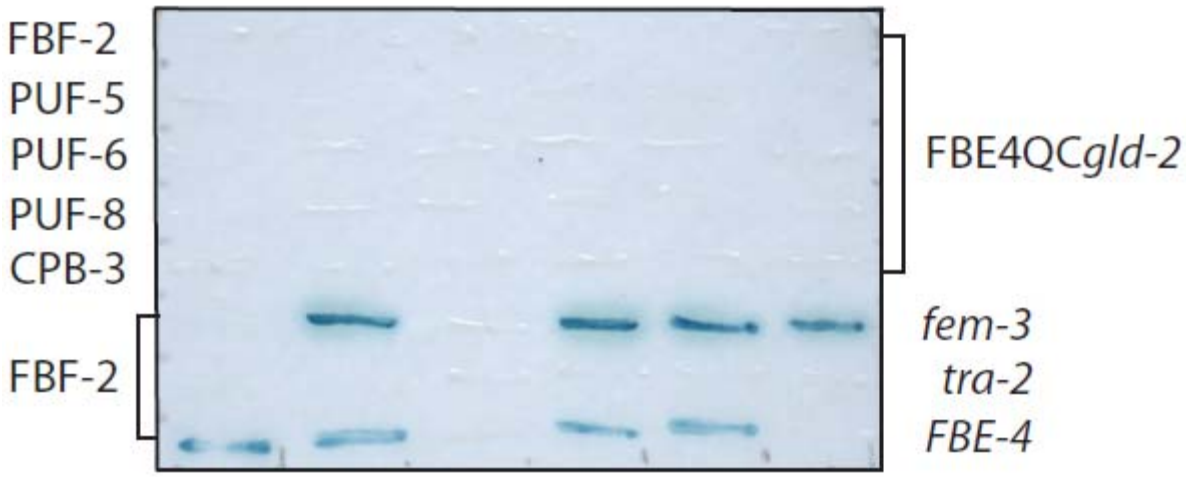

Fig.41: β - galactosidase assay results of interaction analysis of mutated FBE4*gld-2* presumptive binding site and some of the PUF proteins, demonstrating that interaction between FBF-2 and the FBF4*gld-2* site is sequence-dependent. No interaction was found for the mutated FBF4*gld-2* site. For comparison, the wild-type FBE4*gld-2* sequence was used. As a positive control the confirmed interaction between FBF-2 and *fem-3* mRNA was used; as a negative control, FBF-2 and *tra-2* mRNA were used, which are known to not interact with each other. All controls worked as expected.

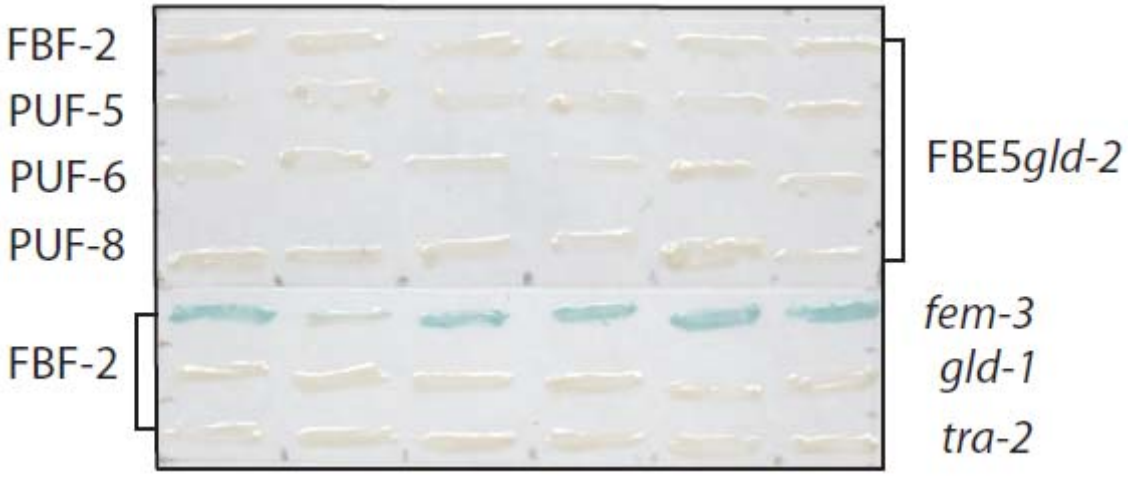

Fig.42: β - galactosidase assay results of interaction analysis of FBE5*gld-2* presumptive binding site and some of the PUF proteins. No interaction was found for this site. As a positive control the confirmed interaction between FBF-2 and *fem-3* mRNA was used; as negative controls FBF-2 with *gld-1* and *tra-2* mRNAs were used, which are known to not interact with each other. All controls worked as expected.

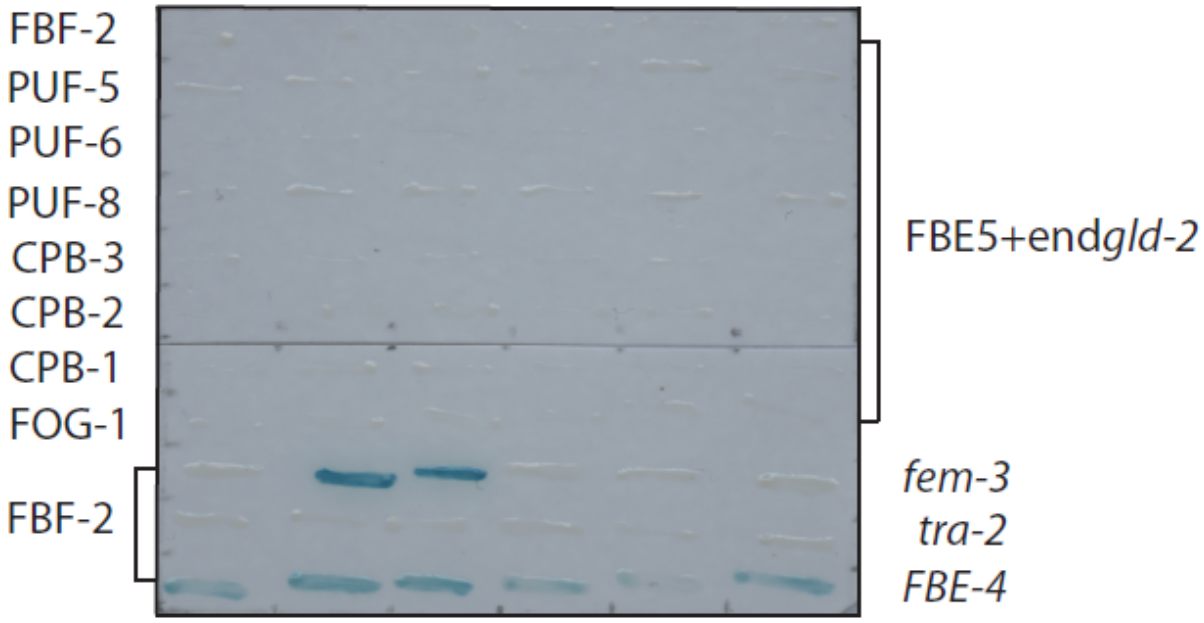

Fig.43: β - galactosidase assay results of interaction analysis of FBE5+end*gld-2* presumptive binding site and some of the PUF and CPB proteins. No interaction was found for this site. As positive controls the confirmed interaction between FBF-2 and *fem-3* and recently found interaction between FBF-2 and FBE4*gld-2* were used; as negative control FBF-2 with *tra-2* mRNA were used, which are known to not interact with each other. All controls worked as expected.

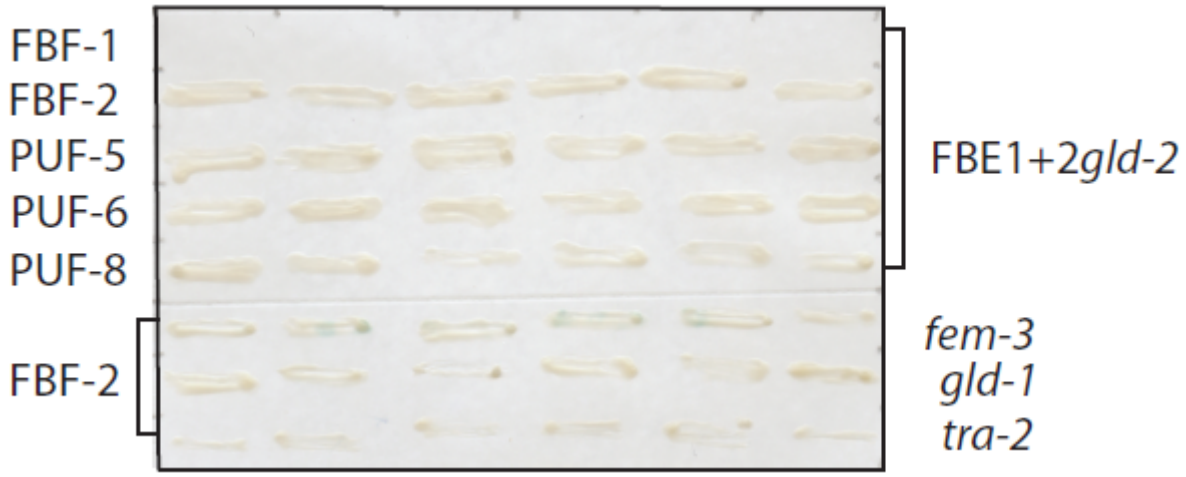

Fig.44: β - galactosidase assay results of interaction analysis of FBE1+2*gld-2* presumptive binding site and some of the PUF proteins. No interaction was found for this site. As a positive control the confirmed interaction between FBF-2 and *fem-3* mRNA was used (blue color is not that strong in this case but still present); as negative controls FBF-2 with *gld-1* and *tra-2* mRNAs were used, which are known to not interact with each other.

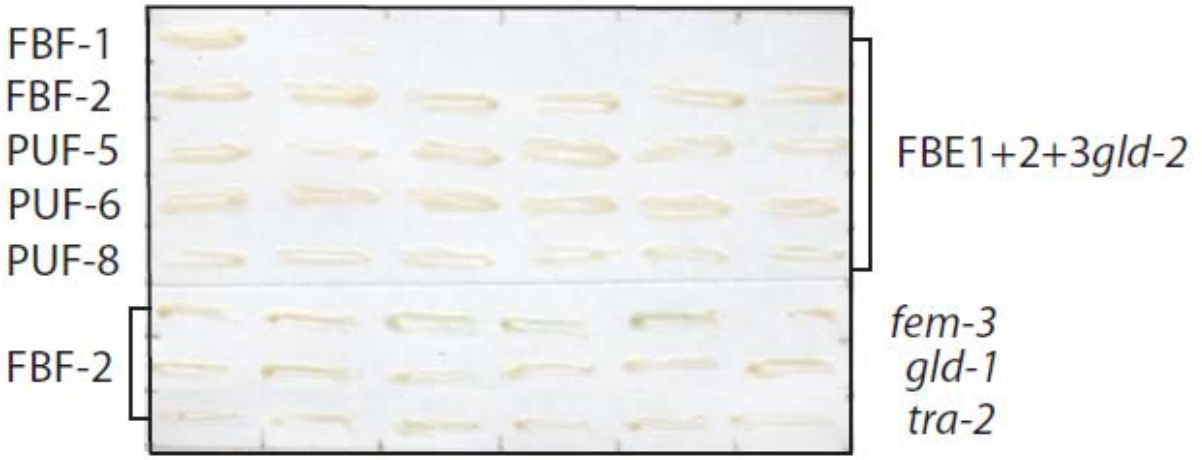

Fig.45: β - galactosidase assay results of interaction analysis of FBE1+2+3*gld-2* presumptive binding site and some of the PUF proteins. No interaction was found for this site. As a positive control the confirmed interaction between FBF-2 and *fem-3* mRNA was used (blue color is not that strong in this case but still present); as negative controls FBF-2 with *gld-1* and *tra-2* mRNAs were used, which are known to not interact with each other.

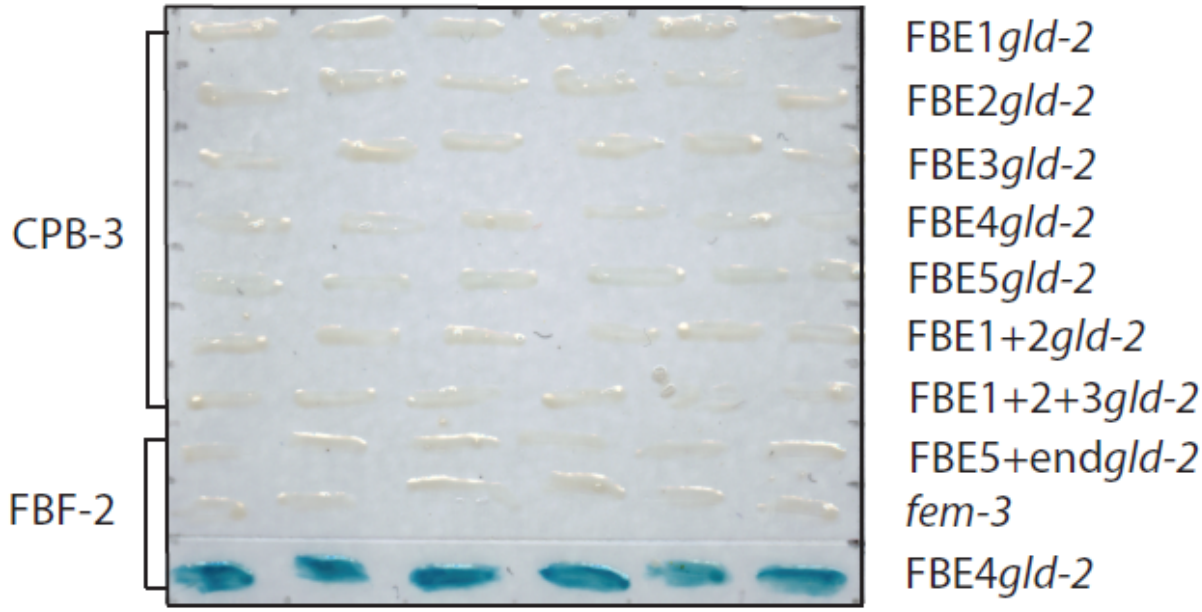

Fig.46: β - galactosidase assay results of interaction analysis of presumptive FBF-binding sites of the 3´UTR of *gld-2* mRNA with CPB-3. No interaction was found for these sites. As positive controls the confirmed interaction between FBF-2 and *fem-3* and recently found interaction between FBF-2 and FBE4*gld-2* were used. The FBF-2 and *fem-3* failed to show a positive result in this case, but FBF-2 and FBE4*gld-2* did.

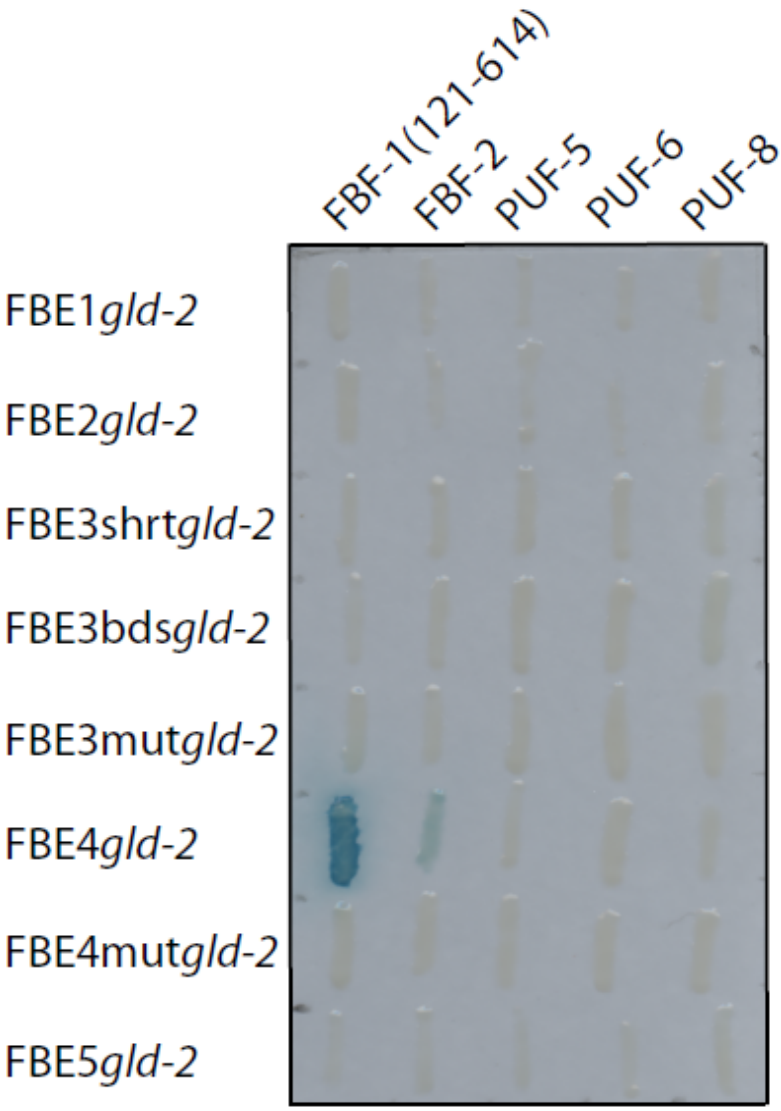

Fig.47: Summary β - galactosidase assay. Results of interaction analysis of presumptive FBF-binding sites predicted in *gld-2* mRNA and some of the PUF proteins. The only found interactions were between FBF proteins [FBF-1(121-614) and FBF-2] and FBE4*gld-2*. Sequence specificity of this interaction was confirmed by mutation of the consensus site (see fig.41).

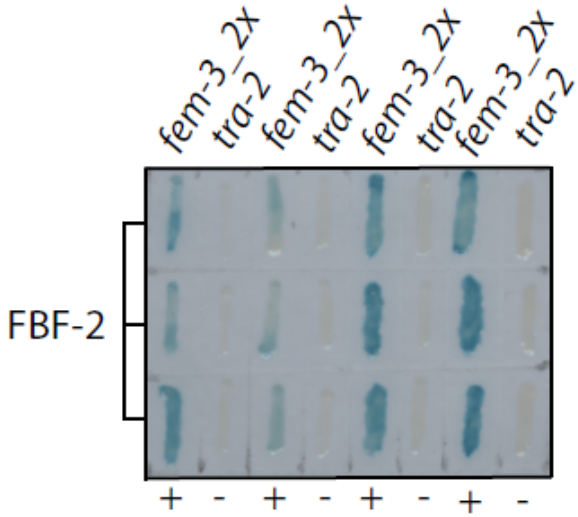

Fig.48: Controls of summary β - galactosidase assay (see fig.47). As a positive control the confirmed interaction between FBF-2 and *fem-3* was used; as negative control FBF-2 with *tra-2* mRNA were used, which are known to not interact with each other. All controls worked as expected.

4.2 Control of expression of tested proteins

From six colonies, streaked on the nitrocellulose membrane, first two were analyzed for protein expression by Western blot. This analysis showed (see fig.49 - 53), that almost all tested proteins were expressed at least in one case - the only exception, which disqualifies it from any conclusion about its interaction, is PUF-6 protein.

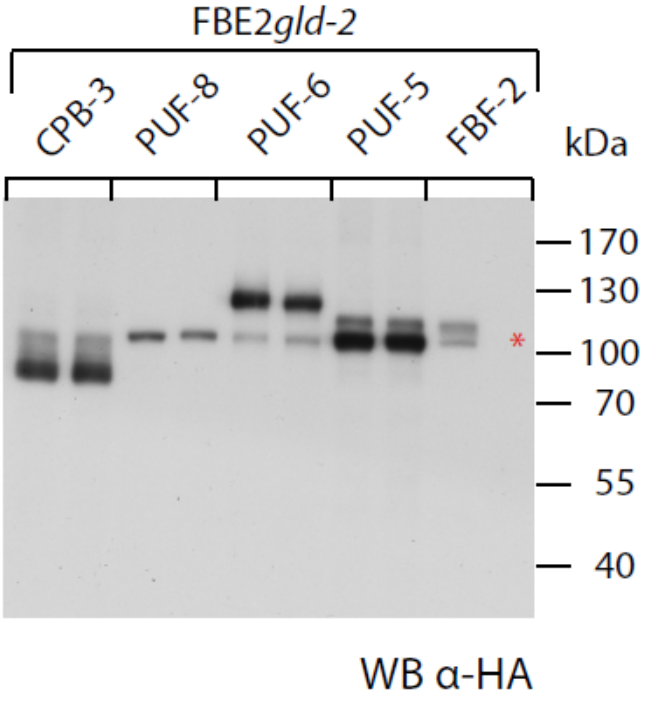

Fig.49: Protein expression from integrated plasmids for FBE2*gld-2*. All bands are of expected size. Red asterisk marks missing band.

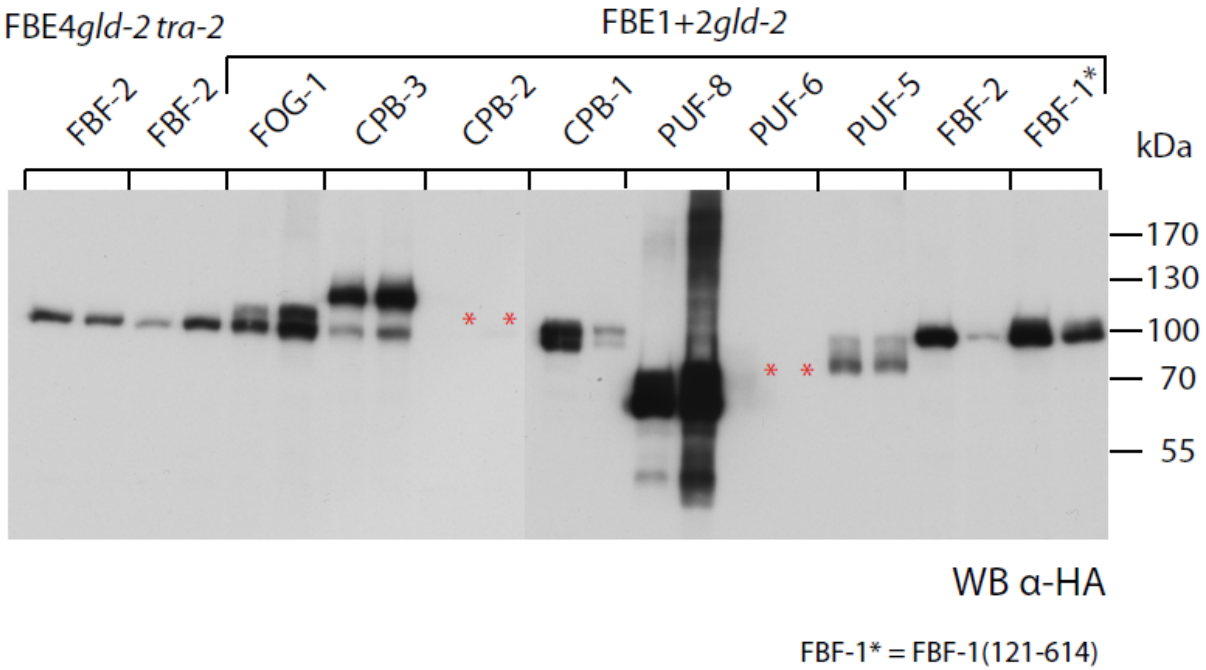

Fig.50: Protein expression from integrated plasmids for FBE1+2*gld-2* and FBE4*gld-2*. CPB-2 and PUF-6 were not expressed when in combination with FBE1+2*gld-2*. All bands are where they are expected. Red asterisks mark missing bands.

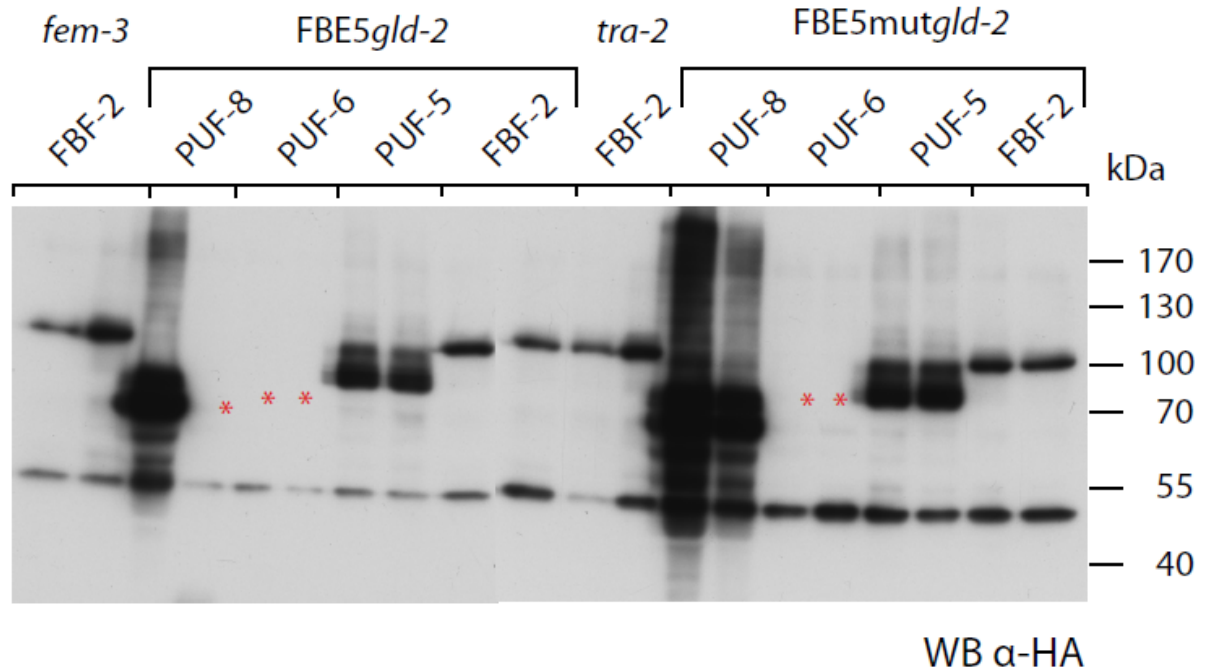

Fig.51: Protein expression from integrated plasmids for FBE5*gld-2* and FBE4*gld-2*. Protein was expressed at least once in all cases. All bands are where they are expected. Red asterisks mark missing bands.

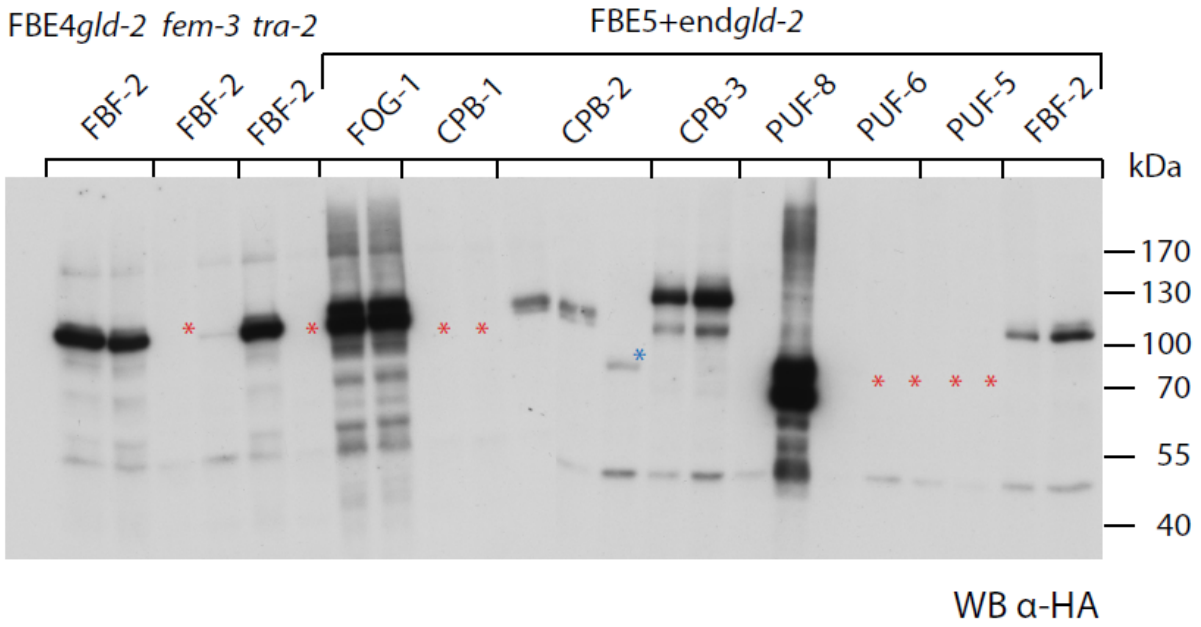

Fig.52: Protein expression from integrated plasmids for FBE5+end*gld-2* and FBE4*gld-2*. PUF-5, PUF-6 and CPB-1 were not expressed when in combination with FBE5+end*gld-2*. All bands are where they are expected (otherwise stated). Red asterisks mark missing bands, blue asterisk marks the unexpected band.

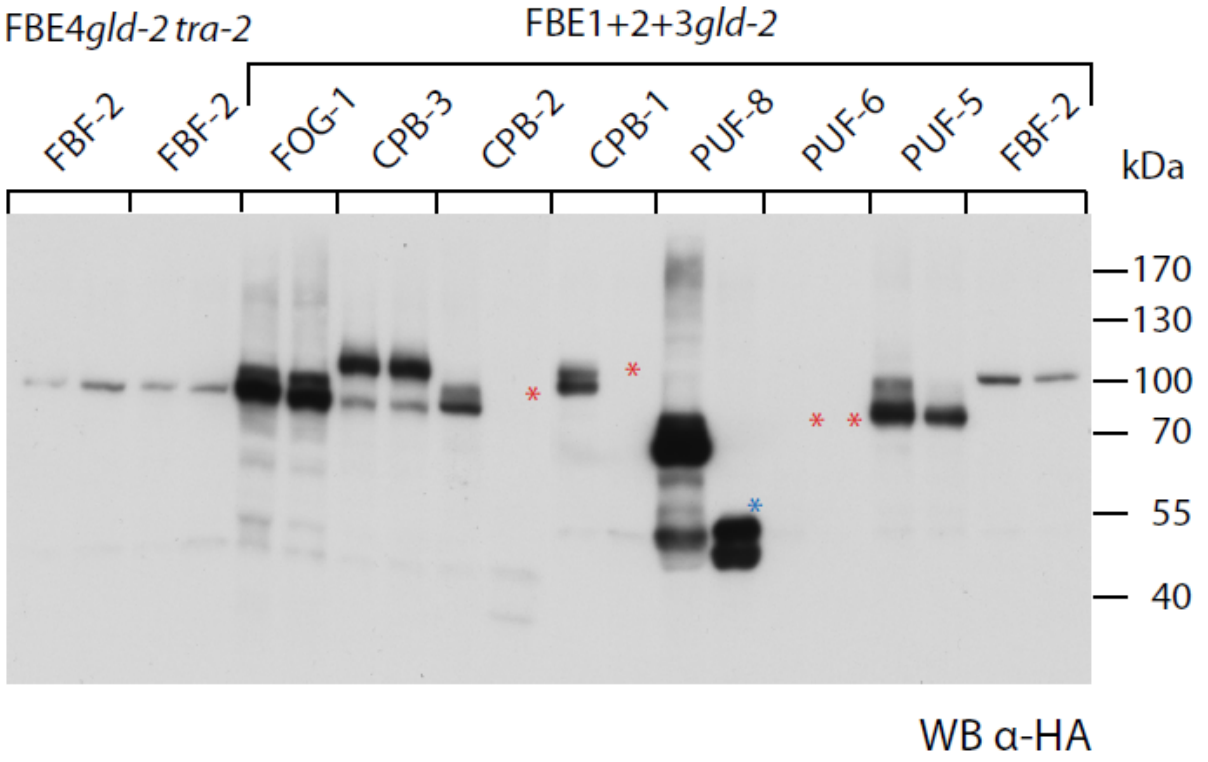

Fig.53: Protein expression from integrated plasmids for FBE1+2+3*gld-2* and FBE4*gld-2*. PUF-6 was not expressed in combination FBE1+2+3*gld-2*. All bands are where they are expected (otherwise stated). Red asterisks mark missing bands, blue asterisk marks the unexpected band.

Discussion

1. Identification of molecular lesions in different *gld-2* mutants

2. Protein expression in *gld-2* mutants

2.1 Potential autoregulatory loop of *gld-2*

3. Protein expression in tumorous and other genetic backgrounds

4. Definition of FBF-binding element (FBE) in the *gld-2* mRNA 3´UTR

1. Identification of molecular lesions in different *gld-2* mutants

To analyze the function of GLD-2 I first began by looking at mutations in GLD-2 and characterizing the molecular lesions of known and novel GLD-2 mutants. The molecular lesions of two known GLD-2 mutants were already characterized - *gld-2(h292)* and *gld-2(q497)*. These were previously identified [57] and eventually confirmed in my experiments. *gld-2(h292)* is a missense mutation, which abolishes its interaction with GLD-3. *gld-2(q497)* is a nonsense mutation and is considered a null mutant.

I have identified the molecular lesions in two additional previously-discovered *gld-2* mutants - *gld-2(q535)* and *gld-2(q540)*. *gld-2(q535)* is a nonsense mutation, placed upstream of *gld-2(q497)*. Cytosine-to-thymidine transition causes the amino acid arginine to change into the premature stop codon (R490*) - see figures 10 and 11 - resulting in a null mutant. *gld-2(q540)* is a missense mutation positioned in the nucleotidyl transferase domain. Cytosine-to-thymidine transition causes change of proline to lysine (P652L) - see figures 12 and 13. In this case, polyadenylation function may be affected, so this could be a unique type of mutation revealing more about the functional role of GLD-2 in addition to that learned from GLD-2 null mutants (*gld-2(q497), gld-2(q535)*) and one structural mutant (*gld-2(h292)*) - this one may present functional mutant.

Finally, the *gld-2(dx32)* mutant, was not previously defined. Even after sequencing of all the exons of *gld-2* and also some of the intronic regions, I was not able to find the molecular lesion of this mutant. This could possibly be either mutation in the promoter region or a mutation affecting splicing of *gld-2* mRNA.

2. Protein expression in *gld-2* mutants

GLD-2 expression in *gld-2(q497)* and *gld-2(h292)* confirmed previous data[57]. Presumptive nonsense mutant, *gld-2(q535)*, was confirmed as another null mutant. Similar behavior was also seen for *gld-2(dx32)*, but the reason for this is - due to the lack of information about its molecular lesion - unknown. I still do not know if part of the protein is expressed (the antibody against GLD-2 is targeting its C-terminal end) or if it is not expressed at all. The second novel mutant, *gld-2(q540)* shows lower expression of GLD-2 than wild-type, but not that low as is in *gld-2(h292)* mutants. Of interest is the role of GLD-3 - in *gld-3(q730)* mutants, GLD-2 protein level was elevated, suggesting potential inhibitory effect on *gld-2* expression. Another possibility is that if GLD-3 protein is missing, there are more *gld-2* mRNA molecules available for binding by FBFs.

GLD-3 seems to have similar - inhibitory - effects on levels of CPB-3 protein. This would be consistent with CPB-3 function in the spermatocyte / oocyte switch and in oocyte production[34]. GLD-3, on the other hand is essential for continued spermatogenesis, and in this process acts as a supressor of FBF[58]. Lower expression of CPB-3 in null mutants *gld-2(q497)* and *gld-2(q535)* together with elevated levels of CPB-3 in the *gld-2(h292)* mutant, which is unable to bind GLD-3, would support the idea of *cpb-3* mRNA expression being positively regulated by GLD-2 in complex with another of its binding partners - RNP-8. This complex is also important for the oogenetic program and *cpb-3* and *rnp-8* mutants exhibit similar phenotypes[59]. Interesting is enrichment of CPB-3 in *gld-2(dx32)* mutants - but without the information about the molecular lesions it is hard to predict what is behind this effect. Results for *gld-2(q540)* are ambivalent and inconclusive, as are the results for PAB-2 proteins due primarily to overly-strong signal for these bands.

2.1 Potential autoregulatory loop of *gld-2*

Neither structural mutation of GLD-2 affecting interaction between GLD-2 and GLD-3, nor mutation in its polyadenylation domain, severely affected GLD-2 protein levels. Only weak negative effect suggesting the presence of the autoregulatory loop was observed. Possible explanation is that GLD-2 function could be substituted by the action of GLD-4 poly(A) polymerase, forming “fail-safe“ system.

To confirm this autoregulatory loop, immunopurification of RNA / protein complexes from worm extracts should be performed to figure out if *gld-2* mRNA is present in GLD-2

RNP complex. To determine the role of GLD-4, analysis of GLD-2 protein levels in double mutants of *gld-4*, *gld-2(h292)* or *gld-4*, *gld-2(q540)* also should be done.

3. Protein expression in tumorous and other genetic backgrounds

Because *fbf-1.2* double mutant germlines are underproliferative and contain only sperm, there is practically no GLD-2 present - see fig.23, where the GLD-2 band for the *fbf-1.2* double mutant is the same as for the *gld-1, gld-2* negative control. In this western blot (WB #2), I used a polyclonal rabbit antibody against GLD-2, which detects GLD-2 even in confirmed null mutant *gld-2(q497)* (unpublished results) but at lower level. For that reason, genetic backgrounds, which prevent premature maturation, were used. *gld-1, gld-2* mutants have tumorous germlines so this double mutant was used as a negative control. As a tumor control, *gld-3, nos-3* double mutant was used. If *gld-2* expression is suppressed by FBF[79], the *gld-3, nos-3* double mutant would show the lowest expression - but this was not the case. With the exception of WB #3 (fig.24) where both *gld-3, nos-3* and *gld-3, nos-3, fbf-1.2* were similar, deletion of FBF proteins caused lower expression of GLD-2. The same was the case when *gld-1* (which is, on the other hand, not tumorous) and *gld-1, fbf-1.2*. Suh and colleagues[69] showed that FBF could act both as a repressor and activator. They also showed that FBF interacts with GLD-2. Together with these data, my results suggest that in adult germline, FBF acts as a positive regulator of *gld-2* expression. This could be done in two ways - either by repression of a *gld-2* repressor, or directly by its activation. FBF´s ability to directly bind GLD-2 suggests a model where FBF binds GLD-2 and this complex is eventually involved in *gld-2* regulation. This idea could be further supported by antagonistic functions of GLD-3 (promotes spermatogenesis) and FBF (promotes oogenesis)[58].

4. Definition of FBF-binding element (FBE) in the *gld-2* mRNA 3´UTR

The 3´UTR contains *cis*-regulatory elements, important for PTR. The average length of human 3´UTRs is 1027.7 nucleotides and even less in other vertebrates (rodents – 607.3; birds – 651.9; others – 446.5)[100]. In *C. elegans*, the mean length of 3′UTRs is 211 nucleotides (median is 140 nucleotides)[43]. The length of the 3´UTR of *gld-2* mRNA - interestingly - is more than 1400 nucleotides long. This fact, together with high conservation of 3´UTRs among three relative worm species (*C. elegans*, *C. briggsae* and *C. remanei*), suggests that it could have important roles in PTR and provide docking sites for different RNA-binding proteins or could play other, structure-dependent roles.

Based on sequence conservation of the 3´UTR sequence among three relative worm species - *C. elegans*, *C. briggsae* and *C. remanei* - five potential FBF-binding elements in the 3´UTRs of *gld-2* mRNA were identified and labeled as *FBE1 - 5* (see fig.35 and 36) (FBE1-5*gld-2*). In Y3H analysis, only one of these sites - called FBE4*gld-2* - was shown as a real FBF-binding site. That this interaction is sequence specific was shown by mutation of this sequence, which abolished its interaction with FBF-2 (fig.41). An octanucleotide sequence, containing *in vitro* derived FBF-binding sequence UGURHHAU (R is either A or G and H is A, C or U), is 100% conserved among all three worm species. The FBE4*gld-2* sequence also differs from "repressive FBE" derived by Merritt and Seydoux[92] (CNUGUVNHAU, where N is any base and V is A, G or C) which further supports the idea of FBF proteins promoting *gld-2* gene expression. The FBE4*gld-2* flanking sequence, which was shown to be also important for the strength of binding [93] is largely conserved (more than 88% conservation in 34 bp long sequence containing *FBE4*) among *C. elegans*, *C. briggsae* and *C. remanei*.

These facts, together with the results of performed Western blots, provide a clue for the role of FBF in PTR of *gld-2* expression. In their genome-wide analysis, Kershner and Kimble[80] found *gld-2* as a putative target of FBF. In this work, I showed a concrete FBF binding site in the 3´UTR of *gld-2* mRNA. Electrophoretic mobility shift assays should be done to confirm this interaction. Furthermore, analysis of other *gld-2* mutations, potentially affecting the 3´UTR would provide another level of evidence.

My results suggest that the role of FBF in promoting *gld-2* expression could be direct and because FBF physically interacts with GLD-2 PAP itself (or uses GLD-3 as a bridge)[69], this complex could positively regulate *gld-2* expression. Because mutation of *gld-3* has a strong positive effect on GLD-2 levels, maybe it acts to repress GLD-2 and in its absence a

positive regulator can bind, or when absent due to decreased competition simply allows more FBF molecules to be bound to *gld-2* mRNA.

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