Polyamine-independent Expression of Caenorhabditis elegans Antizyme*

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Background: Ornithine decarboxylase degradation by the proteasome is promoted by antizyme. Expression of antizyme is positively regulated by rising polyamine concentrations that induce a +1 translational frameshift required for production of the full-length protein. Antizyme itself is negatively regulated by the antizyme inhibitor. In our study, the regulation of Caenorhabditis elegans antizyme was investigated, and the antizyme inhibitor was identified. By applying a novel GFP-based method to monitor antizyme frameshifting in vivo, we show that the induction of translational frameshifting also occurs under stressful conditions. Interestingly, during starvation, the initiation of frameshifting was independent of polyamine concentrations. Because frameshifting was also prevalent in a polyamine auxotroph double mutant, a polyamine-independent regulation of antizyme frameshifting is suggested. Polyamine-independent induction of antizyme expression was found to be negatively regulated by the peptide transporter PEPT-1, as well as the target of rapamycin, but not by the daf-2 insulin signaling pathway. Stress-dependent expression of C. elegans antizyme occurred more slowly than expression in response to increased polyamine levels, pointing to a more general reaction to unfavorable conditions and a diversion away from proliferation and reproduction toward conservation of energy. Interestingly, antizyme expression was found to drastically increase in aging individuals in a postreproductive manner. Although knockout of antizyme did not affect the lifespan of C. elegans, knockdown of the antizyme inhibitor led to a significant reduction in lifespan. This is most likely caused by an increase in antizyme-mediated degradation of ornithine decarboxylase-1 and a resulting reduction in cellular polyamine levels.

Protein degradation represents a crucial step in cellular protein homeostasis. The majority of intracellular proteins are degraded by the ubiquitin-proteasome pathway, which is highly conserved from yeast to mammals (1). Here, multiple moieties of ubiquitin are covalently bound to lysine residues of proteins that are designated for degradation via the proteolytic system of the 26S proteasome. Apart from this, antizyme-mediated degradation represents an alternative, ubiquitin-independent possibility for 26S proteasomal degradation. This is based on a noncovalent antizyme target protein interaction (2, 3).

In contrast to ubiquitin-dependent degradation, which is responsible for degradation of the vast majority of cellular proteins (1), the only verified antizyme target is the rate-limiting enzyme of polyamine biosynthesis, ornithine decarboxylase (ODC)4 (4). Only a handful of other antizyme targets have been proposed, including the transcriptional regulator of the bone morphogenetic pathway SMAD-1 and the mitosis/cell cycle regulatory proteins Cyclin D1 and Aurora A (5–7). However, these have been questioned recently, restricting antizyme function solely to the cellular polyamine metabolism (8). Here, the antizyme functions as a feedback inhibitor of ODC and polyamine transport (9, 10). Briefly, antizyme binds ODC monomers with a higher affinity than ODC monomers show toward each other, resulting not only in the obstruction of the ODC homodimer interface, but also in the exposition of a C-terminal segment of ODC. This C-terminal segment leads to recognition and association with the 26S proteasome, followed by rapid degradation of the ODC monomer (4, 11).

Consistent with its inhibitory role in polyamine metabolism, antizyme expression is positively stimulated by high polyamine concentrations. Antizyme transcripts consist of two open reading frames, ORF1 and ORF2, separated by an internal stop codon. To produce a functional full-length protein, transcription needs to encompass ORF1 as well as ORF2, omitting the internal stop codon by a ribosomal +1 frameshifting event. This characteristic is a key feature of antizyme and is conserved from yeast to mammals (12). Frequency of this usually rare event is drastically increased by polyamines, representing a physiological polyamine sensor as well as constituting an auto-regulatory loop, where rising intracellular polyamine levels lead to an increase in the expression of antizyme, the feedback

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4 The abbreviations used are: ODC, ornithine decarboxylase; AZI, antizyme inhibitor; TOR, target of rapamycin.
inhibitor of cellular polyamine synthesis. It was recently shown that under physiological conditions, only the intracellular spermidine levels induce antizyme expression (13).

Antizyme itself is regulated by antizyme inhibitor (AZI), an ODC-like protein without any apparent enzymatic activity (3, 14). AZI binds antizyme with higher affinity than ODC and can therefore rescue ODC monomers from antizyme-mediated degradation. Furthermore, it stimulates polyamine transport (15, 16). Hence AZI, whose synthesis is negatively regulated by polyamines at transcriptional level, is the antagonist of antizyme in the regulatory interplay of polyamine synthesis and transport. This ensures tight control of the intracellular polyamine levels (3, 17, 18).

Physiological polyamines such as putrescine and spermidine are aliphatic, polycationic amino acid derivatives involved in a myriad of biological processes (19, 20). Consistent with their essential role in the eukaryotic cell cycle, knock-out mutants of polyamine synthesis genes odc and smd-1 (S-adenosylmethionine decarboxylase) were found to be embryonic lethal in mammals (21, 22).

Development of the nematode Caenorhabditis elegans also depends on polyamines. However, here the loss of polyamine synthesis leads to polyamine auxotrophy and hence can be circumvented by dietary polyamines (23, 24). This offers the opportunity to study the role of polyamine synthesis genes beyond embryogenesis. In good agreement with other eukaryotes, polyamine levels in C. elegans are maintained by (i) a synthesis pathway with ODC, SMD-1, and spermidine synthase and (ii) a polyamine uptake system, with the P_5-type ATPase CATP-5 (23–25).

The C. elegans antizyme is encoded by the gene ZK484.1a. As is the case for all known antizymes, the corresponding transcript includes two ORFs that, when fused by a +1 frameshift, result in the full-length polypeptide that, in case of C. elegans antizyme, is composed of 159 amino acids. Polyamine-dependent frameshifting of C. elegans antizyme has been previously demonstrated in a heterologous in vitro system (12).

We have recently demonstrated that a lack of polyamine synthesis combined with an impaired polyamine uptake in C. elegans results in reduced polyamine levels. These were linked to postembryonic phenotypes, including a small body size and a shortened adult lifespan (23). Interestingly, moderate elevation of intracellular polyamine levels by dietary polyamines has been reported to lead to a prolonged lifespan in C. elegans and other eukaryotes (26, 27). In this regard, polyamines were discussed to be involved in stress tolerance. However, in contrast to the situation in plants and prokaryotes, where polyamines are established as key molecules in stress response, the role of polyamines in the metazoan stress metabolism is not yet clear (28, 29).

In the present study, we have investigated the expression of C. elegans antizyme in vivo by employing a reporter gene approach. Consistent with homologous proteins of other organisms, the C. elegans antizyme is controlled by a polyamine-responsive internal ribosomal frameshifting site and by AZI. Furthermore, we have found that antizyme levels are enhanced under food deprivation and other stressful conditions, including heat, UV exposure, and oxidative stress. Most remarkably, this stress-induced increase in antizyme level occurs even in the absence of polyamine synthesis and independent of an increment in the intracellular polyamine concentrations. Interestingly, we observed that the C. elegans antizyme expression is negatively regulated by the target of rapamycin complex 1 (TOR-1) signal pathway. Monitoring intra- and extracellular physiological conditions, the TORC-1 pathway controls cell functions such as protein synthesis, autophagy, cell growth, and cell proliferation. Furthermore, it is involved in aging and stress response (30, 31). Taken together, our findings suggest that the role of C. elegans antizyme is not limited to polyamine regulation but is extended to cellular protein homeostasis under stressful conditions.

**Experimental Procedures**

**Cloning of C. elegans Antizyme and C. elegans AZI for Recombinant Expression and Pulldown Assay**

For cloning of the constructs, whole worm RNA was prepared from C. elegans using TRIzol reagent (Invitrogen) according to the supplier’s protocol. First strand cDNA synthesis was performed according to the manufacturer’s protocol (Thermo Scientific). Amplification of coding sequences was done by PCR using Phusion high fidelity DNA polymerase (Thermo Scientific), following the manufacturer’s instructions. PCR products were cloned into respective vectors using standard protocols. For recombinant expression of full-length antizyme, the internal frameshift site had to be removed. This was done by mutagenesis using the QuikChange™ II XL site-directed mutagenesis kit (Roche).

**Construction of Plasmids for Microinjection**

\( \text{oazp::oaz(177)::gfp—} \) A 3.9-kb promoter fragment of the C. elegans antizyme (oazp) that includes the N-terminal part of the coding region and ends immediately 5’ adjacent to the internal stop codon of the potential frameshift site was amplified by PCR from C. elegans genomic DNA using the Expand Long Template PCR system (Roche). The PCR product was cloned into pPD95.77 provided by A. Fire (Carnegie Institute, Baltimore, MD).

\( \text{oazp::oaz(647FS)::gfp—} \) A 4.4-kb promoter fragment of C. elegans antizyme including 3743 bp of the upstream region and the complete coding region of three exons and two introns was amplified by PCR and subsequently cloned into pPD95.77 using the In-Fusion™ cloning kit (Takara), following the manufacturer’s guidelines. The construct contains the internal stop codon representing the potential ribosomal frameshift site of antizyme.

**C. elegans Culturing, Strains, and Generation of Double Mutants**

C. elegans were maintained on nematode growth medium (NGM) at 20 °C under standard conditions using Escherichia coli OP50 as a food source (32). Worm populations were synchronized by alkaline hypochlorite lysis (33). The following strains were obtained from the Caenorhabditis Genetics Center at the University of Minnesota, which is funded by the National Institutes of Health National Center for Research Resources:
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wild-type N2 Bristol and SF1 *odc-1(pc13::Tc1) V*. Strain FX02772 *smd-1(tm2772)* was obtained from the National Bioresource Project of the Tokyo Women’s Medical University School of Medicine. KLU001 *catp-5(mun1)* has been recently isolated (23). *odc-1(pc13::Tc1), smd-1(tm2772)*, and *catp-5(mun1)* alleles were verified by PCR according to (23).

**Transgenic C. elegans**

Germ line transformation was performed by co-injecting vector constructs (20–50 μg ml⁻¹) with the pRF4 plasmid (80 μg ml⁻¹) encoding the dominant marker gene rol-6 into the germ line of young adults. N2 wild-type worms were injected with *oazp::oaz(177)::gfp* and *oazp::oaz(673FS)::gfp*. The *odc-1(pc13),smd-1(tm2772)* double mutant was transformed with *oaz-1(673FS)*::gfp.

**Analysis of the C. elegans Antizyme Expression Pattern**

To investigate the polyamine and stress-regulated expression of antizyme, the GFP expression pattern was analyzed by fluorescence microscopy in young adult worms. These were grown (i) under standard culture conditions, (ii) in the presence of 5 mM spermidine (Applichem) for up to 16 h, or (iii) following cultivation under standard conditions. Short exposure to UV light (100 J/m²) was followed by isolation (23). *oaz-1(673FS)*::gfp and *smd-1(tm2772)* double mutant was transformed with *oaz-1(673FS)*::gfp.

**Polyamine Determination**

Synchronized worms were cultured for 66 h under standard conditions before being separated on standard NGM plates with OP50 and starvation plates without food bacteria, respectively. After an additional 6 h of incubation at 20 °C, worms samples were harvested and sonicated, and the 10,000 × g supernatants were deproteinized by adding 0.2 × perchloric acid. HPLC analysis was performed as described (23). In short, samples were derivatized with dansyl chloride (2 mg/ml acetone) before being separated by HPLC (Thermo Scientific, Dreieich, Germany) on a Nucleosil 120–5 C₁₈ column (250 mm × 3 mm; Macherey and Nagel, Düren, Germany). Dnsylated polyamines were detected by fluorescence spectrophotometry (Jasco 821-FP; Thermo Scientific).

**Determination of Lifespan and Brood Size**

Phenotype analysis of *C. elegans* antizyme (*oaz*) RNAi worms, antizyme inhibitor (*azi*) RNAi worms, and pl4440 RNAi control worms was performed at 20 °C, as described (34). For brood size determination, worms were cultured individually on NGM plates. Adults were transferred onto new plates daily, until egg production ceased. Plates with progenies were retained and counted once the larvae reached L3/L4. For longevity determination, 100 μg/ml 5-fluoro-2’-deoxyuridine was added to the NGM agar. For each lifespan assay, 3 × 40 worms were distributed equally onto five plates and examined daily. Animals that no longer responded to mechanical sensation were counted as dead. Worms that crawled off the plates or died because of internal hatching of progenies were replaced by worms from parallel substitute plates. Survival plots were compared using the log rank test (GraphPadPrism Software).

**Recombinant Expression and Purification of C. elegans Antizyme and AZI**

Following transformation of the pGEX-4T2::oaz and pTrcHisB::azi expression plasmids into *E. coli* BL21DE3 Star cells (Stratagene), expression of the tagged proteins was initiated by the addition of 1 mM iso-propyl-β-thiogalactopyranoside (Sigma-Aldrich) once the cultures reached OD₆₀₀ = 0.5. Following induction, cells were left to grow for additional 3 h at 37 °C. Cells were harvested by centrifugation, and the resulting bacterial pellets were stored at −20 °C until further use.

**Interaction Analysis via Pulldown Assay**

To investigate possible interactions between antizyme and the antizyme inhibitor, bacterial pellets were resuspended in antizyme buffer (pH 7.4) containing 10 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, and protease inhibitor (Roche); sonified; and centrifuged. Approximately 20 μl of *E. coli* lysate were combined in the following fashion: (i) antizyme inhibitor + antizyme, (ii) antizyme inhibitor + GST only (control), (iii) antizyme lysate only, (iv) antizyme inhibitor lysate only, and incubated for 1 h at room temperature on a rotator (6 rpm). The combined lysates were incubated with 50 μl of glutathione-agarose (Sigma) for 2 h at room temperature by gently shaking, followed by washing with 50 bed volumes of antizyme buffer. Next, the proteins were eluted in GSH elution buffer (10 mM GSH (Sigma)) in 50 mM Tris/HCl, pH 8.0). The bound proteins were analyzed by Western blot using anti-His (Invitrogen) and anti-GST (Dianova) antibodies.

**Genetic Interaction Studies**

To identify metabolic and stress signaling pathways that affect antizyme levels, *oazp::oaz(643FS)::gfp* worms were fed with *E. coli* HT115 bacteria producing double-stranded RNA of *azi, skn-1, pept-1, (geneservice), let-363 (TOR), or daf-2*. Following standard RNAi protocols, *C. elegans* L4 or eggs were transferred to NGM plates containing 2.5 mM iso-propyl-β-thiogalactopyranoside and 50 μg/ml ampicillin that had previously been inoculated with the respective HT115 RNAi strain. Worms were incubated for 96 h (L4 to F1 young adults) and 72 h (eggs to young adults) at 20 °C, before young adult worms were inspected for GFP expression.

**Results**

Physical Interaction between *C. elegans* Antizyme and the Putative Antizyme Inhibitor—Our first goal was to establish whether the regulatory circuit between ODC, antizyme, and antizyme inhibitor for *C. elegans* complies with the well known
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**Figure 1. Interaction analysis of antizyme and antizyme inhibitor via pulldown assay.** Lane 1 shows bead-bound GST:antizyme (45 kDa) detected by anti-GST antibody and retained His::AZI (55 kDa) detected by anti-His antibody. Lane 2 demonstrates that His::AZI is not retained by GST alone. Lane 3 shows the ability of GST:antizyme to bind to GSH-agarose, whereas lane 4 shows that His-tagged AZI is not retained by GSH-agarose. Analysis was performed by SDS-PAGE and Western blotting using anti-GST and anti-His antibodies. Boxes indicate proteins of interest. MW, molecular weight.

standard model (3). Contrary to *odc* and antizyme, an *azi* gene for *C. elegans* has not yet been described. Because protein sequences of ODC and AZI are usually very similar, we identified the gene F53F10.2, which is annotated as a *C. elegans* ODC-1 paralogue, to be the most likely candidate for *C. elegans* AZI. Remarkably, the longest F53F10.2 transcript (isoform a) encodes a protein of 477 amino acids that shows highest sequence similarity with the filarial ODC-like proteins of *Loa loa* and *Brugia malayi* (31% identities, data not shown), followed by the human ODC paralogue (29% identities; data not shown), which has recently been described to be an AZI (35). Furthermore, the F53F10.2a sequence lacks several amino acids described to be crucial for ODC function (14). In accordance to our recent findings, where we demonstrated that lack of amino acids crucial for enzymatic ODC function leads to a complete lack of enzymatic activity of *B. malayi* ODC-like protein, we propose *C. elegans* F53F10.2a to be a putative AZI (36).

Because we most recently have shown physical interaction between CeODC and antizyme *in vitro*, we next investigated whether this was also the case for antizyme and the putative antizyme inhibitor (F53F10.2a), which would be expected if the components of the antizyme regulatory network in *C. elegans* function in the same way as generally accepted (3, 36). To this end, we chose to use a GST-based pulldown assay (Fig. 1). *Lane 1* of Fig. 1 shows bead bound GST:antizyme (45 kDa) detected by anti-GST antibody, as well as His::AZI (55 kDa) detected by anti-His antibody, indicating co-purification. *Lanes 2–4* are controls, where *lane 2* demonstrates that His-tagged ODCs are not retained by GST alone, *lane 3* shows the ability of GST::antizyme to bind to GSH-agarose, and *lane 4* shows that His-tagged ODCs are not retained by GSH-agarose. Taken together, these results indicate a direct physical interaction between *C. elegans* antizyme and antizyme inhibitor.

**Polyamine-dependent in Vivo Expression of Antizyme**—Next we investigated the expression pattern of the antizyme *in vivo*. To visualize the effects of ribosomal frameshifting, we chose to employ two GFP reporter gene constructs under control of the native *oaz* promoter. The first construct includes only the N-terminal part of antizyme fused to GFP omitting the internal frameshift site (*oazp:oaaz(177):gfp*). In the second construct, the whole coding sequence of antizyme was fused to GFP and therefore included the internal frameshifting site as a regulatory element (*oazp:oaaz(643FS):gfp*). Fig. 2A shows the expression pattern of *oazp:oaaz(177):gfp* worms cultivated under control conditions and on plates containing 5 mM spermidine. GFP signals are ubiquitously visible in apparently all tissues of the worm without any notable differentiation in signal strength between both conditions. The observed GFP pattern reflects the activity of the *oaz* promoter, because *oaz* RNAi drastically reduced GFP fluorescence to very low levels with some more prominent fluorescence remaining in the anterior part of the worm surrounding the pharynx. Fig. 2B depicts the expression pattern of *oazp:oaaz(643FS):gfp*. Here the effect of additional transcriptional regulation mediated by the ribosomal frameshifting site led to a stark contrast when compared with Fig. 2A. Worms grown under control conditions showed very miniscule fluorescence, which was restricted to the spermatheca uterine valves. Furthermore, some minor fluorescence was observed in the posterior and anterior parts of the worms. As expected and in good accordance with the reported *in vitro* stimulation of ribosomal frameshifting of *oaz* by polyamines (12), the addition of 5 mM spermidine led to a strong induction of *oazp:oaaz(643FS):gfp* expression, which was restricted to the intestinal parts of the worms. Already after 5 h of incubation, ~80% of the worms showed bright fluorescence signals. Hence, our reporter gene construct *oazp:oaaz(643FS):gfp* represents an appropriate tool to study antizyme expression in a living organism.

**Antizyme Expression Level Is Negatively Regulated by the Putative Antizyme Inhibitor AZI**—We next wanted to know whether the putative AZI (F53F10.2) affects the expression level of antizyme. *oazp:oaaz(643FS):gfp* worms, which were subjected to *azi* (RNAi) exhibited the same strong intestinal expression pattern as worms grown on 5 mM spermidine, indicating that the antizyme inhibitor functions as a negative regulator of antizyme (Fig. 2B).

**Food Deprivation Led to Elevation of Antizyme Level in a Polyamine-independent Manner**—During the course of cultivation of the transgenic *oazp:oaaz(643FS):gfp* strain, we noticed that starvation conditions resulted in increased GFP expression similar to the effect triggered by 5 mM spermidine. Slightly elevated GFP levels were detectable already 5 h after worms were transferred to empty NGM plates without OP50 food, and signals became more intensive after 16 h (Fig. 3A). As a control, we also used plates supplemented with prekilled OP50 bacteria. Here no increased GFP signals were observed. This suggests that the absence of food causes the elevated antizyme levels.

To investigate whether the increase of *oazp:oaaz(643FS):gfp* expression under starvation conditions is linked to an increase in cellular polyamine levels, we determined the spermidine and putrescine content of worms that were beforehand subjected to starvation conditions for 6 h (Fig. 3B). Worms cultivated under control conditions contained ~130 nmol/mg of putrescine and ~210 nmol/mg of spermidine. Surprisingly, the polyamine content of starved worms was slightly lower, with ~120 nmol/mg of putrescine and 200 nmol/mg of spermidine. Therefore no alterations in cellular levels of spermidine and putrescine were detectable when comparing worms grown under control conditions to worms exposed to starvation conditions.
This might suggest that starvation-induced increase in oazp::oaz(643FS)::gfp expression is independent of the cellular spermidine and putrescine concentrations. However, our analytical HPLC method measures the polyamine content of the entire organism and is not able to detect local, tissue-specific changes in polyamine levels that might be responsible for elevated antizyme levels under starvation.

To clarify this, we chose to employ the *C. elegans* double mutant strain odc-1(pc13),smd-1(tm2772) as genetic background to study oazp::oaz(643FS)::gfp expression. This *C. elegans* strain is unable to generate the polyamine precursor putrescine because of the odc-1(pc13) mutation, which impairs ODC function (23, 24). It is also unable to synthesize spermidine from putrescine because of the smd-1(tm2772) mutation, which impairs *S*-adenosylmethionine decarboxylase (23). The oazp::oaz(643FS)::gfp expression was monitored in odc-1(pc13),smd-1(tm2772) worms that were grown under control and starvation conditions, as well as in worms grown on plates supplemented with 5 mM spermidine. Worms grown under control conditions showed only marginal GFP fluorescence (Fig. 4A). In contrast, over 95% of worms grown on plates containing 5 mM spermidine showed GFP fluorescence in their intestinal cells. Intriguingly, intestinal expression of oazp::oaz(643FS)::gfp was also induced by starvation (Fig. 4B). Worms grown on plates containing 5 mM spermidine (95%) showed notably stronger fluorescence when compared with worms grown under starvation conditions (>75%). Taken together, these data strongly suggest that the observed elevation of reporter gene expression levels under food deprivation occurred in a polyamine-independent manner.

**The Antizyme Level Is Regulated by let-363(TOR) and pept-1 but Not by daf-2**—Because the elevated oazp::oaz(643FS)::gfp level under starvation appears not to be accompanied by a change in intracellular polyamine levels, we next investigated whether other upstream regulators could be found for polyamine-independent antizyme regulation. The TOR and the insulin signaling pathway represent two central metabolic regulators that respond to changes in the nutritional status (37, 38). To investigate a potential involvement of either TOR or DAF-2 in the polyamine-independent expression of oazp::oaz (643FS)::gfp, we chose to employ an RNAi approach. Although
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daf-2(RNAi) did not induce the intestinal GFP signal in our oazp::oaz::gfp reporter strain, let-363(RNAi) led to a considerable fluorescence signal, which was localized to the intestine (Fig. 5). Remarkably, let-363(RNAi) also triggered oazp::oaz::gfp expression in the polyamine synthesis-deficient odc-1(pc13),smd-1(tm2772) mutant background, whereas daf-2(RNAi) failed to alter expression of the reporter gene construct in this background (data not shown). Hence, these data indicate that let-363(TOR) functions as a negative regulator of antizyme expression levels.

Lowering the intracellular concentrations of certain amino acids has generally been associated with a decreased TOR pathway signaling, which in turn affects several metabolic processes, e.g. leading to inhibition of protein translation and induction of autophagy (30). Because the oligopeptide transporter PEPT-1 has been demonstrated to be a crucial factor of amino acid metabolism and to act upstream of let-363 (39, 40), we next performed pept-1(RNAi) in our oazp::oaz::gfp reporter gene strain (Fig. 5). Knockdown of pept-1 also resulted in increased GFP levels. In this regard, it is tempting to speculate that a lack of nutrients, most likely amino acids, leads to a PEPT-1/TOR-mediated expression of C. elegans antizyme.

The Antizyme Level Increases under Various Environmental Stress Conditions in a Polyamine-independent Manner—We next investigated the effect of several environmental stressors on oazp::oaz::gfp expression. The conditions applied included mild heat stress (30 °C), oxidative stress (20 mM paraquat), and UV radiation (100 J/m²). In Fig. 3A, the rate of ribosomal frameshifting is given as a percentile value of worms showing GFP fluorescence. After a 5-h incubation time, the percentile fluorescence values for worms grown under control conditions, as well as worms subjected to UV radiation, were very low, generally under 10%. A slight increase in GFP fluorescence was observed in worms grown on plates containing 20 mM paraquat (~15%). Most notably, worms subjected to heat conditions showed a strong induction of GFP fluorescence, with percentile values of ~50 and 60%, respectively. An increase of incubation time to 16 h led to a strong general increase in GFP fluorescence for all stressful conditions, with percentile values of ~90% across the board. Notably, an increase of incubation time did not lead to an increase in GFP fluorescence for worms grown under control conditions.

To investigate whether the observed stress response depends on elevation of polyamine levels, we next tested oazp::oaz::gfp expression in the polyamine synthesis-deficient double mutant odc-1(pc13),smd-1(tm2772). The induction of oazp::oaz::gfp expression was monitored for worms grown under control and mild heat (30 °C) conditions. Evaluation was carried out after 24 h and recorded as percentile values of worms showing fluorescence (Fig. 4A). In contrast to worms grown under control conditions that showed only marginal GFP fluorescence, expression of oazp::oaz::gfp was also induced by heat in a polyamine synthesis-deficient background. Here ~85% of worms showed fluorescence that was localized mainly to the intestine (Fig. 4B). This implies that elevation of antizyme levels under stress conditions is also regulated in a polyamine-independent manner.

Postreproductive Elevation of Antizyme Level—We subsequently investigated the expression pattern of oazp::oaz::gfp in aging individuals (Fig. 6). As mentioned above, in young adult hermaphrodites, GFP expression was restricted to the spermatheca uterine valve. However, from day 5 onwards, expression of oazp::oaz::gfp started in the 20 intestinal cells, and levels increased constantly until day 13. GFP fluorescence was strongest around day 13, after which it decreased again, even while maintaining high GFP levels. Interestingly, with proceeding age of the worms, localization of GFP fluorescence was no longer restricted to the intestinal cells and started...
to become more ubiquitous around day 9. After day 11, GFP fluorescence was widespread, and no specific localization was visible anymore. To summarize, it can be said that under standard culture conditions, the antizyme shows an age-related expanding expression that starts when the reproduction phase of *C. elegans* ceases.

*Knockdown of the Antizyme Inhibitor Affects Lifespan of* *C. elegans*—Cellular polyamine levels are known to impact the lifespan of *C. elegans* (23, 26). Because both antizyme and AZI...
play key roles in cellular polyamine homeostasis, we next investigated their impact on the lifespan of \textit{C. elegans} (Fig. 7). Knockdown of \textit{oaz} did not lead to a noticeable lifespan phenotype, whereas knockdown of \textit{azi} resulted in a drastic reduction in adult lifespan. This reduction in lifespan was notable from day 12 on, after which knockdown of \textit{azi} resulted in the death of over 60\% of the test subjects during the next 3 days. Interestingly, knockdown of neither \textit{oaz} nor \textit{azi} had any effect on \textit{C. elegans} brood size (data not shown). Although reduced cellular polyamine concentrations lead to a shortened adult lifespan, elevation in dietary polyamines has been reported to lead to an increment in lifespan (23, 26). Therefore, it is tempting to speculate, that the effect of \textit{azi} knockdown is due to the lack of the antizyme inhibitor binding to the antizyme and thereby regulating its function.

**Discussion**

The Antizyme Regulatory Network in \textit{C. elegans}—In the current study, we established a novel \textit{in vivo} system to monitor \textit{oaz} mRNA frameshifting in \textit{C. elegans}. This enabled us to monitor antizyme expression in a whole, living organism and look into the circumstances leading to the induction of frameshifting. To achieve a better understanding of the regulation of antizyme expression, we first needed to identify the \textit{C. elegans} antizyme inhibitor because up to now, no AZI was reported for this organism (41, 42). Our computational and experimental results presented in this work suggest the gene product of the \textit{C. elegans} locus F53F10.2 to be the putative antizyme inhibitor of \textit{C. elegans}. Blastp search showed highest sequence homologies with the proposed filarial antizyme inhibitors of \textit{L. loa} and \textit{B. malayi}. Protein sequence alignment with the \textit{C. elegans} ODC, although showing high similarity of the two sequences, revealed amino acid substitutions in positions crucial for ODC enzymatic activity, a key feature of all known antizyme inhibitors (14, 43).

Furthermore, we were able to verify the interaction of the putative antizyme inhibitor with the antizyme in an \textit{in vitro} pulldown assay. Application of RNAi-mediated knockdown of the F53F10.2 mRNA \textit{in vivo} resulted in an increase in the expression of the \textit{oazp::oaz(643FS);gfp} reporter gene construct, indicating negative regulation of the antizyme by the putative antizyme inhibitor. Interestingly, the putative antizyme inhibitor seems to regulate the antizyme in a manner different to that described for the mammalian antizyme inhibitor AZI-one. Although in mammalia, the antizyme inhibitor binds the antizyme, hence freeing ODC from antizyme suppression, it also does not promote but rather protects it from degradation (3). Here it is important to note that the putative \textit{C. elegans} AZI shows higher homologies to the mammalian ODC parologue also termed antizyme inhibitor AZI-two than toward the mammalian AZI-one (35). AZI-two expression has been found in testis and brain and more recently also in secretory tissues and other cell types (44, 45). The physiological role of AZI-two is mostly unknown and a different way of regulating antizymes by diverging antizyme inhibitors could be possible (46). In this case, the proposed \textit{C. elegans} AZI would either negatively influence synthesis of the antizyme or promote the degradation of the protein. Taken together, our results present compelling evidence that the \textit{C. elegans} locus F53F10.2 encodes for the putative antizyme inhibitor.

Antizyme Expression in Vivo Is Induced by Polyamines—The antizyme promotes ubiquitin-independent degradation of ODC, the rate-limiting enzyme of polyamine synthesis, by the 26S proteasome (2, 4). In general, expression of antizyme itself is dependent on the cellular level of polyamines. Higher polyamine levels lead to a programmed ribosomal frameshift in the \textit{oaz} mRNA, which is required for expression of the full-length protein (47). For \textit{Saccharomyces cerevisiae} antizyme, it was
shown that polyamine binding to the nascent ribosome-associated antizyme polypeptide promotes completion of its synthesis, whereas at low polyamine levels, the emerging antizyme polypeptide inhibits the completion of its synthesis by causing a ribosome pile-up on antizyme mRNA (48). For the C. elegans antizyme, it was shown that the addition of spermidine increases the occurrence of frameshifting when expressing the protein in rabbit reticulocyte lysate (12). In the present study, we confirmed this polyamine-dependent induction of ribosomal frameshifting in vivo by utilizing two GFP reporter gene constructs in C. elegans.

Not surprisingly, omitting the ribosomal frameshifting site led to a constitutive expression of the GFP reporter gene construct in all tissues of the worm, without differentiation between control conditions and conditions with elevated spermidine concentrations. In contrast to this, a GFP reporter gene construct that included the ribosomal frameshifting site showed no significant expression under control conditions, with the exception of some elevated expression in the spermatheca uterine valve. A possible cause for this regionally limited expression could be a generally higher polyamine concentration in the aforementioned compartment. The spermatheca uterine valve serves as passageway of fertilized oocytes from the spermatheca to the uterus (49). Interestingly, this localized expression of the reporter gene construct seems to dissipate with ongoing age of the worms (Fig. 6), restricting the observed effect to a time before passage of fertilized embryos from the spermatheca. In this context, it is interesting to note that seminal fluid of mammals is known to have high concentrations of the polyamines spermidine and spermine (2).

Earlier investigations on the effects of polyamines on frameshifting events were carried out in heterologous systems (12). Although a considerable effect on the frameshifting frequency (an ~2-fold increase in frameshifting efficiency) was observed using rabbit reticulocyte lysate, only a low number of actual frameshifting events (~1.5% after addition of spermidine (12)) could be demonstrated. The in vivo effect in C. elegans was much more pronounced. Application of dietary polyamines, in our case 5 mM spermidine, led to a very strong induction of reporter gene expression, which was predominantly restricted to the intestinal apparatus of the animal, most likely because of the uptake of spermidine with the food source.

Postreproductive Elevation of Antizyme Expression—As mentioned above, expression of the antizyme was restricted to the spermatheca uterine valve in young C. elegans adults grown under control conditions. This changed drastically in aging individuals. Here, expression levels rose most notably in the intestinal cells from day 5 onwards. Finally, expression was widespread, without distinct localization, with a maximum at day 13 and slightly lower but still significant levels post day 13. Interestingly, the rise in antizyme expression level coincides with the end of embryogenesis and egg laying. It is tempting to speculate that this is due to an increased need for polyamine production (and hence reduced antizyme levels) during oogenesis, embryogenesis, and development of the young adult worms in the first 5 days of their lifespan. In this regard, it is interesting to note that if polyamine supply is disrupted in L4 larvae, they reproduce normally, but the resulting eggs develop into embryos that arrest at early stage 3, indicating the importance of polyamines for embryogenesis (24). A reduction in ODC activity caused by an increased antizyme expression in aging individuals after the reproductive phase could be caused by a stop of proliferation and a reduction in polyamine-dependent gene expression. No postreproductive up-regulation of oaz mRNA levels was observed in a transcriptional profile of aging in C. elegans (50), suggesting regulation at the level of translation.

Effects of Antizyme and Antizyme Inhibitor Knockdown on Adult Lifespan—Theoretically, RNAi-mediated depletion of oaz and azi levels could have several effects on lifespan. Knockdown of the antizyme could lead to a situation where intracellular polyamine levels increase. An increase of dietary spermidine has been shown to increase C. elegans lifespan by up to 15% (26). On the other hand, knock-out of the oaz locus leads to sterility and confers embryonic lethality in C. elegans (National BioResource Project). Remarkably RNAi-mediated knockdown of oaz had no notable effect on C. elegans adult lifespan. One might argue that the severe effects of oaz knock-out could be masked by the fact that these are all in correlation with reproduction and the monitored animals were adults. However, knockdown of oaz also did not lead to any notable differences in brood size when compared with wild-type animals (data not shown). A possible explanation for the divergence between knock-out and RNAi-mediated knockdown of oaz could be that oaz mRNA suppression by RNAi is not 100% effective and that the remaining antizyme protein level is sufficient to negate the drastic consequences observed in knock-out mutants. In this context, it is important to note that RNAi-mediated suppression of oaz during induction of oaz::oaz(643FS):gfp did not completely abolish GFP fluorescence (Fig. 1).

In contrast to this, knockdown of the antizyme inhibitor resulted in a significant reduction of C. elegans adult lifespan from day 12 on. This matches the expectations, because knockdown of azi should increase the effect of antizyme-mediated degradation of CeODC, which in turn would lead to a reduction in cellular polyamine levels. Fittingly, reduced availability of cellular polyamines is known to negatively affect C. elegans lifespan, and selective depletion of intracellular polyamines is known to induce apoptosis (23, 52).

Antizyme Expression Is Induced by Lack of Nutrition and Regulated by let-363 as Well as pept-1 but Not daf-2—Interestingly, during the course of our experiments, we noticed that antizyme expression in vivo gets up-regulated not only by dietary polyamines with a subsequent increase in polyamine concentrations but also by the application of nutritional stress, i.e. starvation of the animals. No increase in the levels of the polyamines putrescine and spermidine was detectable after application of starvation conditions, although antizyme expression was clearly induced. Although one might argue this to be the result of our HPLC measurements not being able to record tissue- and cell-specific changes in polyamine concentrations, the induction of antizyme expression under starvation conditions was prevalent, albeit weaker when compared with induction by 5 mM spermidine. This induction of oazp::oaz(643FS):gfp expression was visible even in the odc-1(pcs13),smd-1(tm2772)
double mutant, which is unable to synthesize putrescine as well as spermidine and hence is polyamine auxotroph.

Because starvation abolishes the supply of amino acids, the potential role of the peptide transporter PEPT-1 as the upstream regulator of polyamine-independent expression of the antizyme was investigated. PEPT-1, which is expressed in intestinal cells, is a proton-coupled transporter of small di- and tripeptides and has been shown to function in nutrient sensing and signaling (39). Knockdown of pept-1 leads to a clear induction of oazp::oaz(643FS)::gfp reporter gene expression, indicating that antizyme expression under starvation conditions is regulated by PEPT-1 nutrient sensing. PEPT-1 is known to be an upstream regulator of LET-363 (TOR), as well as being interwoven with the DAF-2-mediated insulin/IGF signaling pathway (37, 39, 40). Intriguingly, RNAi-mediated knockdown of let-363 induced the expression of the oazp::oaz(643FS)::gfp reporter gene construct in the wild type as well as in odc-1(pc13),smd-1(tm2772) double mutant background. In contrast, daf-2 RNAi did not increase the antizyme expression. This leads us to the conclusion that TOR negatively regulates antizyme levels and that TOR inhibition induces antizyme expression independent of the cellular polyamine biosynthesis. In this context, it is interesting to note that recent findings in cell culture experiments have shown the amino acids asparagine and glutamine to regulate rat antizyme1 expression by differential activation of mammalian TORC-1 and TORC-2 (53, 54). Here it was shown that amino acid starvation leads to inhibition of TORC-1 signaling, whereas TORC-2 is activated. This results in the inhibition of global protein synthesis, whereas antizyme 1 expression is increased in a cap-independent mechanism. It is tempting to speculate that our observation of negative regulation of antizyme expression by TOR is also based upon an inactivation of TOR caused by starvation, in particular amino acid starvation.

TOR kinases are known to serve in nutrient regulation of cell growth and proliferation by regulating several cellular processes, especially the initiation of mRNA translation and ribosome synthesis (55). In the case of C. elegans, TOR is known to be an upstream regulator of overall mRNA translation (56). It would not be surprising that inactivation of TOR caused by nutritional deficits leads to an expression of antizyme, which in itself is a regulator of cellular proliferation by reducing cellular polyamine biosynthesis. However, our results show an activation of antizyme expression without any alterations observed in whole worm polyamine levels and, furthermore, in a polyamine-deficient double mutant. This poses the question as to how translational frameshifting of oaz mRNA, which has been shown to be polyamine-dependent, is initiated in C. elegans under these conditions.

It is conceivable that frameshifting is mediated by a yet unknown interface, which is regulated by more signals than just increasing polyamine concentrations. The reduction of intracellular polyamine synthesis by TOR-dependent activation of antizyme expression during starvation would serve the need of the animal to conserve energy for the maintenance of basal cellular processes. In this regard, it is interesting to note that polyamines (e.g. spermidine) are involved in eukaryotic translation and are required for the formation of hypusine in the translation initiation factor eIF5A, which is involved in the translation of specific RNAs. On the other hand, TOR regulates the binding of eIF-4E to eIF-4G to form the complex eIF-4F, which is needed for the translation of mRNAs possessing highly structured 5' UTRs (19, 56). In this way, the deactivation of TOR caused by starvation would have a 2-fold effect on cellular.
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translation. Polyamines have been shown to critically impact cellular proliferation in C. elegans, and deprivation of polyamines in odc null mutants results in an arrest of embryogenesis at stage 3 and with a behavioral phenotype that resembles that of starvation (24). Furthermore, our findings indicate that starvation-induced expression of antizyme is independent of the daf-2 insulin/IGF signaling pathway.

Expression of Antizyme Is Increased by Several Different Stress Conditions in a Polyamine-independent Manner—Antizyme expression is up-regulated not only by nutritional but also by several other stress conditions, including mild heat (30 °C), oxidative stress (20 mm parquat), and UV (100 J/m²). The fluorescence observed in oazp::oaz(643FS)::gfp worms caused by polyamines was more prominent and showed maximal response earlier than stress-dependent expression. Interestingly, stress-dependent induction was not only slower but also seems to differ in strength depending on the nature of the stressor. Microarray experiments of heat and oxidative stressed worms show no difference in the transcriptional level of oaz (57, 58).

Polyamines, as already stated, have an important role in cellular proliferation and also have a high impact on reproductive capability and viability. A reduction of the cellular polyamine pool induced by a stress-dependent expression of antizyme could represent an adaption of the animal to unfavorable conditions, where conservation of energy toward the survival of the animal is more important than an investment into differentiation and reproduction. Furthermore, although induction of antizyme expression caused by starvation seems to be clearly linked to the nutrient sensing pathway of PEPT-1 and TOR, it is not known whether this is also the case for other stressors investigated and should be the aim of further studies. Our findings led us to propose the model presented in Fig. 8 for polyamine-dependent and -independent induction of antizyme expression. Investigations of the mechanistic links between antizyme expression and stress stimuli are currently underway.

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References
1. Glickman, M. H., and Ciechanover, A. (2002) The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. Physiol. Rev. 82, 373–428
2. Coffino, P. (2001) Regulation of cellular polyamines by antizyme. Nat. Rev. Mol. Cell Biol. 2, 188–194
3. Kahana, C. (2009) Antizyme and antizyme inhibitor, a regulatory tango. Cell Mol. Life Sci. 66, 2479–2488
4. Murakami, Y., Matsufuji, S., Kameji, T., Hayashi, S., Igarashi, K., Tamura, T., Tanaka, K., and Ichihara, A. (1992) Ornithine decarboxylase is degraded by the 26S proteasome without ubiquitination. Nature 360, 597–599
5. Gruendler, C., Lin, Y., Farley, J., and Wang, T. (2001) Proteosomal degradation of Smad1 induced by bone morphogenetic proteins. J. Biol. Chem. 276, 46533–46543
6. Newman, R. M., Mobasher, A., Mangold, U., Koike, C., Diah, S., Schmidt, M., Finley, D., and Zetter, B. R. (2004) Antizyme targets cyclin D1 for degradation. A novel mechanism for cell growth repression. J. Biol. Chem. 279, 41504–41511
7. Lim, S. K., and Gopalan, G. (2007) Antizyme mediates AURKAIP1-de-}

dependent degradation of Aurora-A. Oncogene 26, 6593–6603
8. Bercovich, Z., Snapir, Z., Keren-Paz, A., and Kahana, C. (2011) Antizyme affects cell proliferation and viability solely through regulating cellular polyamines. J. Biol. Chem. 286, 33778–33783
9. Mitchell, J. L., Judd, G. G., Bareyal-Leyser, A., and Ling, S. Y. (1994) Feedback repression of polyamine transport is mediated by antizyme in mammalian tissue-culture cells. Biochem. J. 299, 19–22
10. Suzuki, T., He, Y., Kashiwagi, K., Murakami, Y., Hayashi, S., and Igarashi, K. (1994) Antizyme protects against abnormal accumulation and toxicity of polyamines in ornithine decarboxylase-overproducing cells. Proc. Natl. Acad. Sci. U.S.A. 91, 8930–8934
11. Li, X., and Coffino, P. (1993) Degradation of ornithine decarboxylase: exposure of the C-terminal target by a polyamine-inducible inhibitory protein. Mol. Cell Biol. 13, 2377–2383
12. Ivanov, I. P., Matsufuji, S., Murakami, Y., Gesteland, R. F., and Atkins, J. F. (2000) Conservation of polyamine regulation by translational frameshifting from yeast to mammals. EMBO J. 19, 1907–1917
13. Ray, R. M., Bhattacharya, S., Bavaria, M. N., Viar, M. J., and Johnson, L. R. (2014) Spermidine, a sensor for antizyme 1 expression regulates intracellular polyamine homeostasis. Amino Acids 46, 2005–2013
14. Mangold, U. (2006) Antizyme inhibitor: mysterious modulator of cell proliferation. Cell Mol. Life Sci. 63, 2095–2101
15. Mitchell, J. L., Simkus, C. L., Thane, T. K., Tokarz, P., Bonar, M. M., Frydman, B., Valasinas, A. L., Reddy, V. K., and Marton, L. I. (2004) Antizyme induction mediates feedback limitation of the incorporation of specific polyamine analogues in tissue culture. Biochem. J. 384, 271–279
16. Keren-Paz, A., Bercovich, Z., Porat, Z., Erez, O., Brener, O., and Kahana, C. (2006) Overexpression of antizyme-inhibitor in NIH3T3 fibroblasts provides growth advantage through neutralization of antizyme functions. Oncogene 25, 5163–5172
17. Ivanov, I. P., Loughran, G., and Atkins, J. F. (2008) uORFs with unusual translational start codons autoregulate expression of eukaryotic ornithine decarboxylase homologs. Proc. Natl. Acad. Sci. U.S.A. 105, 10079–10084
18. Murakami, Y., Suzuki, J., Samejima, K., Kikuchi, K., Hascilowicz, T., Murai, N., Matsufuji, S., and Oka, T. (2009) The change of antizyme inhibitor expression and its possible role during mammalian cell cycle. Exp. Cell Res. 315, 2301–2311
19. Childs, A. C., Mehta, D. J., and Gerner, E. W. (2003) Polyamine-dependent gene expression. Cell Mol. Life Sci. 60, 1394–1406
20. Casero, R. A., Jr., and Marton, L. J. (2007) Targeting polyamine metabolism and function in cancer and other hyperproliferative diseases. Nat. Rev. Drug Discov. 6, 373–390
21. Nishimura, K., Nakatsu, F., Kashiwagi, K., Ohno, H., Saito, T., and Igarashi, K. (2002) Essential role of S-adenosylmethionine decarboxylase in mouse embryonic development. Genes Cells 7, 41–47
22. Pendeville, H., Carpinio, N., Marine, J. C., Takahashi, Y., Muller, M., Martial, J. A., and Cleveland, J. L. (2001) The ornithine decarboxylase gene is essential for cell survival during early murine development. Mol. Cell Biol. 21, 6549–6558
23. Heinick, A., Urban, K., Roth, S., Spies, D., Nunes, F., Phanstiel, O., 4th, Liebau, E., and Lüersen, K. (2010) Caenorhabditis elegans PSB-type ATPase CATP-5 operates in polyamine transport and is crucial for norsesmidine-mediated suppression of RNA interference. FASEB J. 24, 206–217
24. MacRae, M., Kramer, D. L., and Coffino, P. (1998) Developmental effect of polyamine depletion in Caenorhabditis elegans. Biochem. J. 333, 301–315
25. Forsen, K., Eschbach, M. L., Liebau, E., and Walter, R. D. (2004) Functional GATA-and initiator-like-elements exhibit a similar arrangement in the promoters of Caenorhabditis elegans polyamine synthesis enzymes. Biol. Chem. 385, 711–721
26. Eisenberg, T., Knauer, H., Schauer, A., Büttner, S., Runkenstuhl, C., Carmona-Gutierrez, D., Ring, J., Schroeder, S., Magnes, C., Antonacci, L., Fussi, H., Deszcz, L., Hartl, R., Schraml, E., Criollo, A., Magnes, C., Antonacci, L., Minois, N., Kroemer, G., and Madeo, F. (2009) Induction of autophagy by spermidine promotes longevity. Nat. Cell Biol. 11, 1305–1314
27. Soda, K., Dobashi, Y., Kano, Y., Tsujinaka, S., and Konishi, F. (2009) Poly-
amine-rich food decreases age-associated pathology and mortality in aged mice. *Exp. Gerontol.* **44**, 727–732

28. Alcázar, R., Altabello, T., Marco, F., Bortolotti, C., Reymond, M., Koncz, C., Carrasco, P., and Tiburcio, A. F. (2010) Polyamines: molecules with regulatory functions in plant abiotic stress tolerance. *Planta* **231**, 1237–1249

29. Rhee, H. J., Kim, E. I., and Lee, J. K. (2007) Physiological polyamines: simple primordial stress molecules. *J Cell Mol. Med.* **11**, 685–703

30. Evans, D. S., Kapahi, P., Hsueh, W. C., and Kockel, L. (2011) TOR signaling never gets old: aging, longevity and TORC1 activity. *Ageing Res. Rev.* **10**, 225–237

31. Wang, L., Fraley, C. D., Faridi, J., Kornberg, A., and Roth, R. A. (2003) Inorganic polyphosphate stimulates mammalian TOR, a kinase involved in the proliferation of mammary cancer cells. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 11249–11254

32. An, J. H., Vranas, K., Lucke, M., Inoue, H., Hisamoto, N., Matsumoto, K., and Daniel, H., and Spanier, B. (2014) Intestinal amino acid availability via PEPT-1 affects TORC1/2 signaling and the unfolded protein response. *J. Proteome Res.* **13**, 3685–3692

33. Lewis, J. A., and Fleming, J. T. (1995) in *Caenorhabditis elegans: Modern biological analysis of an organism* (Epstein, H. F., and Shakes, D. C., eds) pp. 3–29, Academic Press, San Diego, CA

34. Kanerva, K., Mäkitie, L. T., Pelander, A., Heiskala, M., and Andersson, L. C. (2009) Expression of antizyme inhibitor 2 in mast cells and role of polyamines as selective regulators of serotonin secretion. *PLoS One* **4**, e6858

35. Kanerva, K., Lappalainen, J., Mäkitie, L. T., Virolainen, S., Kovanen, P. T., and Andersson, L. C. (2009) Expression of antizyme inhibitor 2 in mast cells and role of polyamines as selective regulators of serotonin secretion. *PLoS One* **4**, e6858

36. Ramos-Molina, B., Lambertos, A., Lopez-Contreras, A. I., Kasprzak, J. M., Czerwoniec, A., Bujnicki, J. M., Cremades, A., and Peñafiel, R. (2014) Structural and degradative aspects of ornithine decarboxylase antizyme inhibitor 2. *FEBS Open Bio.* **4**, 510–521

37. Jia, K., Chen, D., and Riddle, D. L. (2004) The TOR pathway interacts with the insulin signaling pathway to regulate *C. elegans* larval development, metabolism and life span. *Development* **131**, 3897–3906

38. Kimura, K. D., Tissenbaum, H. A., Liu, Y., and Ruvkun, G. (1997) daf-2, an insulin receptor-like gene that regulates longevity and diapause in *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 36739–36740

39. Kalderon, D. (1984) The role of RNA and DNA in the control of gene expression. In *Modern Genetics* (Epstein, H. F., and Shakes, D. C., eds) pp. 3897–3906

40. Geillinger, K. E., Kuhlmann, K., Eisenacher, M., Giesbertz, P., Meyer, H. E., Daniel, H., and Spanier, B. (2014) Intestinal amino acid availability via PEPT-1 affects TORC1/2 signaling and the unfolded protein response. *J. Proteome Res.* **13**, 3685–3692

41. Ivanov, I. P., Gesteland, R. F., and Atkins, J. F. (2000) Antizyme expression: a subversion of triplet decoding, which is remarkably conserved by evolution, is a sensor for an autoregulatory circuit. *Nucleic Acids Res.* **28**, 3185–3196

42. Macrae, M., Plasser, R. H., and Coffino, P. (1995) The ornithine decarboxylase gene of *Caenorhabditis elegans* cloning, mapping and mutagenesis. *Genetics* **140**, 517–525

43. Ivanov, I. P., Firth, A. E., and Atkins, J. F. (2010) Recurrent emergence of catalytically inactive ornithine decarboxylase homologous forms that likely have regulatory function. *J. Mol. Evol.* **70**, 289–302

44. Ramos-Molina, B., Lopez-Contreras, A. I., Cremades, A., and Peñafiel, R. (2012) Differential expression of ornithine decarboxylase antizyme inhibitors and antizymes in rodent tissues and human cell lines. *Amino Acids* **42**, 539–547

45. Kanerva, K., Lappalainen, J., Mäkitie, L. T., Virolainen, S., Kovanen, P. T., and Andersson, L. C. (2009) Expression of antizyme inhibitor 2 in mast cells and role of polyamines as selective regulators of serotonin secretion. *PLoS One* **4**, e6858

46. The Antizyme from *C. elegans*