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ORIGINAL ARTICLE

Multiple forms of mouse antizyme inhibitor 1 mRNA differentially regulated by polyamines

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Abstract Antizyme inhibitor 1 (Azin1), a positive regulator of cellular polyamines, is induced by various proliferative stimuli and repressed by polyamines. It has been reported that the translational repression of Azin1 by polyamines involves an upstream open reading frame on the mRNA, but little has been known about polyamine effect on its transcription or splicing. We found multiple forms of *Azin1* transcripts formed by alternative splicing and initiation of transcription from putative alternative start sites. One of the novel splice variants, Azin1-X, has a premature termination codon on 5′ extension of exon 7, encodes a C-terminal truncated form of protein (Azin1ΔC), and is subject to nonsense-mediated mRNA decay. 2-Difluoromethylornithine (DFMO), an inhibitor of polyamine synthesis, increased both transcription from the canonical transcription start site and the ratio of the full-length mRNA to Azin1-X mRNA, whereas polyamines show the opposite effect. Thus, polyamines regulate two novel steps of *Azin1* expression, namely the transcription and a particular splicing pattern, both of which may affect the level of mRNA encoding the full-length active Azin1 protein.

Keywords Antizyme inhibitor 1 · Polyamines · Transcription · Alternative splicing · Nonsense-mediated mRNA decay · Cycloheximide

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Abbreviations

AZ	Antizyme
Azin	Antizyme inhibitor
CHX	Cycloheximide
DFMO	2-Difluoromethylornithine
MEF	Mouse embryonic fibroblast
NMD	Nonsense-mediated mRNA decay
ODC	Ornithine decarboxylase
Put	Putrescine
siRNA	Small interfering RNA
Spd	Spermidine
Spm	Spermine
SSAT	Spermidine/spermine N^1-acetyltransferase
uORF	Upstream open reading frame

Introduction

Antizyme inhibitor (Azin) is a catalytically inactive homolog of ornithine decarboxylase (ODC) and positively regulates cellular polyamines by inhibiting antizyme (AZ) (Murakami et al. 1996; Mangold 2006; Kahana 2009). One of the two Azin isoforms in mammals, Azin1, is distributed in most of the tissues. Azin1 is induced by various proliferative stimuli (Fujita et al. 1982; Murakami et al. 1989; Nilsson et al. 2000) and implicated in tumorigenesis (Jung et al. 2000; Choi et al. 2005) or hepatic fibrogenesis (Paris et al. 2011).

Azin1 is repressed by polyamines, forming a negative feedback loop (Nilsson et al. 2000; Ivanov et al. 2008, 2010; Murakami et al. 2009). It has been reported that the repression is mainly at the translational level involving a short upstream open reading frame (uORF) with a non-AUG start codon (Ivanov et al. 2008). In addition, the stability of Azin1 increases by binding with AZ (Bercovich

and Kahana 2004) and the interaction between Azin1 and AZ1 is changed spatiotemporally (Mangold et al. 2008; Murakami et al. 2009). However, it has not been clear whether polyamines regulate expression of the *Azin1* gene at the transcription or RNA processing step.

We previously reported a line of gene trap mice in which the *Azin1* gene was disrupted (Tang et al. 2009). Homozygous *Azin1* gene trap mice showed partial lethality with decreased tissue levels of ODC and putrescine. However, significant amounts of Azin1 mRNA were detected in their tissues, raising the possibility that alternative forms of Azin1 mRNA are transcribed to skip the trapping insertion. To address this issue, we first analyzed alternative forms of Azin1 mRNA in both the mutant mice and wild-type controls. Then we examined the effect polyamines on the levels of the different forms of Azin1 mRNA. The results demonstrate that polyamines regulate at least two steps of *Azin1* expression, namely the transcription and selection of splicing acceptor sites for the exon 7, both may affect the level of mRNA that encodes the full-length active Azin1 protein.

Materials and methods

Animals

The *Azin1*gene trap mice (B6 × CB) F1-*Azin* $^{GT(pU17)}$ (Tang et al. 2009) were backcrossed for more than 10 generations onto BALB/cAJcl (CLEA) and C57BL/6 J (CLEA) mice separately. Heterozygous mice were interbred to produce homozygous mutant mice. Mice were housed in an animal cabinet at 23.5 °C and 40–60 % humidity on a 12-h light/dark cycle. All animal experiments were carried out in accordance with protocols approved by the experimental animal ethics committee at The Jikei University School of Medicine.

RNA extraction and analysis

Total RNA was isolated from cultured cells or tissues using the RNA-Bee (TEL-TEST, INC) or TRizoL Reagent (Invitrogen) and treated with DNase I (Ambion) according to the manufacturer's instructions. The first-strand cDNA was obtained using the GeneRacer kit (Invitrogen), and the amplification of cDNA ends was performed with the GeneRacer primer and an *Azin1* gene-specific primer (5′-GCTCATCACAGGATGGACCCCAAA). The nested PCR was performed with the GeneRacer 5′ Nested primer and an *Azin1* gene-specific nested primer (5′- GTGAAGCCTCCACCGATGTCTAAC). PCR-amplified products were cloned and sequenced using the Zero Blunt TOPO PCR Cloning Kit for Sequencing (Invitrogen).

DNase-treated RNAs were reverse-transcribed into cDNA using the RNA PCR Kit (AMV, TaKaRa) or the PrimeScript RT reagent kit (TaKaRa). PCR was performed using KOD DNA polymerase (Toyobo). The amplified products were either quantified with ImageQuant LAS 4000 (GE Healthcare) or isolated using 2 % agarose gel electrophoresis and cloned into TOPO vector (Invitrogen) for sequencing. The following primers were used for RT-PCR. T1 Forward 5′-TATGATAGTTGTGTCACTGGAATGG; T2 Forward 5′-TTTATCACTCAGATTCCCTGTGG; T2 Reverse 5′-CCACAGGGAATCTGAGTGATAAA; T3 Forward 5′-TGGAAGATAAGTCGGTGCTCT; T4 Forward 5′-TTCTCTTTCATTTTCATCAATAGACAG; T5 Forward 5′-CTATTGAGAGTAGGATTCCTGTGTAT; Azin1 exon 1(E1) Forward 5′-CGCCTTTCTTCTTTTTCTCG; Azin1 E2 Forward 5′-CTACCCAGAAGGCCTAGGAGA; Azin1 E3 Forward 5′-CACTTCAGAAACCCAAGACTCA; Azin1 E4 Forward 5′-AGGACCCAGACATAGGCTTG; Azin1 E5 Forward 5′-TTTTTGTGGGGGATCTTGG; Azin1 E6 Forward 5′-TGGCTTTAGTGCAAGAATTGG; Azin1 E7 Reverse 5′-ACATCAAGTETCCTTGGCACA; Azin1 E7ext Reverse 5′-CTAAGAGAAGGGGAAGATGA; E9 Reverse 5′-TGAAGCCTCCACCGATGTCTAAC; Azin1 E13 Reverse 5′-TTAAGCTTCAGTGGAAAAGC.

Cell culture and transfection

Mouse embryonic fibroblasts (MEFs) were isolated from the fetuses at the day 13.5–15 of pregnancy and cultured in Dulbecco's modified Eagle's minimum essential medium (DMEM, GIBCO) supplemented with 10 % FCS and 1 % penicillin/streptomycin (GIBCO) for 2–3 passages. The cells were plated in 6-well plates at a density of 3.3×10^5 cells per well for the RNA or protein analyses. NIH3T3 cells were maintained in DMEM supplemented with 10 % calf serum. For transfection of siRNA, 3×10^5 of NIH3T3 cells were plated in each well of a six-well plate and cultured overnight. The next day, siRNA were transfected at a final concentration of 100 nM of Upf1 siRNA (Sigma) or 100 nM of control siRNA (Cosmo Bio), using Lipofectamine RNAiMAX (Invitrogen) following the manufacturer's direction. Cells were harvested 48 h after the transfection.

Western blot analysis

MEFs of each genotype were pooled from three individual embryos. The pooled MEFs were cultured for 6 h in 6-well plate with fresh medium containing putrescine (5 mM), spermine plus aminoguanidine (1 mM each), or 2-difluoromethylornithine (DFMO), an irreversible inhibitor of ODC (5 mM). Cells were washed three times with PBS and suspended in 0.2 ml of lysis buffer containing 20 mM

Tris–HCl (pH7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1 % NP-40, 1 % sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin and 0.2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (Sigma). After 30 min of incubation on ice, the cell suspension was sonicated for 20 s and centrifuged at 12,000*g* for 20 min at 4 °C. Protein concentration of the supernatant was determined with BCA protein assay reagent (Pierce) and 80 μg of the supernatant protein per lane was separated on 11.5 % polyacrylamide-SDS gel. Immunoblotting and immunodetection were performed using monoclonal anti-rat Azin1, HI-12, as reported previously (Murakami et al. 2009). Non-specific band was used as a loading control. Images were captured by Light Capture II (ATTO).

Statistical analysis

Data are presented as mean ± standard deviations. Significance was estimated using unpaired one-tailed Student's *t* tests. Difference with $p < 0.05$ was considered significant.

Results

Expression of multiple forms of Azin1 mRNA

The *Azin1* gene on Entrez Gene and RefSeq databases consists of 13 exons (Fig. 1a, top), being transcribed into 2 mRNA variants, with and without exon 3, but encoding the identical protein since the protein coding region (1,344 nucleotides encoding 448 amino-acids protein) spans exon 4 to exon 13. The position of the trapping insertion is located within intron 2. RT-PCR followed by sequencing revealed a number of additional forms of Azin1 transcripts both in wild-type and homozygous *Azin1* gene trap mice (Fig. 1a, bottom). We noted a novel exon, exon 2b, which is mostly utilized in a mutually exclusive manner with exon 2. Other types of alternative splicing, namely extension of exon 7 to the 5′ direction and 5′ truncation of exon 11 by 3 nucleotides (Azin1c.905_907del, p. Val302del) were also found. In addition, the splicing of the last 3 introns was extensively variable with frequent changes of splicing acceptor or donor sites. In some cases, intron 11 was entirely retained. One particular splicing variant around this region has been implicated in hepatitis C-related hepatic fibrogenesis in human (Paris et al. 2011). The 5′-GeneRacer PCR as well as conventional RT-PCR revealed that, in homozygous mutant mice, most of transcripts with exon 1 used an alternative splicing donor site for intron 1, resulting in a lack of 78 nucleotides of the 3′-end of exon 1 and therefore a loss of regulatory function of the uORF (Fig. 1a, bottom) (Ivanov et al. 2008).

Figure. 1b shows results of RT-PCR in various tissues of wild-type and the mutant mice. In homozygous mutant mice, exon 1- and exon 2-containing transcripts significantly decreased, but instead transcript with exon 3, which is rarely utilized in wild-type mice, markedly increased, due probably to the trapping insertion.

Some of the bands amplified by E3-E13 to E6-E13 appeared double bands (Fig. 1b). Sequencing analysis showed that the upper band is a transcript containing a 5′ extension (152 bases) of exon 7. This transcript, referred to as Azin1-X, encodes a 154 amino acid product (Azin1ΔC) that is terminated at the premature termination codon (PTC) on the extension (Fig. 1a, bottom).

There were large differences in the levels of E1-E13, E2-E13 and E3-E13 amplified products between wild-type and mutant mice (Fig. 1b). In contrast, the difference in the E4-E13, E5-E13 and E6-E13 amplified products were smaller in most tissues, suggesting the existence of additional transcription start sites (TSSs) downstream to the trapping insertion site. We searched Database of Transcriptional Start Sites (DBTSS) and found four potential alternative TSSs (T1, T2, T4 and T5) (Fig. 2a). In addition, presence of an EST (dbEST 21033007) with a 5′ extension of exon 4 suggested another TSS within intron 3 (T3). The position of T3 was determined to be 72 nucleotides upstream of intron 3-exon 4 border with a series of RT-PCR with forward primers set gradually upstream (Fig. 2a). Existence of T1 and T3 was supported by that RT-PCR with reversed primers at the corresponding positions and that primer E1 did not amplify products. T2 is on exon 2b, but it is likely to be an alternative TSS since the amount of RT-PCR product with forward T2-E4 was much larger than that with E1-reverse T2.

Although T1, T2 and T3 are all upstream of exon 4 where the initiating AUG codon is located, the regulating uORF is missing from the corresponding transcripts. The transcript from T4 encodes an N-terminal truncated form of Azin1 (Azin1ΔN, 354 amino acids). Note that the region necessary for binding to antizyme is encoded on exon 6 and retained by both Azin1ΔN and Azin1ΔC. As shown in Fig. 2b, transcripts from T1, T2 and T3 were tissue specific and increased in the mutant mice.

Effects of DFMO and polyamines on the expression of *Azin1*

Next we examined the effect of polyamines and DFMO, an inhibitor of polyamine synthesis, on the level of Azin1 mRNA in wild-type MEFs. RT-PCR with E1-E-13 primers detects two types of the full-length mRNA starting at the canonical TSS with and without exon 3. As shown in

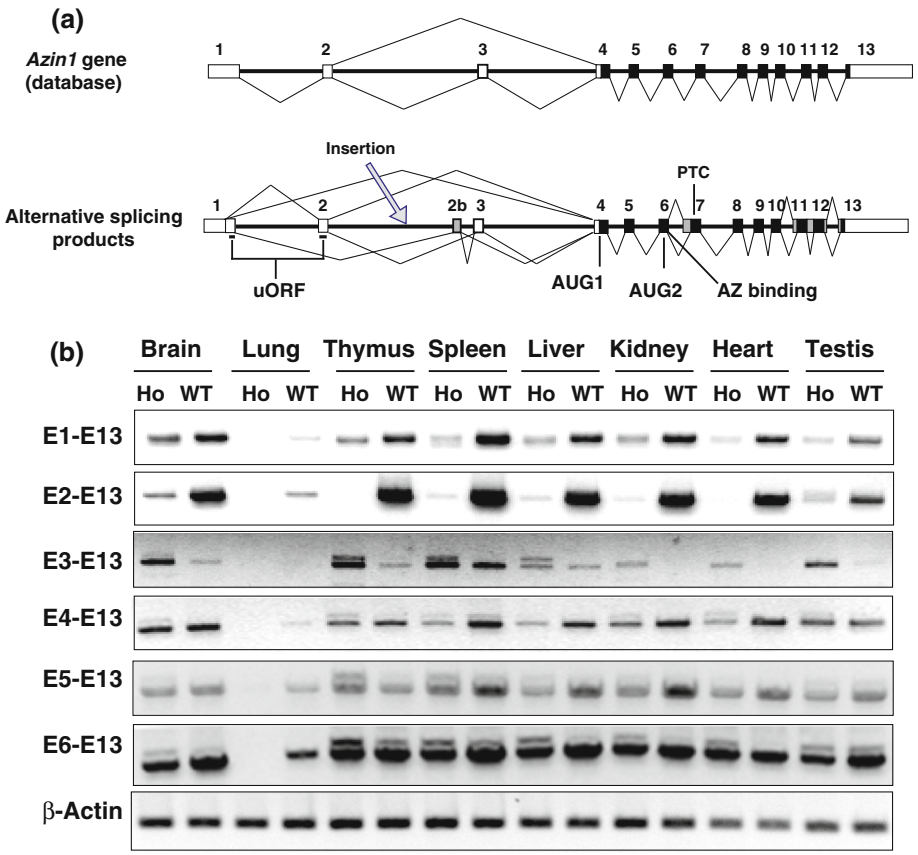

Fig. 1 Expression and alternative splicing of Azin1 mRNA. **a** *Azin1* gene in the databases consists of 13 exons. Two splicing patterns, with and without exon 3, have been reported. The *arrow* indicates the site of trapping insertion in the gene trap mice. RT-PCR analysis detected a number of novel splicing forms. They include various splicing forms involving exons 1–4, novel exon 2b, extension of exon 7 to the 5′ direction (Azin1-X), and various splicing forms involving exons 10–13. *Open box*, untranslated region; *black box*, coding region; *gray box*, new exon; *uORF,* upstream open reading frame; *PTC*, premature termination codon. **b** Splicing pattern was tissue specific and affected by the insertion of trapping vector. Total RNA was isolated from tissues of homozygous (Ho) and wild-type (WT) female or male 40 ± 2 days old mice. For the tissues other than testis, only samples from a single pair of female littermate mice were used. For testis, three mice for each genotype were analyzed. A representative result of multiple analyses is shown. The expression of *Azin1mRNA* variants were analyzed by RT-PCR using primer sets indicated. E3–E13 to E6–E13 products show double band. Sequencing analysis revealed that the upper band of the E3–E13 to E6–E13 products is the transcript containing 5′ extension (152 nucleotides) of exon7 (Azin1-X)

Fig. 3a, DFMO increased both types of Azin1 mRNA. To test whether DFMO increased the mRNA synthesis, we employed MEFs from the heterozygous gene trap mice in which the *Azin1* (exon 1 and 2)-β-galactosidase-neomycin fusion gene (*Azin1-Geo*) is transcribed under the direction of the endogenous *Azin1* promoter. As shown in Fig. 3b, *Azin1-Geo* transcript was significantly increased by DFMO and decreased by simultaneous addition of DFMO and spermidine. Thus, transcription of the full-length Azin1 mRNA is under the negative control of polyamines. Effects of polyamines of the expression of Azin1 were also examined at the protein level using Western blotting of extracts of MEFs from wild-type and homozygous gene trap mice (Fig. 3c). Two bands, a major 49 kDa and a minor 40 kDa bands, were detected in both wild-type and homozygous MEFs. The size of the minor band corresponds to Azin1ΔN which is transcribed from T4 TSS (note that the monoclonal antibody for the Western detection recognizes a C-terminal half of Azin1). Western blot analysis using diluted extracts revealed that the 49 kDa band in untreated homozygous mutant MEFs was 25–30 % of untreated wild-type MEFs (data not shown). The 49 kDa band decreased with polyamines (putrescine or spermine) and increased with DFMO treatment in both MEFs. However, the repression was much less evident in the mutant MEFs than in wild-type. This could be explained by that effectively all the exon 1-containing mRNA in homozygous *Azin1* gene trap mice lacks 78 bases of the 3′-end of exon 1 and therefore devoid of the regulatory uORF as described above. It is also noteworthy that the uORF-lacking mRNA is still subject of repression by polyamines, implying an additional mechanism for regulation by polyamines (see below).

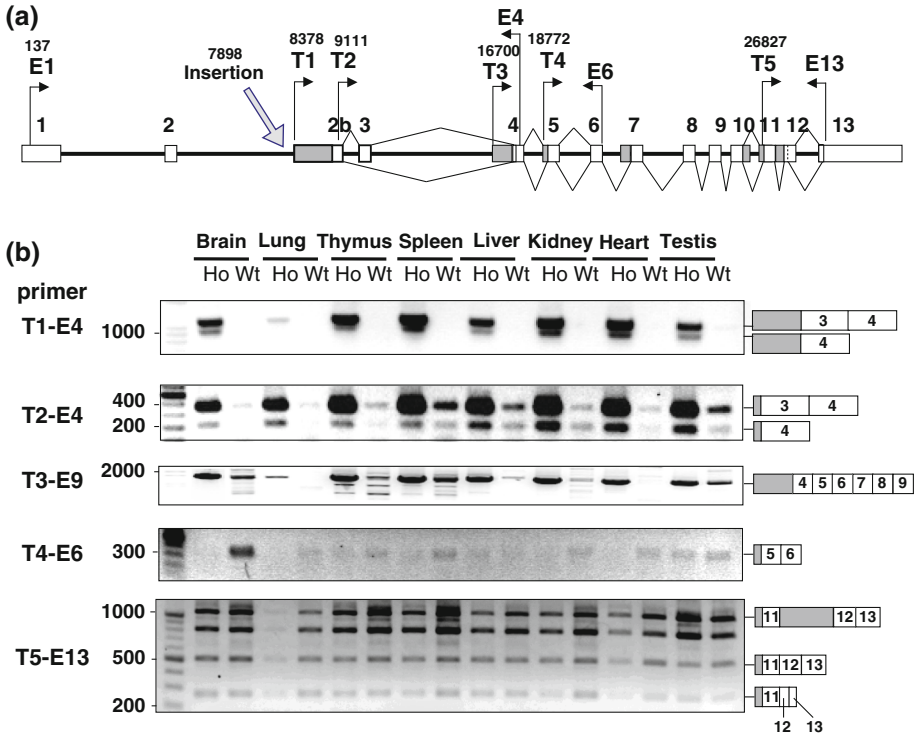

Fig. 2 Expression of *Azin1* transcripts with alternative splicing and additional transcription start sites in tissues. **a** Various forms of *Azin1* transcripts were amplified by RT-PCR using primers designed for *Azin1* transcription starting sites (T1, T2, T4, and T5). The *arrowheads* indicate the positions of the primers used. **b** Tissue-specific pattern, and difference between wild-type and mutant mice of transcripts from T1–5. The identical set of samples in Fig. 1b was used

Azin1-X is a target for nonsense-mediated mRNA decay

We looked into the novel splice variants, Azin1-X, that contains a 5′ extension of exon 7, since first its relative amount was significant (Fig. 1b), second it has a PTC and is a potential target of nonsense-mediated mRNA decay (NMD) (Isken and Maquat 2007), and third its protein product, Azin1ΔC, retains the antizyme-binding site (Fig. 1a-bottom). Two primer sets were used to measure Azin1-X on RT-PCR; E4-E7ext (reverse primer on the extension of exon7) amplifies only Azin1-X, and E4-E9 simultaneously amplifies Azin1-X and the full-length Azin1 mRNA. We tested the effect of cycloheximide (CHX) on the level of Azin1-X in wild-type MEFs or NIH3T3 cells since CHX is known to inhibit NMD which is a co-translational process. As shown in Fig. 4, the level of Azin1-X was increased by CHX in both cells. In wild-type MEFs (Fig. 4a), the level of Azin1-X (the upper band of the middle gel) was 1.00 ± 0.18 (mean ± SD) and 3.42 ± 0.08 in the absence and presence of CHX, respectively. The full-length Azin1 mRNA (lower band of middle gel) was increased by CHX from 7.27 ± 0.75 to 10.49 ± 0.49. Consequently the relative amount of Azin1-X (Azin1-X/Total Azin1 mRNA) was increased by CHX from 12.1 to 24.6 %. Similarly, in NIH3T3 (Fig. 4b), the mean expression of Azin1-X was 1.0 without CHX and 11.5 with CHX, and the relative amount of Azin1-X was 4.3 % without CHX and 20.4 % with CHX. Note that CHX also increased the level of the full-length Azin1 mRNA but less extent compared to Azin1-X.

To confirm the involvement of NMD pathway, an essential cellular NMD factor Upf1 was knocked down by RNA interference. As shown in Fig. 4c, introduction of Upf1 siRNA lowered cellular Upf1 less than half, and significantly increased Azin1-X mRNAs but not the full-length Azin1 mRNA (Fig. 4c). These results indicated that Azin1-X mRNA is a target for NMD. It is also suggested that the transcription or the stability of the full-length Azin1 mRNA or both is regulated by a labile protein as discussed below.

Transcriptional and posttranscriptional regulation of Azin1 mRNA by polyamines

As mentioned above (Fig. 3), expression of Azin1 mRNA is negatively regulated by polyamines at the transcriptional level. We further examined if polyamines affect the selection of 5′ splice site of exon 7 (splicing donor site in splicing of intron 6), which determines the relative amount of the full-length Azin1 mRNA and Azin1-X. As shown in Fig. 5, absolute level and relative content of Azin1-X were both decreased by DFMO and increased by simultaneous addition of putrescine or spermidine, indicating that the selection of 5′ splice site of exon 7 is regulated so that mRNA processing to produce the full-length Azin1 mRNA is repressed by polyamines. The negative effect of

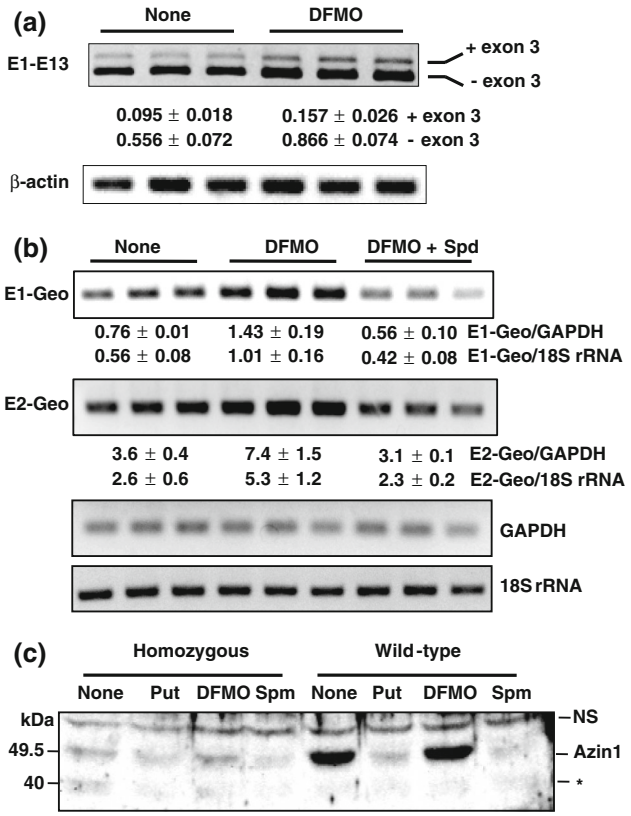

Fig. 3 Effect of DFMO and polyamines on the level of Azin1 mRNA and protein. **a** Wild-type MEF were cultured for 4 days with or without DFMO. Total RNA was extracted and mRNA levels were analyzed by semi-quantitative RT-PCR. Values are the mean ± standard deviation. Intensities of both the upper and lower bands are significantly different between the two conditions ($p < 0.05$). **b** MEF of heterozygous gene trap mice were cultured for 4 days with or without DFMO, or with DFMO plus spermidine. Total RNA was extracted and *Azin1-Geo* fusion mRNA level were analyzed by semi-quantitative RT-PCR. Intensities of E1-Geo and E2-Geo bands, normalized with both GAPDH and 18S rRNA, are significantly different between with and without DFMO, and between with DFMO and DFMO plus spermidine ($p < 0.05$). **c** Effect of polyamines on the level of Azin1 protein in MEFs of homozygous gene trap mice and wild-type mice. The experiment was carried out in duplicate and a representative result is shown. *NS* non-specific band and *asterisk* denotes the minor 40 kDa band possibly corresponding to Azin1ΔN

polyamines on the level of full-length Azin1 mRNA was also confirmed.

Finally, we examined if a labile protein is necessary for the regulation of the splicing by polyamines. Wild-type MEFs were incubated with DFMO, DFMO plus spermidine, or none, and then treated with CHX for 6 h. As shown in Fig. 6a, CHX largely increased Azin1-X mRNA, but the effects by polyamines and DFMO on the level of Azin1-X were still observed in the presence of CHX. Similar experiments were carried out three times, and the effects of CHX and DFMO on Azin1-X mRNA were reproducible although effects on Full-Azin1 mRNA were somewhat variable. This result suggests that the regulation of splicing steps by polyamine is not mediated by a labile protein.

Discussion

The primary aim of this study was to determine whether polyamines regulate expression of the *Azin1* gene at the transcription or RNA processing step. We first analyzed Azin1 mRNA both in gene trap mice and wild-type mice, and found multiple forms of *Azin1* transcripts formed by alternative splicing or initiation of transcription from putative alternative start sites. One of the novel splice variants, Azin1-X, is of particular interest since it contains a 5′ extension of exon 7 leading to premature termination of translation. Next, we examined the effects of polyamines on the levels of canonical full-length Azin1 mRNAs and Azin1-X. The full-length mRNAs was increased by DFMO, whereas Azin1-X mRNA was decreased by DFMO. *Azin1-Geo* fusion gene that had been created by the trapping insertion and can be used as a reporter of the transcription from the canonical TSS was increased by DFMO and decreased by polyamines. We conclude that polyamines regulate two steps of *Azin1* expression, namely the transcription and selection of splicing acceptor sites for the exon 7, both of which may affect the level of mRNA encoding the full-length active Azin1 protein (Fig. 6b).

It is noteworthy that, in the homozygous *Azin1* gene trap mice, almost all the Azin1 mRNA encoding the full-length Azin1 protein does not have the regulatory uORFs either by the lack of 78 nucleotides of the 3′-end of exon 1 or by the use of alternative TSSs (T1-3), but still the level of the full-length Azin1 protein was partially regulated by polyamines (Fig. 3c). This can be explained by the polyamines regulation on transcription from the canonical TSS (at the 5′ end of exon 1) and selection of 5′ splice site of exon 7 to create Azin1-X.

Azin1-X is expressed in both wild-type and mutant mice. It encodes a 154 amino acid product, Azin1ΔC, which lacks the almost two-thirds of the C-terminal region, but retains the antizyme-binding site. Azin1-X is rapidly degraded by NMD pathway (Fig. 4), but if Azin1-X could escape from NMD, Azin1ΔC might function as a regulator of polyamines by interfering the binding of Azin1 and AZs.

Azin1 and Spermidine/spermine N^1-acetyltransferase (SSAT) share some translational and posttranslational regulatory mechanism although their impacts on cellular polyamines are opposite: Azin1 is a positive regulator of cellular polyamines whereas SSAT is the key catabolic enzyme for cellular polyamines. A particular alternative splicing of *SSAT* mRNA is regulated by polyamines, but the direction of the regulation is opposite to that for Azin1 (Wang et al. 1998; Hyvönen et al. 2006; Pegg 2008). Our

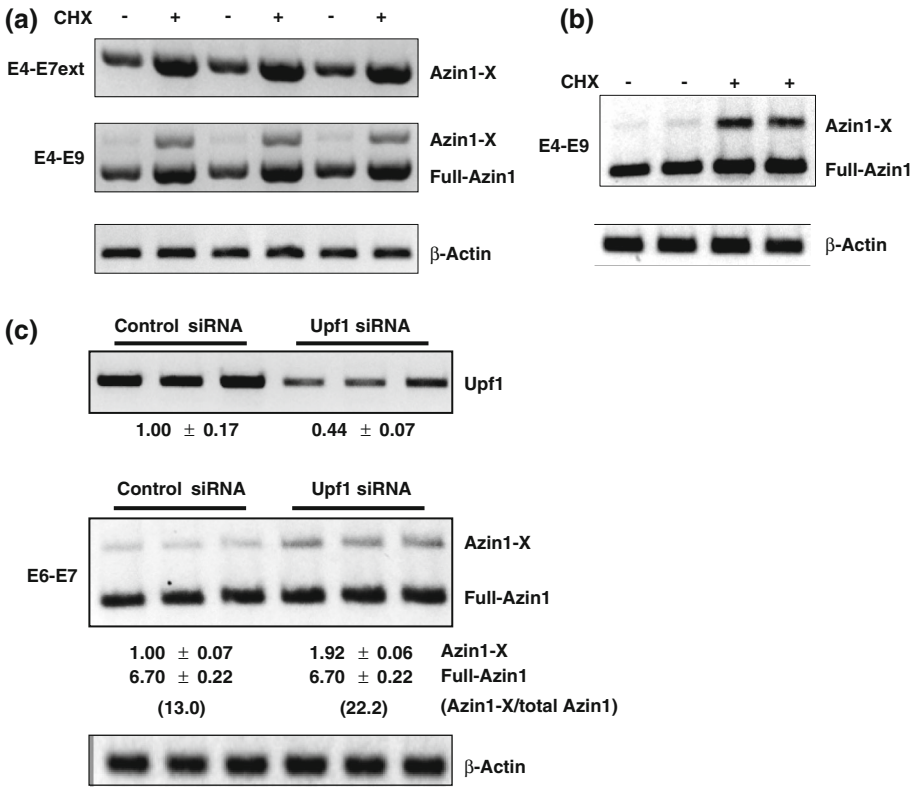

Fig. 4 Effects of CHX and Upf1 knockdown on the levels of *Azin1* transcripts. **a** Wild-type MEFs at growing stage were cultured in the presence or absence of CHX (10 μg/ml) for 6 h. Total RNA was extracted and mRNA levels were measured by semi-quantitative RT-PCR with Azin1-X specific primer set (E4-E7ext) or E4-E9. The expression level was normalized against β-actin. The experiment was performed at least twice, giving an essentially the same result. **b** Similar experiment was carried out in NIH3T3 cells. Experimental condition was identical except that the concentration of CHX was 25 μg/ml. The experiment was performed twice, giving an essentially the same result. **c** Effects of Upf1 knockdown on the levels of *Azin1* transcripts in NIH3T3 cells. The levels of Upf1 and Azin1-X, as well as Azin1-X to total Azin1 ratio are significantly different between the two conditions ($p < 0.05$)

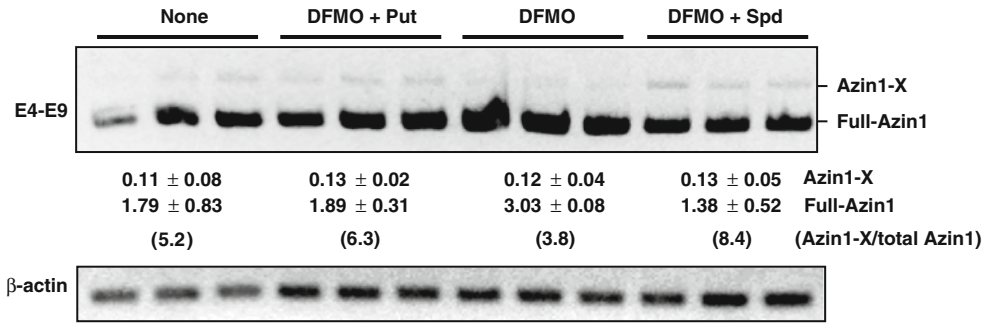

Fig. 5 Effects of polyamines on the level of Azin1 mRNA. Wild-type MEFs were cultured with or without 2.5 mM DFMO, 100 μM putrescine (Put), 1 mM spermidine supplemented with 1 mM aminoguanidine (Spd) for 96 h. Total RNA was extracted and mRNA levels were measured by semi-quantitative RT-PCR with E4–E9 primer set. The expression level was normalized against β-actin. The normalized level of Full-Azin1 is significantly different between DFMO and the other three conditions (None vs DFMO, DFMO vs DFMO + Put, and DFMO vs DFMO + Spd) ($p < 0.05$)

results also suggest that a labile protein(s) regulates transcription and/or the stability of the full-length Azin1 mRNA (Figs. 4, 6a). It has been reported that a labile regulatory protein(s) is also involved in the regulation of SSAT (Fogel-Petrovic et al. 1996; Hyvönen et al. 2006). More detailed mechanism of Azin1 regulation as well as its

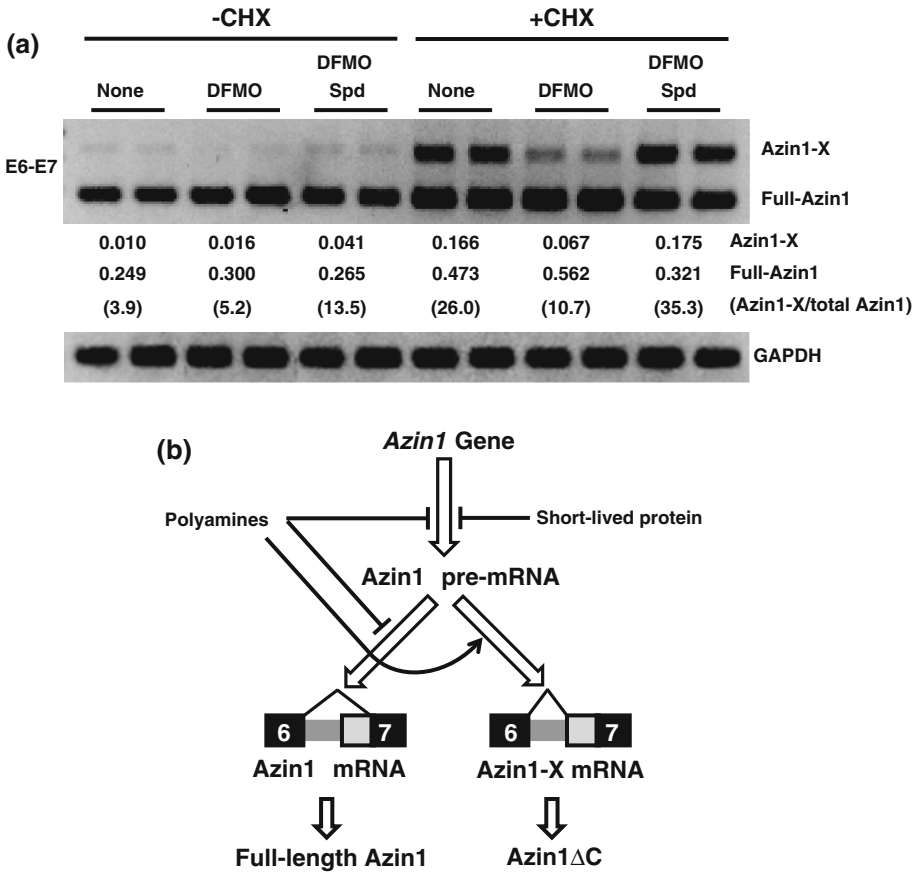

Fig. 6 Effects of polyamines and CHX on the expression of Azin1-X. **a** Wild-type MEFs were cultured in multiple wells with or without DFMO (2.5 mM) for 48 h. Then, the culture medium was changed with a same fresh medium. A half of DFMO wells were supplemented with 1 mM spermidine and 1 mM aminoguanidine (DFMO + Spd). Forty-eight hours later, the culture medium was changed again with the same fresh medium containing CHX (10 μg/ml). After 6 h incubation, cells were harvested and total RNA was extracted and mRNA levels were measured by semi-quantitative RT-PCR with E6–E7 primer set. The expression level was normalized against GAPDH. Similar experiments were carried out three times and a representative result is shown. **b** Scheme of polyamine regulation of transcription and splicing of *Azin1*. A short-lived protein is involved in the regulation of transcription but not the regulatory alternative splicing

relationship with that of SSAT should be clarified in future study.

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Conflict of interest The authors declare that they have no conflict of interest.

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