Molecular Cell

Supplemental Information

Polyamine Control of Translation Elongation Regulates Start Site Selection on the Antizyme Inhibitor mRNA via Ribosome Queuing

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Figures S1 to S6

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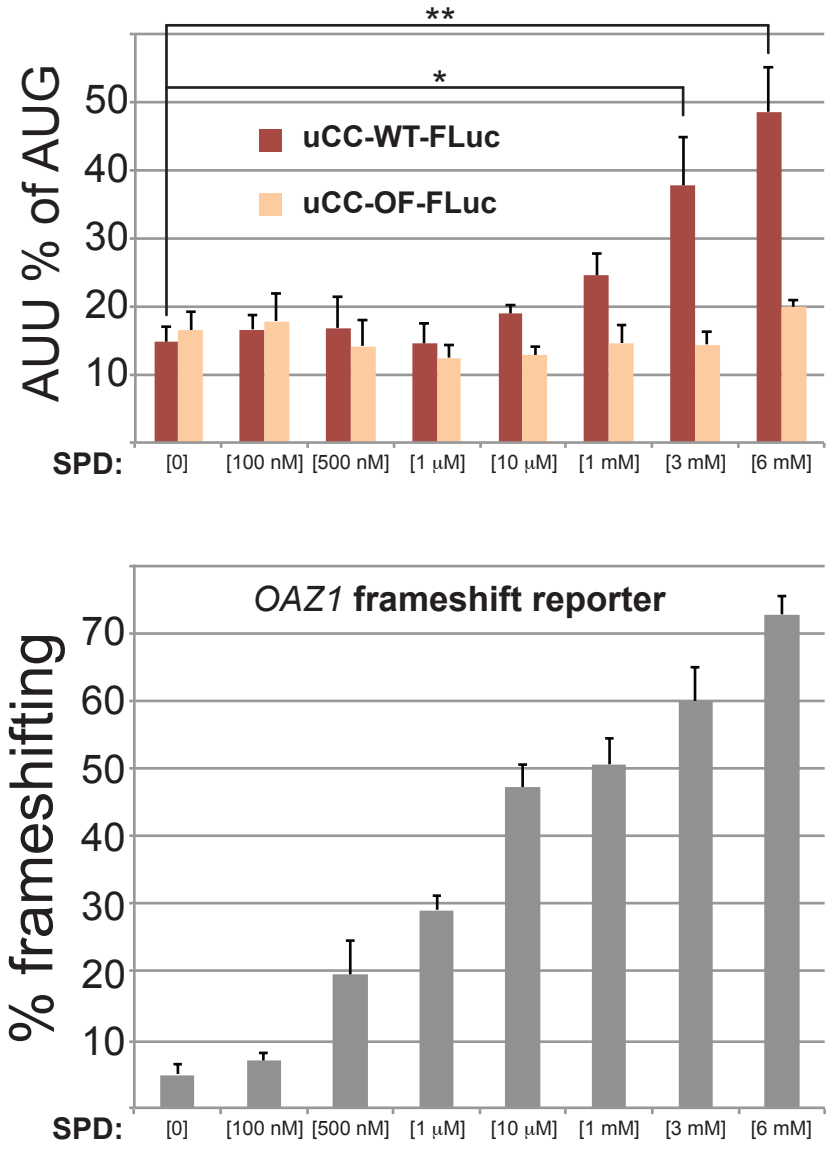

Figure S1. Dose-dependent stimulation of AZIN1 uCC translation and antizyme frameshifting by spermidine – Related to Figure 1A

The indicated uCC-Luc reporters from Figure 1A (upper panel) or a human antizyme OAZ1 dual-luciferase frameshift reporter (lower panel) were transfected in DFMO-treated HEK293T cells and then incubated in the presence of the indicated concentrations of spermidine.

(Upper panel) The percent AUU initiation and normalization to a co-transfected Renilla luciferase reporter initiated by AUG in perfect context were calculated as described in Figure 1A.

(Lower panel) +1 frameshifting was calculated relative to an in-frame reporter. Each data point represents the mean of four biological replicates, each done in duplicate. Error bars denote standard deviation. *p<0.05, **p<0.01 (Student's two-tailed t-test, n=4).

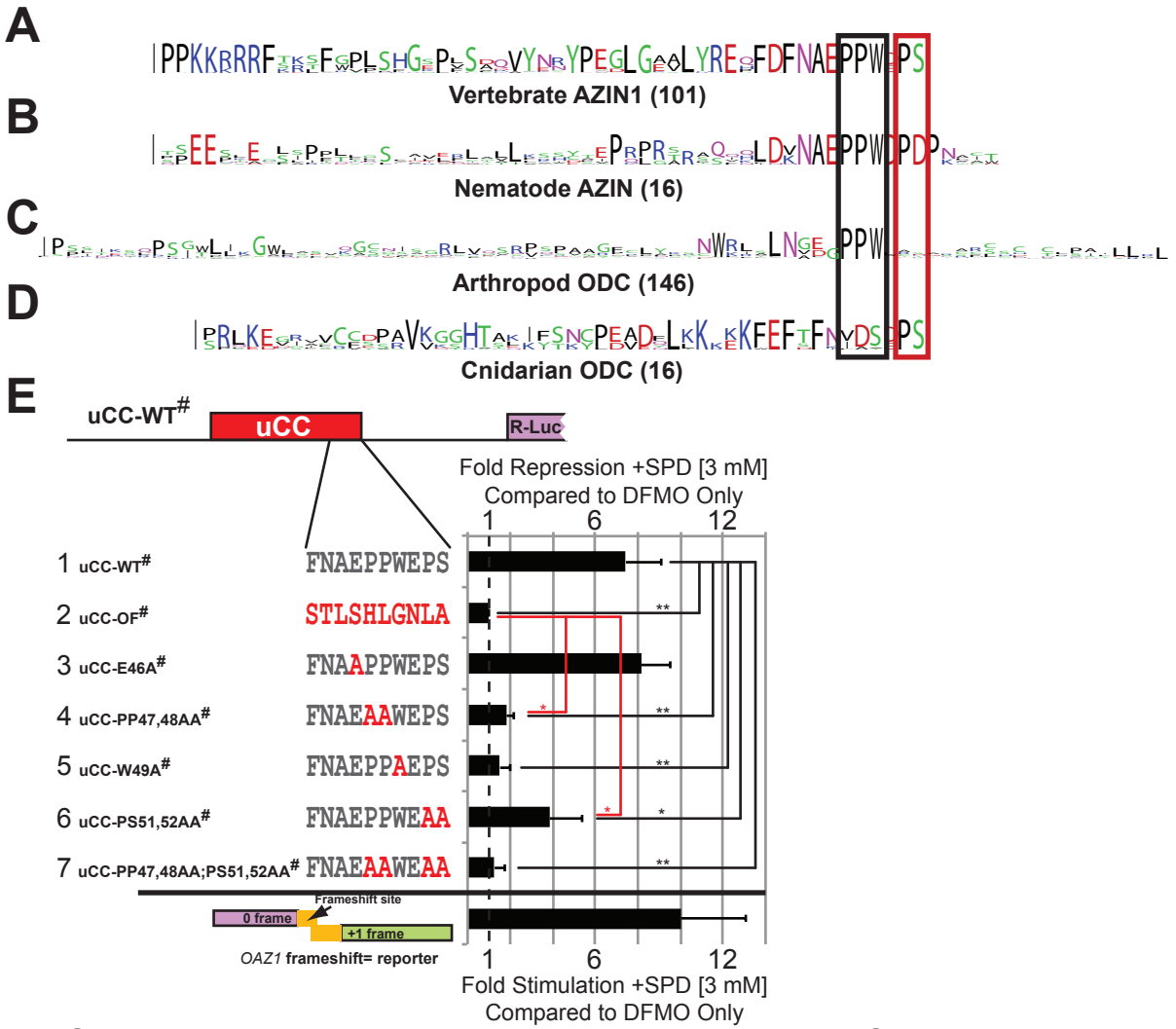

Figure S2. Phylogenetic comparisons reveal that PPW and PS-stop are highly conserved, but discrete, motifs in metazoan homologs of ODC/AZIN and these motifs mediate polyamine regulatory effects of the AZIN1 uCC – Related to Figures 1B, 1C

(A) Weblogo representation of amino acid conservation of uCCs in 101 orthologs of vertebrate AZIN1.

(B) Conservation of uCCs in 16 homologs of AZIN in nematodes. Though the nematode uCC is an ortholog of the AZIN1 uCC, the nematode AZIN is not a true ortholog of vertebrate AZIN1 and instead arose as a result of a gene duplication that occurred independently in the nematode lineage (Ivanov et al., 2010b; Kurosinski et al., 2013).

(C) Conservation of uCCs in 146 arthropod ODC mRNAs.

(D) Conservation of uCCs in 16 cnidarian ODC mRNAs. In all WebLogos the starting residue is shown as isoleucine (corresponding to the AUU start codon); however, presumably translation is initiated by Met-tRNAiMet. The PPW and PS-stop motifs are boxed in black and red, respectively.

(E) (Top) Schematic of the AZIN1 reporter; the # symbol in the names of the reporters indicates that AUG start codons of the three conventional uORFs were mutated to AAA. The full 5' leader of mouse azin1 mRNA with the AUG start codons of the three conventional uORFs mutated to AAA was fused upstream of Renilla luciferase. The "wild type" and "out-of-frame" reporters were described in Figure 6B, and for each reporter the amino acid changes relative to the wild type uCC are in red. HEK293T cells were pretreated, transfected and incubated as in Figure 1, and reporter normalization was performed as in Figure 6B. The fold repression in the presence of 3 mM SPD was calculated relative to the reporter in which the last 10 codons are out-of-frame (set at "1"). (Bottom) The human antizyme OAZ1 dual-luciferase frameshift reporter was examined in parallel and +1 frameshifting was calculated relative to an in-frame reporter. The fold stimulation indicates the increase in percent frameshifting in 3 mM vs. no SPD. Each data point represents the mean of four biological replicates, each done in duplicate. Error bars denote standard deviation. *p<0.05, **p<0.01 (Student's two-tailed t-test, n=4).

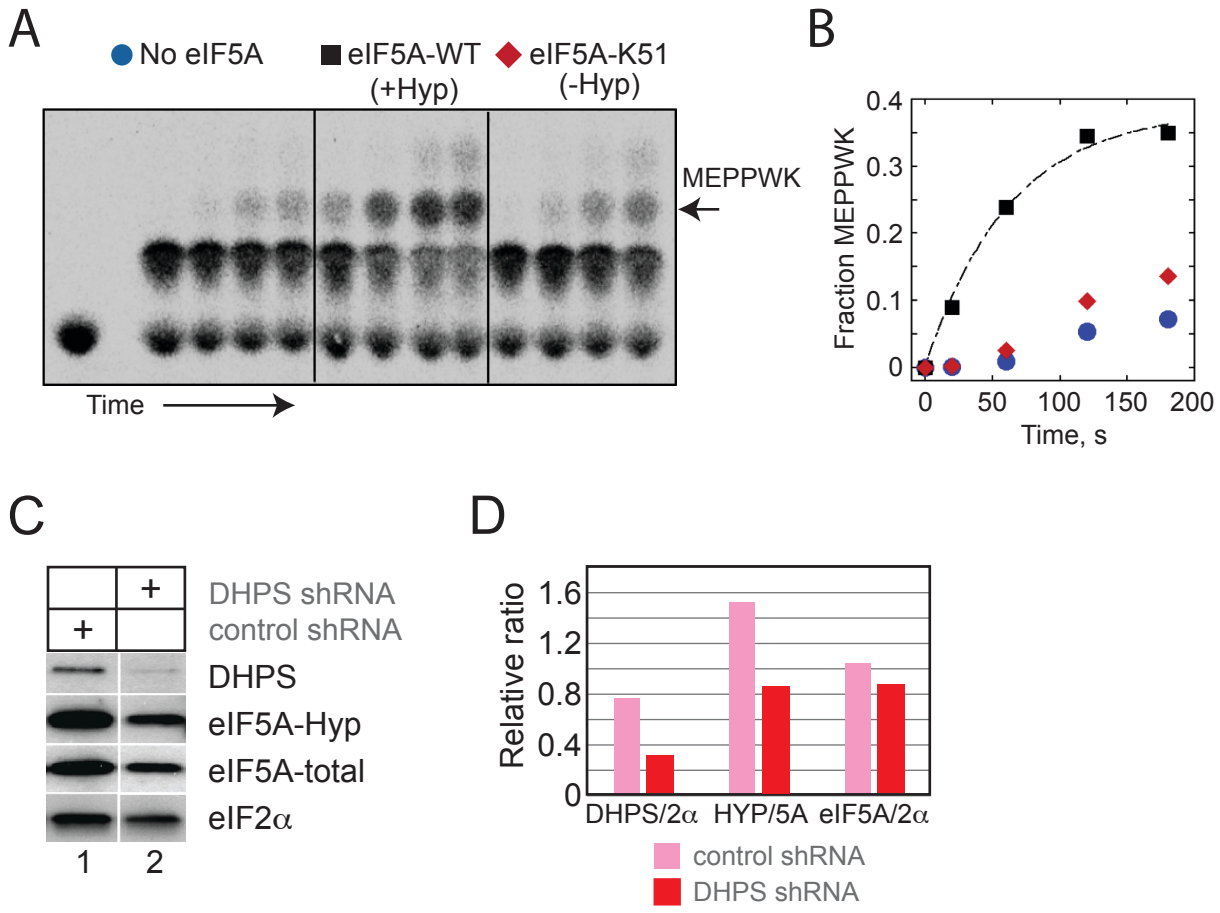

Figure S3. Hypusination of eIF5A is required for in vitro synthesis of MEPPWK peptide and *DHPS* shRNA transfections reduce hypusination of eIF5A in cells – Related to Figures 4A,4G

(A) Electrophoretic thin-layer chromatography (TLC) analysis of MEPPWK peptide synthesis in yeast in vitro reconstituted elongation assays performed in the presence of no eIF5A, hypusinated eIF5A (eIF5A-WT) or unmodified form of eIF5A (eIF5A-51K), as indicated.

(B) Fraction of MEPPWK synthesis in each reaction was plotted and fit to a single exponential equation (black squares, +WT hypusinated eIF5A; red diamonds, +unmodified eIF5A-K51; blue circles, -eIF5A).

(C) Western blot analysis of whole cell lysates from HEK293T cells pre-treated in 1 mM DFMO for 4 days, then transfected with control shRNAs or shRNAs against human DHPS. Transfectants were incubated an additional 48 hr in DFMO and then incubated for another 24 hr in DFMO supplemented with 1 M SPD. Immunoblot analysis of whole cell lysates was performed using antibodies against DHPS, hypusinated eIF5A, total eIF5A, or total eIF2α, as indicated.

(D) Relative levels of DHPS, hypsinated eIF5A, total eIF5A, and total eIF2α from panel (C) were determined by quantitative densitometry and NIH ImageJ software.

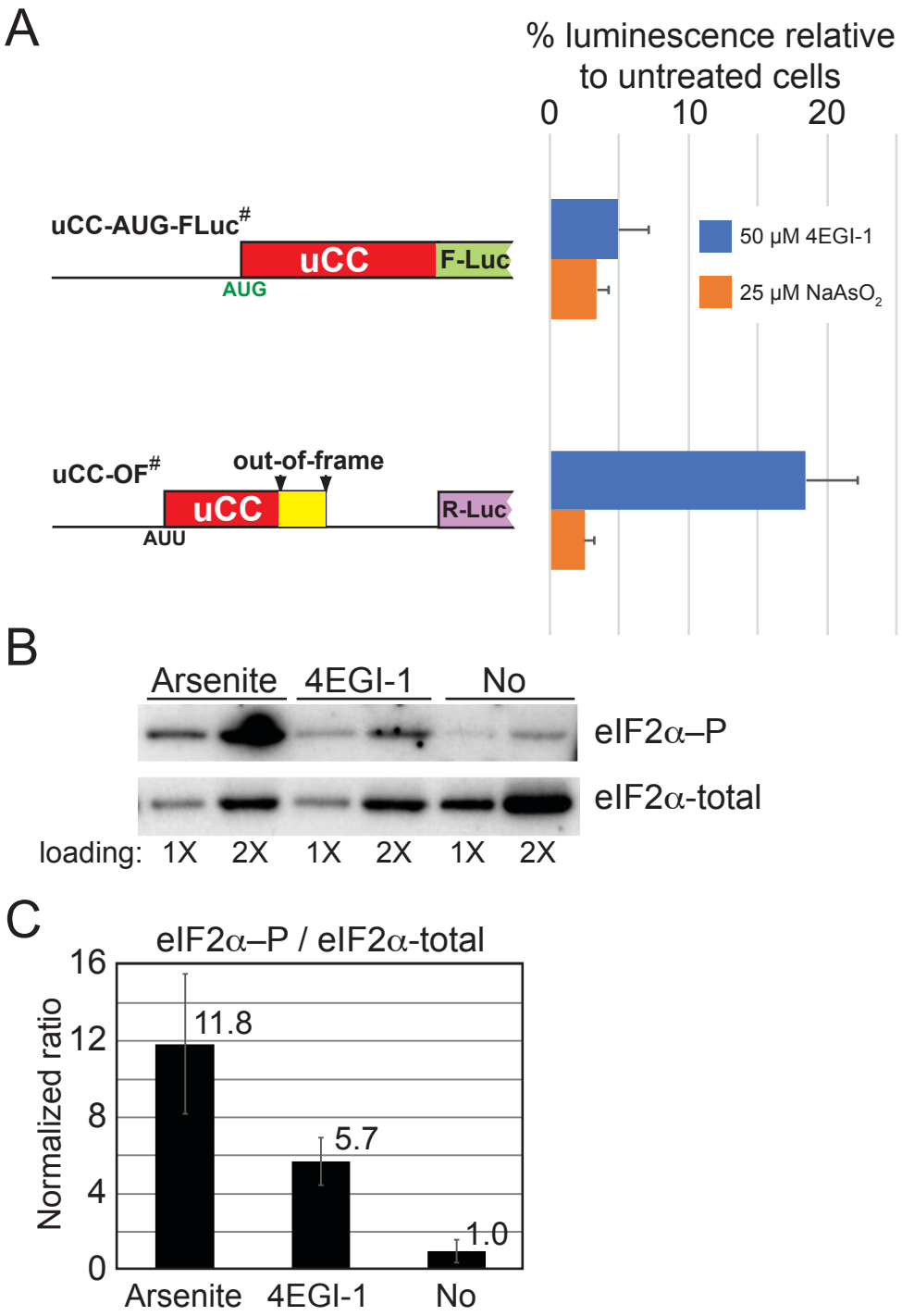

Figure S4. Arsenite and 4EGI-1 treatments substantially impair luciferase activities from control reporters and impact eIF2α phosphorylation – Related to Figures 6 and S8

(A) The indicated control reporters, AUG-initiated uCC firefly luciferase fusion and "out-of-frame" uCC upstream of Renilla luciferase, which were used in Figures 6A-C and Figure S5A, respectively, were transfected in HEK293T cells. Luminescence of lysates from cells treated with 25 μM arsenite or 50 μM 4EGI-1 were compared to untreated cells.

(B) Western blot analysis of whole cell lysates from HEK293T cells treated with either 50 μM 4EGI-1, as in Figures 6A-C, or 25 μM arsenite, as in Figure S5B. Two different amounts of each cell extract differing by a factor of 2 were loaded in successive lanes and subjected to immunoblot analysis using rabbit monoclonal anti-eIF2α (phospho-Ser51) or rabbit monoclonal anti-eIF2α antibodies.

(C) Relative levels of phosphorylated and total eIF2α from panel (A) were determined by quantitative densitometry and NIH ImageJ (1.46r) software and are shown as the average of the quantifications obtained from the two dilutions. Error bars are standard deviations; n=2 for arsenite and 4EGI-1, and n=4 for no treatment.

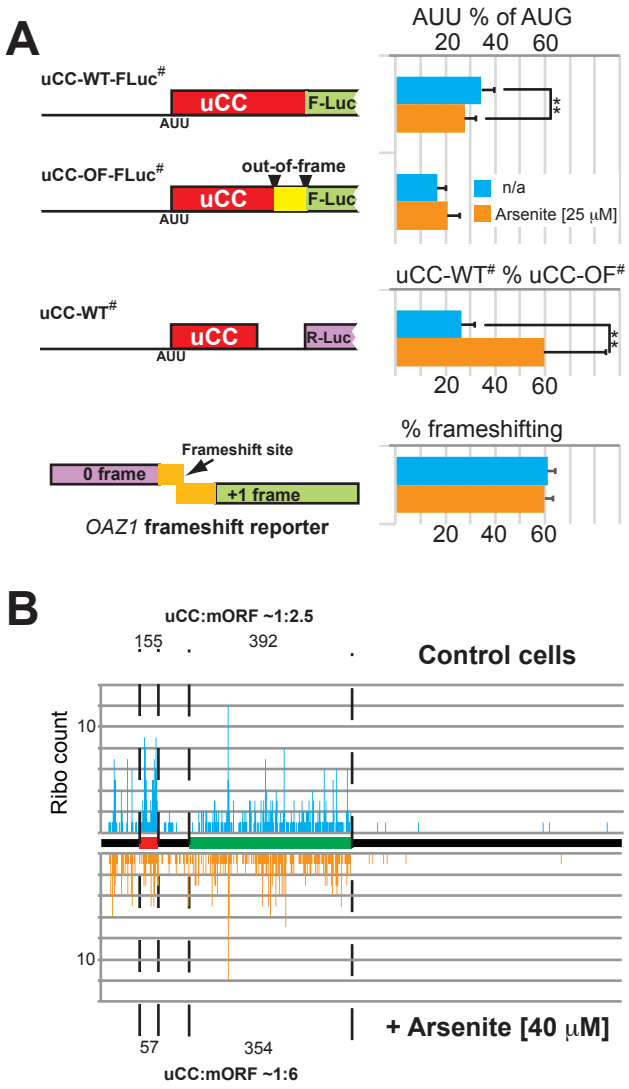

Figure S5. Arsenite induces derepression of AZIN1 mORF translation – Related to Figure 6A-C
(A) (Top two constructs) To monitor uCC translation, HEK293T cells were transfected with the indicated uCC-Luc reporters and incubated for 18 hr in DMEM supplemented with 1 mM aminoguanidine and 1 mM SPD in the absence or presence of the 25 μM $NaAsO_2$. Percent AUU initiation was calculated as described in Figure 1A, except that no normalizing co-transfected Renilla reporter was used. The # symbol in the names of the reporters indicates that the AUG start codon of uORF1 was mutated to AAA. (Third construct) To monitor AZIN1 synthesis, the full 5' leader of mouse azin1 mRNA with the AUG start codons of the three conventional uORFs mutated to AAA (as denoted by the # symbol in the name of the reporter) was fused upstream of Renilla luciferase. HEK293T cell transfectants were grown -/+ 25 μM $NaAsO_2$ as described above. Wild type reporter activity was calculated as a percent of a reporter containing the out-of-frame uCC mutation (i.e. constitutively derepressed). (Bottom construct) To monitor levels of free polyamines, a dual-luciferase human antizyme OAZ1 frameshifting reporter was assayed in cells treated as described above. Percent +1 frameshifting was calculated relative to a dual-luciferase in-frame reporter. Each data point represents the mean of eight biological replicates, each performed in triplicate. Error bars denote standard deviation. **p<0.01 (Student's two-tailed t-test, n=8).
(B) Ribosome footprint raw data was obtained from (Andreev et al., 2015). Ribosome protected fragments from HEK293T cells grown under control conditions or in the presence of 40 μM sodium arsenite for 30 min were mapped to the mRNA of AZIN1. Total (not normalized) fragment counts were aligned to the AZIN1 mRNA assuming a 15-nt offset from the 5' end of protected fragments to the ribosomal A-site. Quantified fragment counts mapping to the uCC and mORF, red and green rectangles, respectively, and their relative ratio under each condition are indicated above (control conditions) and below (arsenite conditions) the ribosome profiles.

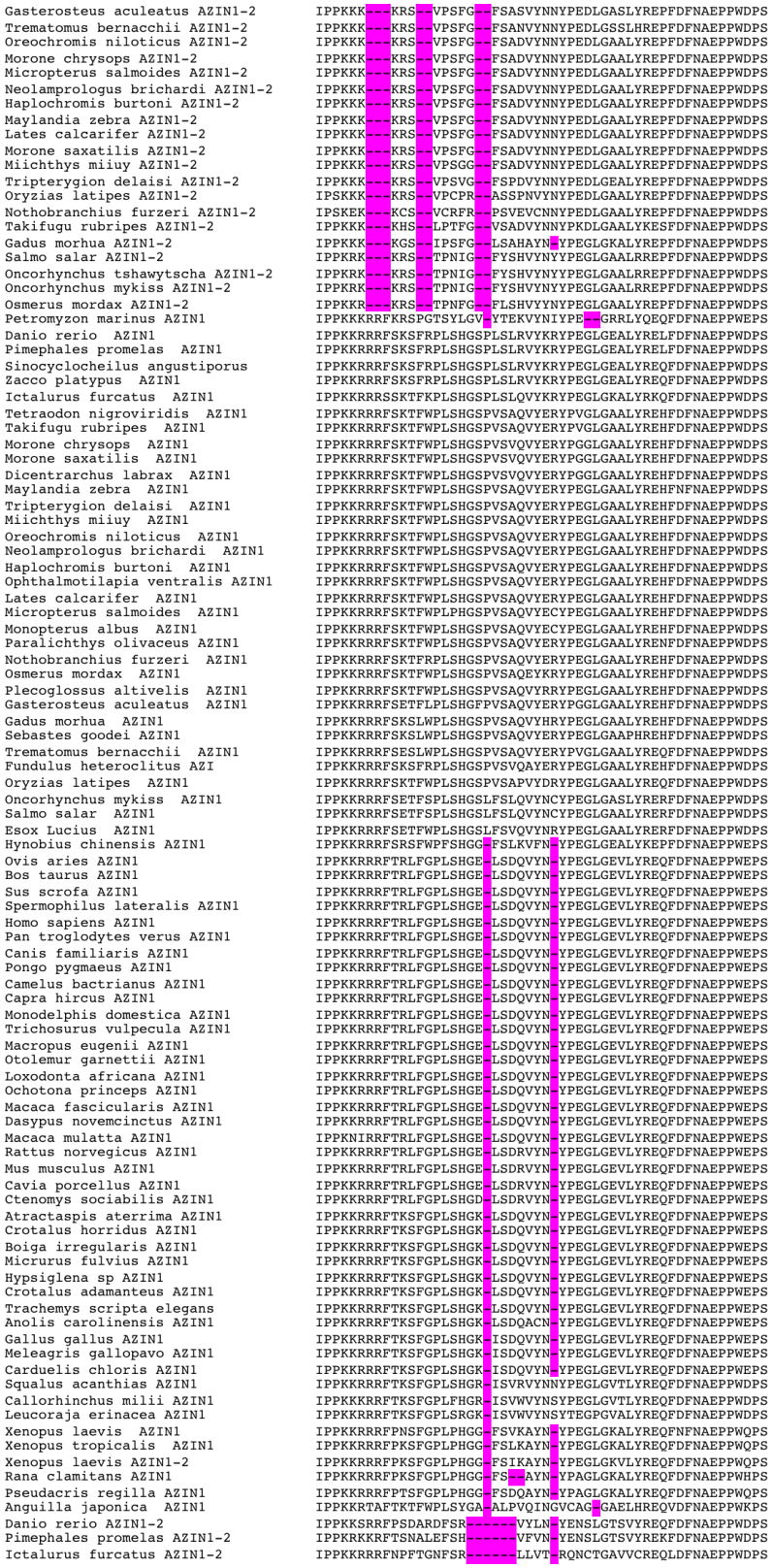

Figure S6. uCCs from AZIN1 orthologs in vertebrates contain naturally occurring deletions/ insertions between the start codon and conserved C-terminus – Related to Figures 1B and 5

Amino acid sequence alignment, generated by ClustalX, of 101 vertebrate AZIN1 uCCs. Species names are on the left. The uCCs in the two paralogs of AZIN1 in boney fish differ in length by 7 residues. The starting residue is shown as isoleucine (corresponding to the AUU start codon); however, presumably translation is initiated by Met-tRNA$_i^{Met}$. Deletions are represented by dashes highlighted in magenta.

Table S1. Relative reporter mRNA levels – Related to Figures 1A, 1C

			FFLuc RNA levels (860 DFMO set at 1)		FFLuc mRNA levels (all DFMO values set at 1)	
plasmid #	plasmid name	treatment		stdev		stdev
854	uCC-WT-Fluc AUU	DFMO	1.19	0.23	1.00	0.19
854	uCC-WT-Fluc AUU	3 mM SPD	5.37	4.00	4.51	3.36
860	uCC-WT-Fluc AUG	DFMO	1.00	0.06	1.00	0.06
860	uCC-WT-Fluc AUG	3 mM SPD	4.08	1.23	4.07	1.23
862	uCC-OF-Fluc AUU	DFMO	1.17	0.17	1.00	0.14
862	uCC-OF-Fluc AUU	3 mM SPD	3.43	0.15	2.92	0.13
868	uCC-OF-Fluc AUG	DFMO	1.10	0.03	1.00	0.03
868	uCC-OF-Fluc AUG	3 mM SPD	2.99	1.28	2.71	1.16
2171	uCC-PP47,48AA-FLuc AUU	DFMO	0.65	0.08	1.00	0.13
2171	uCC-PP47,48AA-FLuc AUU	3 mM SPD	2.84	1.18	4.39	1.82
2138	uCC-PP47,48AA-FLuc AUG	DFMO	0.87	0.07	1.00	0.08
2138	uCC-PP47,48AA-FLuc AUG	3 mM SPD	3.08	0.14	3.52	0.16

Relative reporter mRNA levels for experiments in Figure 1A,C determined by qPCR. Normalization controls set at 1 are highlighted in yellow. Standard deviation based on two biological replicates.

Table S2. Relative reporter mRNA levels – Related to Figure 3B

plasmid #	plasmid name	treatment	FFLuc RNA levels (2260 no Arg set at 1)	stdev	FFLuc mRNA levels (all no Arg values set at 1)	stdev
2255	uCC-AAP-Fluc AUU	no Arg	0.80	0.08	1.00	0.10
2255	uCC-AAP-Fluc AUU	25 mM Arg	1.52	0.41	1.90	0.51
2260	uCC-AAP-Fluc AUUG	no Arg	1.01	0.17	1.00	0.17
2260	uCC-AAP-Fluc AUG	25 mM Arg	2.06	0.60	2.05	0.60
2256	uCC-AAP-D12N-Fluc AUU	no Arg	0.51	0.08	1.00	0.16
2256	uCC-AAP-D12N-Fluc AUU	25 mM Arg	0.70	0.16	1.39	0.31
2261	uCC-AAP-D12N-Fluc AUG	no Arg	0.86	0.09	1.00	0.11
2261	uCC-AAP-D12N-Fluc AUG	25 mM Arg	1.89	0.23	2.19	0.26

Relative reporter mRNA levels for experiments in Figure 3B determined by qPCR. Normalization controls set at 1 are highlighted in yellow. Standard deviation based on two biological replicates.

Table S3. Relative reporter mRNA levels – Related to Figures 6A, 6B, 6D

plasmid #	plasmid name	treatment	FFLuc RNA levels (2324 no 4EGI-1 set at 1)	stdev	FFLuc mRNA levels (all no 4EGI-1 values set at 1)	stdev
2109	uCC-WT-Fluc# AUU	no 4EGI-1	1.09	0.44	1.00	0.40
2109	uCC-WT-Fluc# AUU	50 μM 4EGI-1	1.80	1.25	1.65	1.15
2324	uCC-WT-Fluc# AUG	no 4EGI-1	1.00	0.04	1.00	0.04
2324	uCC-WT-Fluc# AUG	50 μM 4EGI-1	1.07	0.55	1.07	0.55
5475	uCC-OF-Fluc# AUU	no 4EGI-1	1.04	0.34	1.00	0.33
5475	uCC-OF-Fluc# AUU	50 μM 4EGI-1	1.36	0.01	1.31	0.01
5476	uCC-OF-Fluc# AUG	no 4EGI-1	1.14	0.21	1.00	0.18
5476	uCC-OF-Fluc# AUG	50 μM 4EGI-1	1.73	0.36	1.52	0.32

plasmid #	plasmid name	treatment	RLuc RNA levels (5320 no 4EGI-1 set at 1)	stdev	RLuc mRNA levels (all no 4EGI-1 values set at 1)	stdev
5320	uCC-WT#	no 4EGI-1	1.00	0.07	1.00	0.07
5320	uCC-WT#	50 μM 4EGI-1	2.15	0.05	2.14	0.05
5321	uCC-OF#	no 4EGI-1	0.76	0.19	1.00	0.26
5321	uCC-OF#	50 μM 4EGI-1	1.82	0.19	2.41	0.25

plasmid #	plasmid name	treatment	RLuc RNA levels (5817 DFMO set at 1)	stdev	RLuc mRNA levels (all DFMO values set at 1)	stdev
5802	AAA-AUU WT	DFMO	0.53	0.17	1.00	0.33
5802	AAA-AUU WT	6 mM SPD	2.45	0.17	4.67	0.32
5803	AUU-AAA WT	DFMO	0.39	0.02	1.00	0.06
5803	AUU-AAA WT	6 mM SPD	2.14	0.04	5.50	0.11
5817	AAA-AAA OF	DFMO	1.00	0.89	1.00	0.89
5817	AAA-AAA OF	6 mM SPD	1.06	0.02	1.06	0.02

Relative reporter mRNA levels for experiments in Figure 6A (plasmids 2109-5476), Figure 6B (plasmids 5320-5321), and Figure 6D (plasmids 5802-5817) determined by qPCR. Normalization controls set at 1 for each group are highlighted in yellow. Standard deviation based on two biological replicates.

Table S4. Oligonucleotides used in this study – Related to STAR Methods

Name	Sequence
AZIUTR/S2	CCAAAGCTTCTCTCGCCGCGGTGTTTCCG
UTRD2/A m5 BamHI	CCAGGATCCAGCTAGGTTCCCAAGGTGGCTC
E50del/A	CCAGGATCCCCAAGGTGGCTCAGCGTTG
PPAA/A	CCAGGATCCGCTAGGTTCCCAAGCTGCCTCAGCGTTGAAG
PSAA/A	CCAGGATCCGGCAGCTTCCCAAGGTGGCTCAGCG
WA/A	CCAGGATCCGCTAGGTTCCGCAGGTGGCTCAGCGTTGAAG
EA/A	CCAGGATCCGCTAGGTTCCCAAGGTGGCGCAGCGTTGAAGTCG
AAPLNG/A	CCAGGATCCGCTGTTGAGAGCTCTCCAGAGATGATCGCTCAGGTAAT
AAP/A	CCAGAGATGATCGCTCAGGTAATCCTGTGATGTCAGCACTTCTCCTAGGCCTTC
AAPM/A	CCAGAGATGATCGCTCAGGTAATTCTGTGATGTCAGCACTTCTCCTAGGCCTTC
OCC1197	ACGTCGTCCCCGCAGCCTAAAGCCCAGGCCGCCTTGGGTATT
OCC1198	AATACCCAAGGCGGCCTGGGCTTTAGGCTGCGGGGACGACGT
AZIUTR/S2	CCAAAGCTTCTCTCGCCGCGGTGTTTCCG
AZIM2/A	CCACTCGGGTTTCATCTCAGCCGTATTCCACAAAGCCGAAAGTTTTAAACCAGGA
AUU/S	CATCCCTCTTTAAAAATTCCGCCGAAAAAGA
AUU/A	TCTTTTTCGGCGGAATTTTTAAAGAGGGATG
M5M6/S	GAACCTAGCTTGAAAATAGGGGGTTCCA
M5M6/A	TGGAACCCCCTATTTTCAAGCTAGGTTC
M1M2M6PPAA/S	CTTCAACGCTGAGGCAGCTTGGGAACCTAGCTGAAAATAGGGGGTTC
M1M2M6PPAA/A	GAACCCCCTATTTTCAGCTAGGTTCCCAAGCTGCCTCAGCGTTGAAG
M1M2M6PSAA/S	CCACCTTGGGAAGCTGCCTGAAAATAGGGGGTTCCATCTCC
M1M2M6PSAA/A	GGAGATGGAACCCCCTATTTTCAGGCAGCTTCCCAAGGTGG
M1M2M6W49A/S	CTGAGCCACCTGCGGAACCTAGCTGAAAATAGGGGGTTCC
M1M2M6W49A/A	GGAACCCCCTATTTTCAGCTAGGTTCCGCAGGTGGCTCAG
M1M2M6E46A/S	CTTCAACGCTGCGCCACCTTGGGAACCTAGCTGAAAATAGGGGGTTCC
M1M2M6E46A/A	GGAACCCCCTATTTTCAGCTAGGTTCCCAAGGTGGCGCAGCGTTGAAG
M1M2M6PPPSAAAA/S	CAACGCTGAGGCAGCTTGGGAAGCTGCCTGAAAATAGGGGGTTCC
M1M2M6PPPSAAAA/A	GGAACCCCCTATTTTCAGGCAGCTTCCCAAGCTGCCTCAGCGTTG
M1M2M6W49ASP/S	CGCTGAGCCACCTGCGGAACCTCCATGAAAATAGGGGGTTCC
M1M2M6W49ASP/A	GGAACCCCCTATTTTCATGGAGGTTCCGCAGGTGGCTCAGCG
hAZ1WT/S	GTCCCTCGAGGGTCTCCCTCCACTGCTGTAGTAAC
hAZ1WT/A	GTCCAGATCTTGAAAGATTGTGATCCCTCTGACTATT

Table S5. Plasmids used in this study – Related to STAR Methods

Construct #	Construct name	start	template	S primer	AS primer/s
862	uCC-OF-FLuc (AUU)	**AUU**	phRL-M5	AZI/UTR-S2	UTRD2/A m5 BamHI
868	uCC-OF-FLuc (AUG)	**AUG**	phRL-M4M5	AZI/UTR-S2	UTRD2/A m5 BamHI
2121	uCC-PS51,52DD-FLuc (AUU)	**AUU**	phRL-WT	AZI/UTR-S2	E50del/A
2171	uCC-PP47,48AA-FLuc (AUU)	**AUU**	phRL-WT	AZI/UTR-S2	PPAA/A
2125	uCC-PS51,52AA-FLuc (AUU)	**AUU**	phRL-WT	AZI/UTR-S2	PSAA/A
2127	uCC-W49A-FLuc (AUU)	**AUU**	phRL-WT	AZI/UTR-S2	WA/A
2326	uCC-E50A-FLuc (AUU)	**AUU**	phRL-WT	AZI/UTR-S2	EA/A
2135	uCC-PS51,52DD-FLuc (AUG)	**AUG**	phRL-M4	AZI/UTR-S2	E50del/A
2138	uCC-PP47,48AA-FLuc (AUG)	**AUG**	phRL-M4	AZI/UTR-S2	PPAA/A
2140	uCC-PS51,52AA-FLuc (AUG)	**AUG**	phRL-M4	AZI/UTR-S2	PSAA/A
2141	uCC-W49A-FLuc (AUG)	**AUG**	phRL-M4	AZI/UTR-S2	WA/A
2361	uCC-E50A-FLuc (AUG)	**AUG**	phRL-M4	AZI/UTR-S2	EA/A
2255	uCC-AAP-FLuc (AUU)	**AUU**	phRL-WT	AZI/UTR-S2	AAPLNG/A + AAP/A
2256	uCC-AAP-D12N-FLuc (AUU)	**AUU**	phRL-WT	AZI/UTR-S2	AAPLNG/A + AAPM/A
2260	uCC-AAP-FLuc (AUG)	**AUG**	phRL-M4	AZI/UTR-S2	AAPLNG/A + AAP/A
2261	uCC-AAP-D12N-FLuc (AUG)	**AUG**	phRL-M4	AZI/UTR-S2	AAPLNG/A + AAPM/A
2109	uCC-WT-FLuc#	**AUU**	phRL-M1	AZI/UTR-S2	UTRD2/A BamHI
2324	uCC-WT-FLuc#	**AUG**	phRL-M1M4	AZI/UTR-S2	UTRD2/A BamHI
5375	uCC-OF-FLuc#	**AUU**	uCC-OF-FLuc (AUU)	OCC1197	OCC1198
5376	uCC-OF-FLuc#	**AUG**	uCC-OF-FLuc (AUG)	OCC1197	OCC1198
5320	uCC-WT#	N/A	phRL-M1M2M3M6	AZIUTR/S2; AUU/S	AUU/A; AZIM2/A
5321	uCC-OF#	N/A	phRL-M1M2M5	AZIUTR/S2; M5M6/S	M5M6/A; AZIM2/A
5325	uCC-E46A#	N/A	phRL-M1M2	AZIUTR/S2; M1M2M6E46A/S	M1M2M6E46A/A; AZIM2/A
5322	uCC-PP47,48AA#	N/A	phRL-M1M2	AZIUTR/S2; M1M2M6PPAA/S	M1M2M6PPAA/S; AZIM2/A
5324	uCC-W49A#	N/A	phRL-M1M2	AZIUTR/S2; M1M2M6W49A/S	M1M2M6W49A/A; AZIM2/A
5323	uCC-PS51,52AA#	N/A	phRL-M1M2	AZIUTR/S2; M1M2M6PSAA/S	M1M2M6PSAA/A; AZIM2/A
5326	uCC-PP47,48AA;PS51,52AA#	N/A	phRL-M1M2	AZIUTR/S2; M1M2M6PPPSAAAA/S	M1M2M6PPPSAAAA/A; AZIM2/A
3007	hAZ1-WT	N/A	AZ-1wt	hAZ1WT/S	hAZ1WT/A
3008	hAZ1-IF	N/A	AZ-1if	hAZ1WT/S	hAZ1WT/A
5802	uCC-AAA-AUU-WT#	N/A	see Table S6	see Table S6	see Table S6
5803	uCC-AUU-AAA-WT#	N/A	see Table S6	see Table S6	see Table S6
5817	uCC-AAA-AAA-OF#	N/A	see Table S6	see Table S6	see Table S6

Start, initiating codon for uCC; S (sense) and AS (antisense) primers for PCR.

Table S6. Synthetic inserts used to generate 5' extended *azin1* uCC reporters – Related to STAR Methods

Plasmid name (#)	Synthetic sequence cloned between HindIII and AccI sites of plasmids "5320" or "5321"
uCC-AAA-AUU-WT# (5802)	AAGCTTAGTTAAAAAAACCGAGTAAAACTGGTGGCTCTTCAAAATTTGATTGGGCTGCTCGCTTCAGCC CCAAAGTTAGCCTACCTAAAACAAAACTATCTCTTCCAGGGTCTTCTAAGTCAGAGACATCAAAACCTG GACCTTCTGGATTACAGGCCAAATTAGCAAGTTTAAGAAAGTCCACCAAGAAACGCAGAAAAATTCCGC CGAAAAAGAGAAGACGCTTTACCCGACTCTTTGGGCCGTTATCTCACGTAAGTATCAAGGTTACAAGAC AGGTTTAAGGAGACCAATAGAAACTGGGCTTGTCGAGACAGAGAAGACTCTTGCGTTTCTGATAGGCAC CTATTGGTCTTACTGACATCCACTTTGCCTTTCTCTCCACAGGGCGAACTTTCTGACCGAGTATAC
uCC-AUU-AAA-WT# (5803)	AAGCTTAGTTAAAAATTCCGAGTAAAACTGGTGGCTCTTCAAAATTTGATTGGGCTGCTCGCTTCAGCC CCAAAGTTAGCCTACCTAAAACAAAACTATCTCTTCCAGGGTCTTCTAAGTCAGAGACATCAAAACCTG GACCTTCTGGATTACAGGCCAAATTAGCAAGTTTAAGAAAGTCCACCAAGAAACGCAGAAAAAACCGC CGAAAAAGAGAAGACGCTTTACCCGACTCTTTGGGCCGTTATCTCACGTAAGTATCAAGGTTACAAGAC AGGTTTAAGGAGACCAATAGAAACTGGGCTTGTCGAGACAGAGAAGACTCTTGCGTTTCTGATAGGCAC CTATTGGTCTTACTGACATCCACTTTGCCTTTCTCTCCACAGGGCGAACTTTCTGACCGAGTATAC
uCC-AAA-AAA-OF# (5817)	AAGCTTAGTTAAAAAAACCGAGTAAAACTGGTGGCTCTTCAAAATTTGATTGGGCTGCTCGCTTCAGCC CCAAAGTTAGCCTACCTAAAACAAAACTATCTCTTCCAGGGTCTTCTAAGTCAGAGACATCAAAACCTG GACCTTCTGGATTACAGGCCAAATTAGCAAGTTTAAGAAAGTCCACCAAGAAACGCAGAAAAAACCGC CGAAAAAGAGAAGACGCTTTACCCGACTCTTTGGGCCGTTATCTCACGTAAGTATCAAGGTTACAAGAC AGGTTTAAGGAGACCAATAGAAACTGGGCTTGTCGAGACAGAGAAGACTCTTGCGTTTCTGATAGGCAC CTATTGGTCTTACTGACATCCACTTTGCCTTTCTCTCCACAGGGCGAACTTTCTGACCGAGTATAC

Extension sequence derived from *Trip12* is shown in blue letters. Sequence of synthetic intron within the uCC is shown in red letters. Nucleotides of the uCC are highlighted in yellow. AUU near-cognate start codons are highlighted in green. Non-cognate AAA codons are highlighted in red. *Hin*dIII and *Acc*I cloning sites are underlined.