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# The perplexities of the ZC3H12A self-mRNA regulation*

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**The mechanisms regulating transcript turnover are key processes in the regulation of gene expression. The list of proteins involved in mRNAs' degradation is still growing, however, the details of RNase-mRNAs interactions are not fully understood. ZC3H12A is a recently discovered inflammation-related RNase engaged in the control of proinflammatory cytokine transcript turnover. ZC3H12A also regulates its own transcript half-live. Here, we studied the details of this regulation. Our results confirm the importance of the 3'UTR in ZC3H12A-dependent *ZC3H12A* mRNA degradation. We compared the mouse and human stemloop structures present in this region and discovered that the human conserved stem-loop structure is not sufficient for ZC3H12A-dependent degradation. However, this structure is important for the *ZC3H12A* mRNA post-transcriptional regulation. Our studies emphasize the importance of the neighboring features of the identified stem-loop structure for its biological activity. Removal of this region together with the stem-loop structure greatly inhibits the ZC3H12A regulation of the investigated 3'-untranslated region (3'UTR).**

**Key words**: ZC3H12A/MCPIP1; RNase; transcript turnover; inflammation.

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## INTRODUCTION

ZC3H12A (zinc finger CCCH-type containing 12A) is a recently discovered protein involved in the attenuation of inflammation (Matsushita *et al.*, 2009). Its importance in this process was shown by the use of a mouse model. *Zc3h12a*$^{-/-}$ mice spontaneously develop severe autoimmune inflammatory disease and most of them die within 12 weeks of birth (Matsushita *et al.*, 2009). The negative regulation of the inflammatory response by ZC3H12A depends on its dual RNase and deubiquitinase activities which allow this single protein to act at two levels of the cellular response – signal transduction (ZC3H12A negatively regulates JNK and NF-κB signaling by deubiquitinating the TRAF proteins) and mRNA decay (Liang *et al.*, 2010; Matsushita *et al.*, 2009). Its RNase activity specifically shortens the half-life of mRNAs of a subset of proinflammatory cytokines such as interleukins: IL1β, IL-6, IL-12p40 and IL-2, or transcription factor c-Rel (Li *et al.*, 2012; Matsushita *et al.*, 2009; Mizgalska *et al.*, 2009; Uehata and Akira, 2013). Transcripts are not the only targets of this RNase. ZC3H12A also cleaves terminal loops in precursor miRNAs (pre-miRNAs) (Suzuki *et al.*, 2011) and viral RNA (Lin *et al.*, 2013; Lin *et al.*, 2014; Liu *et al.*, 2013).

The level of *ZC3H12A* mRNA is rapidly upregulated in macrophages in response to Toll-like receptors (TLRs) and IL-1β receptor activation (Matsushita *et al.*, 2009). Additionally, signaling from TLRs and IL-1R influences the ZC3H12A protein level. Stimulation of these receptors leads to the activation of the IKK complex resulting in phosphorylation and subsequent ubiquitination, followed by proteasomal degradation of ZC3H12A. The protein reappears within two hours after stimulation (Iwasaki *et al.*, 2011). Such mechanism allows for temporal synthesis of proinflammatory cytokines within the period of time when ZC3H12A is degraded and then ZC3H12A-dependent degradation of proinflammatory transcripts can be switched on.

The mechanisms responsible for mRNAs' degradation by ZC3H12A have been studied as well. It is documented that the CCCH-type zinc finger domain is engaged in the interaction of ZC3H12A with RNA. Following binding, another domain, the NYN/PIN-like domain, is involved in endonucleolitic activity of the protein (Iwasaki *et al.*, 2011; Matsushita *et al.*, 2009; Mino *et al.*, 2015; Yokogawa *et al.*, 2016). ZC3H12A was reported to associate with a set of mRNAs containing a stemloop structure within their 3'UTRs, with *IL-6* to be the most prominent target (Matsushita *et al.*, 2009). Results obtained by our group confirmed a direct interaction of ZC3H12A with *IL-6* mRNA in a single cell (Kochan *et al.*, 2015). However, our knowledge about the features of the sequences present in 3'UTR which influence the binding is very poor.

Recently, ZC3H12A involvement in the regulation of its own mRNA turnover has been identified, presenting an intrinsic auto-regulatory mechanism (Iwasaki *et al.*, 2011; Mino *et al.*, 2015; Mizgalska *et al.*, 2009). To understand exactly which elements are crucial for ZC3H12A-dependent degradation, in the paper presented here we performed detailed analyses of this transcript focused not only on the stem-loop structures but also on their neighboring features. Unexpectedly, our results suggest, in contrast to published data, that the conserved stem-loop structure present in human *ZC3H12A* 3'UTR is not sufficient for ZC3H12A-dependent degradation.

## MATERIALS AND METHODS

**Cell culture**. HeLa cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 1 g/L Dglucose

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**Abbreviations**: IL, interleukin; UTR, untranslated region; TLR, Toll-like receptor; ZC3H12A, Zinc finger CCCH-type containing 12A.

(Lonza, Belgium), supplemented with 2 mM L-glutamine (Sigma-Aldrich, USA), and 10% (v/v) FBS (Biowest, France), at 37°C in a humidified atmosphere containing 5% $CO_2$.

**Construct preparation**. The pLuc-*ZC3H12A*-3'UTR construct encoding the firefly luciferase transcript fused with the *ZC3H12A* 3'UTR fragment, was prepared by insertion of PCR amplified *ZC3H12A* 3'UTR (NM_025079.2) into the pmirGLO vector (Promega, USA). Briefly, the 3'UTR fragment of *ZC3H12A* was amplified from cDNA prepared from HeLa cells using PfuUltra II Fusion HS DNA Polymerase (Agilent, USA), and the following primers: forward 5'ATCGCTAGCGCTGCCTGTGGCTGG-3', and reverse 5'ATCTCTAGAGATGTGTTACAGGAGGTAAGGACT-3'. After agarose gel electrophoresis, the PCR product corresponding to *ZC3H12A* 3'UTR fragment was excised, purified, cleaved with NheI and XbaI (NEB, USA), and inserted using T4 DNA ligase (NEB, USA) into gelpurified pmirGLO vector linerized with NheI and XbaI and dephosphorylated with CIP (NEB, USA). The obtained construct was verified by restriction digestion and Sanger sequencing (Genomed, Polska).

The plasmids encoding the firefly luciferase transcript fused with the *ZC3H12A* 3'UTR fragment devoid of stem-loop structures 1(ΔSL1), 2(ΔSL2) or both(ΔSL1+ΔSL2), were prepared by site-directed mutagenesis using QuikChange II XL Site-Directed Mutagenesis Kit (Agilent, USA), according to the manufacturer's instructions, with pLuc-*ZC3H12A*-3'UTR as template for ΔSL1 and ΔSL2 constructs, or ΔSL1 for the ΔSL1+ΔSL2 construct. The following primers were used: dSL1 sense 5'GAAGCGATCACCCTGTTGCTCTGTAGTTTAAGGAGA-3', dSL1 anti-sense 5'TCTCCTTAAACTACAGAGCAACAGGGTGATCGCTTC-3', dSL2 sense 5'CTATTCCTTATCCTGCTGTCAAGGGCCCTCCGTCTA-3' and dSL2 anti-sense 5'TAGACGGAGGGCCCTTGACAGCAGGATAAGGAATAG-3'.

The plasmids encoding the firefly luciferase transcript fused with 3' truncations of the *ZC3H12A* 3'UTR, were prepared by restriction digestion using sites naturally occurring within *ZC3H12A* 3'UTR. Briefly, the pLuc-*ZC3H12A*-3'UTR plasmid was digested with NheI and ApaI (D1), or BsmBI (D2) or DraIII (D3). The digestion products were resolved using agarose gel electrophoresis and the DNA bands corresponding to the truncated 3'UTR fragments were excised, purified, blunted with T4 DNA polymerase (NEB, USA), and inserted using T4 DNA ligase into the pmirGLO vector which was linearized with NheI and XbaI, blunted using T4 DNA polymerase and dephosphorylated with CIP. The obtained constructs were verified by restriction digestion and Sanger sequencing.

The plasmid for expression of human ZC3H12A protein was prepared by insertion of PCR-amplified *ZC3H12A* coding sequence (CDS) into the pcDNA6.2/cTC-Tag-DEST vector (Invitrogen, USA), using the Gateway cloning system (Invitrogen, USA). Briefly, the *ZC3H12A* CDS was amplified as described above using the following primers: forward 5'CACCATGAGTGGCCCCTGTGGAGAG-3' and reverse 5'CTCACTGGGGTGCTGGGACTTG-3'. The PCR product corresponding to *ZC3H12A* CDS fragment was gel-purified and inserted into pENTR/D-TOPO vector using the pENTR™/D-TOPO® Cloning Kit (Invitrogen, USA), according to the manufacturer's instructions. *ZC3H12A* CDS was subsequently shuttled from the entry vector pENTR/D-TOPO into the destination vector pcDNA6.2/cTC-Tag-DEST using the LR Clonase II Enzyme Mix (Invitrogen, USA), according to the manufacturer's instructions. The expression vector coding for RNase-inactive mutant of ZC3H12A, referred to as ZC3H12A(D141N) was obtained with QuikChange II XL site directed mutagenesis kit (Agilent, USA) using plasmid coding for WT form as a template and the following primers: M1-D141Nsense: 5'AGACCAGTGGTCATCAATGGGAGCAACGTGG-3' and M1-D141Nantisense: 5'CCACGTTGCTCCCATTGATGACCACTGGTCT-3'. The final vectors were verified by Sanger sequencing.

**RNA isolation and cDNA preparation**. Total RNA was isolated from HeLa cells using the Chomczynski method (Chomczynski and Sacchi, 2006). Briefly, cells were lysed with the GTC buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% (w/v) sarkosyl and 0.1 M 2-mercaptoethanol) and total RNA was isolated using subsequent acid phenol-chloroform extraction and isopropanol precipitation. RNA quality was assessed using standard agarose gel electrophoresis under denaturing conditions. RNA quantity was measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). For cDNA preparation, 1 μg of the obtained total RNA was incubated with M-MLV reverse-transcriptase (Promega, USA), $oligo(dT)_{15}$ primer (Genomed, Poland), and processed according to the manufacturer's instructions.

**Transfection and reporter gene assays**. HeLa cells were seeded in 24-well plates at density of $1 \times 10^5$ cells/well. 24-h later, the cells were transfected with plasmid DNA using the Lipofectamine 2000 reagent (Invitrogen, USA), according to the manufacturer's instructions. The total amount of 0.8 μg of plasmid DNA per well was used, including 0.7 μg of the luciferase-coding reporter vector and 25 ng of the ZC3H12A expression vector or the empty control plasmid. The quantity of DNA/well was normalized using an empty pcDNA3.1/mycHisA vector (mock DNA; Invitrogen, USA). 24-hs post-transfection, the cells were lysed and firefly and *Renilla* luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega, USA), according to the manufacturer's instructions. For transfection efficiency normalization, the *Renilla* luciferase encoded on the same plasmid as the firefly luciferase (pmirGLO) was used. Luciferase data are presented as relative luciferase activity (firefly/*Renilla*) and in some cases for clearer presentation normalized to 1.0 for the control sample. Experiments were conducted in technical duplicates and represent at least two biological replicates.

## RESULTS AND DISCUSSION

To assess the influence of ZC3H12A on its own mRNA in the human system, we cotransfected HeLa cells with the pLuc-*ZC3H12A*-3'UTR construct (coding for the firefly luciferase transcript fused with the human *ZC3H12A* 3'UTR) and human ZC3H12A expression plasmid. We observed a decrease in luciferase activity when ZC3H12A was expressed (Fig. 1). The regulation seems to be specific, as no effect of ZC3H12A over-expression is observed when luciferase without additional 3'UTR was used (pmirGLO; Fig. 1). Thus, our data indicate that ZC3H12A regulates its own mRNA in the human system. Further, we tested whether the influence of ZC3H12A on its own transcript depends on the presence of a wild-type NYN domain. Over-expression of mutant, RNase-inactive form of ZC3H12A, ZC3H12A(D141N), does not lead to a decrease in lu-

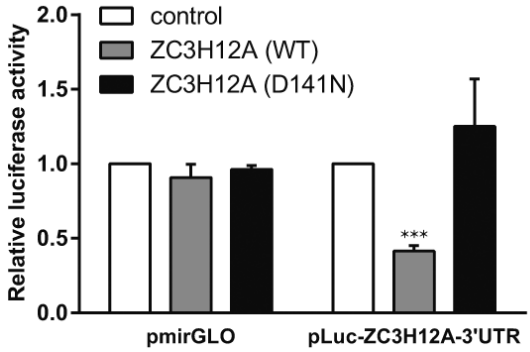


**Figure 1. ZC3H12A downregulates transcripts containing ZC3H12A 3'UTR.**
HeLa cells were co-transfected with pmirGLO or pLucZC3H12A-3'UTR and an empty vector (control) or expression vectors coding for wild-type (WT) or RNase-inactive variant (D141N) of the ZC3H12A expression vector. The plot shows mean results from two independent experiments ± S.D., ***$p<0.001$. Two-way ANOVA with Bonferroni *post-hoc* test was used.

ciferase activity, although it was expressed at the same level as the wild type ZC3H12A (not shown), clearly indicating that the NYN domain is crucial for this activity (Fig. 1). Similarly to wild-type variant, no effect of ZC3H12A(D141N) over-expression on the empty pmirGLO was observed.

To specify exactly which fragment of the human *ZC3H12A* 3'UTR is responsible for the observed ZC3H12A-dependent regulation, we aligned *ZC3H12A* 3'UTR fragments from various mammalian species using ClustalW2 (Larkin *et al.*, 2007) and visualized their secondary structures using the mfold web server (Zuker, 2003) (Fig. 2). The analysis of the sequences and secondary structures reveals that the stem-loop identified by Iwasaki and others (2011) as the one responsible for ZC3H12A-dependent regulation in the murine model is also present in human *ZC3H12A* 3'UTR (SL1). However, the 3'UTRs differ slightly as an additional stem-loop structure (SL2), similar to SL1, is present only in the human 3'UTR and not in the murine one (Fig. 2B). The similarity between both stem-loops (SL1 and SL2) in human *ZC3H12A* 3'UTR and their close proximity, prompted us to verify whether the ZC3H12A-dependent regulation of *ZC3H12A* mRNA relies solely on SL1, as was shown in the murine model, or maybe also on the additional SL2, which is present only in the 3'UTR of human origin.

In order to identify the role of each stem-loop within the *ZC3H12A* 3'UTR in our system, we prepared reporter constructs lacking each stem-loop (ΔSL1 or ΔSL2) or lacking both of them (ΔSL1+ΔSL2), and assessed whether they are also regulated in a ZC3H12Adepenedent manner (Fig. 3). Surprisingly, removal of neither SL1 nor SL2 abrogated the decrease in luciferase activity observed upon ZC3H12A over-expression. The effect was also unchanged when both of the stem-loops were deleted (ΔSL1+ΔSL2; Fig. 3C). Although all of the constructs we used did not stop to respond to ZC3H12A over-expression, the SL1 is of great importance, as removal of this stem-loop in both single (ΔSL1) and double (ΔSL1+ΔSL2) mutant constructs increased the luciferase activity approximately 5-times. The SL2 did not show such an effect, as its removal did not change the luciferase activity (Fig. 3D). This indicates that SL1, conserved between the analyzed species, is important in *ZC3H12A* mRNA post-transcriptional regulation, but seems to be dispensable for ZC3H12A-protein dependent regulation in the human system.

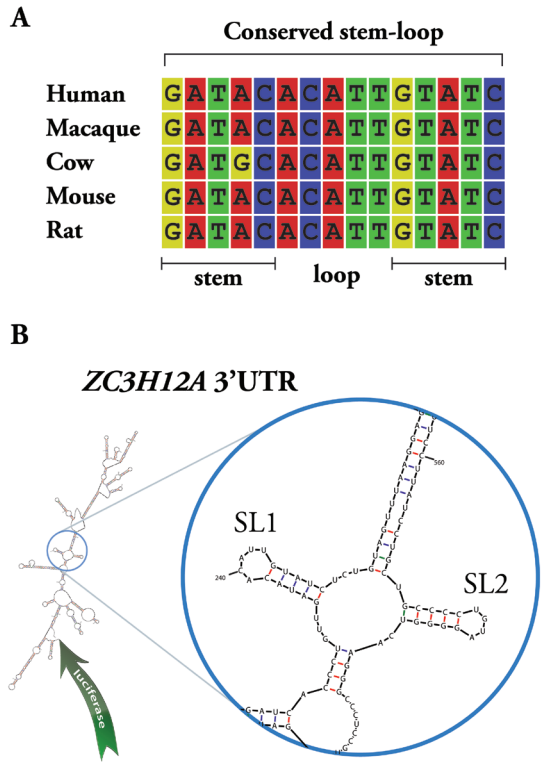


**Figure 2. Folding of human ZC3H12A 3'UTR.**
**(A)** Alignment of *ZC3H12A* 3'UTR conserved element of five various mammalian species revealing the presence of a structurally conserved stem-loop. The alignment was prepared using ClustalW2 (Larkin *et al.*, 2007) with default settings and visualized in JalView2 (Waterhouse *et al.*, 2009). Graphical adjustments were done in CLC Sequence Viewer 6 (CLC bio A/S, Denmark) and Adobe Illustrator CS4 (Adobe Systems, USA). **(B)** Secondary structure of human *ZC3H12A* 3'UTR fragment cloned into the reporter vector adjacent to the luciferase CDS folded using the mfold web server (Zuker, 2003). Enlarged are two stem-loop, hairpin-like structures. SL1 is corresponding to the one present in the murine *Zc3h12a* 3'UTR, shown by Iwasaki *et al.* (2011) to be responsible for the destabilization of the *Zc3h12a* mRNA by the Zc3h12a protein. SL2 is a second stemloop structure present only in the human, and not the murine, *ZC3H12A* 3'UTR.

As removal of SL1 or SL2 did not result in irresponsiveness of *ZC3H12A* 3'UTR to ZC3H12A over-expression, we decided to prepare a series of truncations of the 3'UTR from the 3' side and test which fragment will no longer be regulated in a ZC3H12A-dependent manner (Fig. 4A). The obtained results clearly indicate that removal of the fragment between 220257 nts within the 3'UTR, greatly diminishes the responsiveness to ZC3H12A over-expression. Surprisingly, this fragment contains the SL1 stem-loop, which when solely removed (ΔSL1) did not abrogate the 3'UTR regulation. However, both constructs, D3 and ΔSL1, indicate that the region containing SL1 is crucial for the regulation of *ZC3H12A* 3'UTR (Fig. 3D and Fig. 4B).

Our studies indicate that the mechanisms underlying the regulation of transcript stability by ZC3H12A still needs to be elucidated. Although the removal of the SL1 structure does not disturb the observed regulation, the truncation studies reveal that the 220–257 nts fragment containing this structure is crucial for ZC3H12A-dependent regulation in the human system. The stem-loop structure corresponding to the human SL1 was already

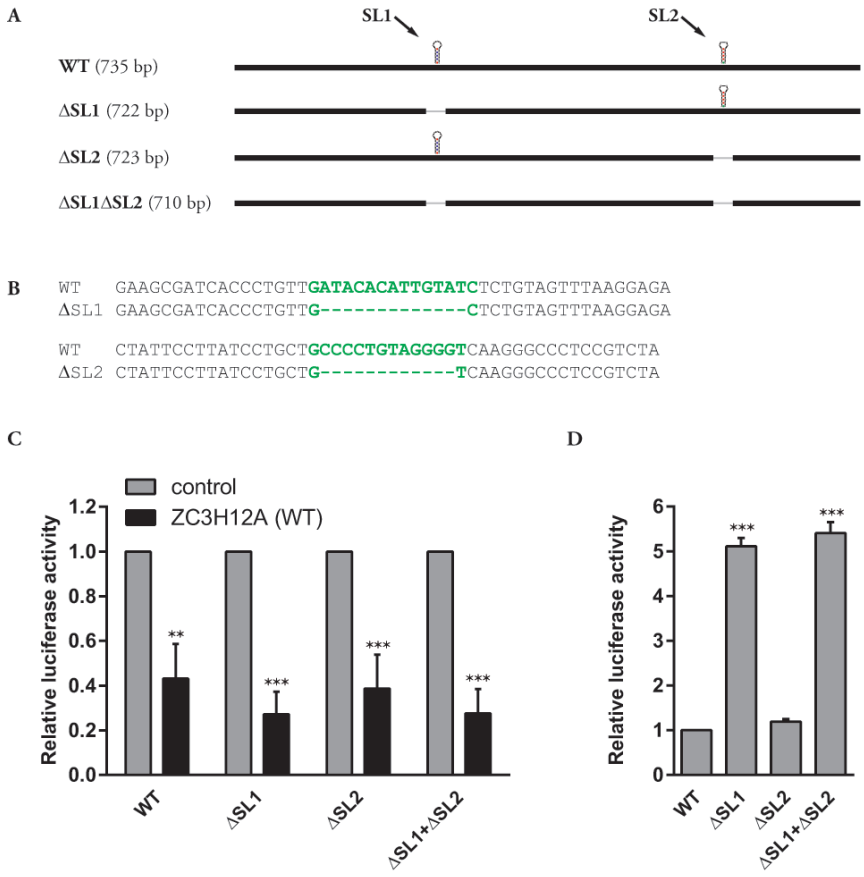


**Figure 3. ZC3H12A regulates its own transcript regardless of the absence of identified stem-loop structures.**
**(A, B)** To uncover the exact element within the *ZC3H12A* 3'UTR required for ZC3H12Adependent regulation, we used site-directed mutagenesis to remove the stem-loop structures indicated in Figure 2. The obtained constructs are schematically depicted in **(A)** along with modified sequences presented in **(B)**. **(C)** HeLa cells were co-transfected with wildtype pLucZC3H12A-3'UTR, pLuc-ZC3H12A-3'UTRΔSL1, pLuc-ZC3H12A-3'UTRΔSL2 or pLuc-ZC3H12A-3'UTRΔSL2+ΔSL2 (constructs schematically depicted in **A**) and ZC3H12A expression vector or empty control vector. 24 hrs after transfection, the firefly and *Renilla* luciferase activity in cell lysates was measured. Luciferase data are presented as relative luciferase activity (firefly/*Renilla*) and for clearer presentation normalized to 1.0 for the control samples. Removal of the SL1 structure does not suppress the effect of ZC3H12A over-expression on its own transcript. Removal of the SL2, absent in the murine transcript, also does not impede the observed effect. Nor does the removal of both structures. **(D)** However, the SL1 is clearly important in the post-transcriptional transcript regulation as is visible when non-normalized relative luciferase values for control samples from (**C**) are plotted. Plots show mean results from three independent experiments ± S.D., $^{**}p<0.01$, $^{***}p<0.001$. Two-way ANOVA with Bonferroni *post-hoc* test **(C)** or two-tailed *t*-test **(D)** was used.

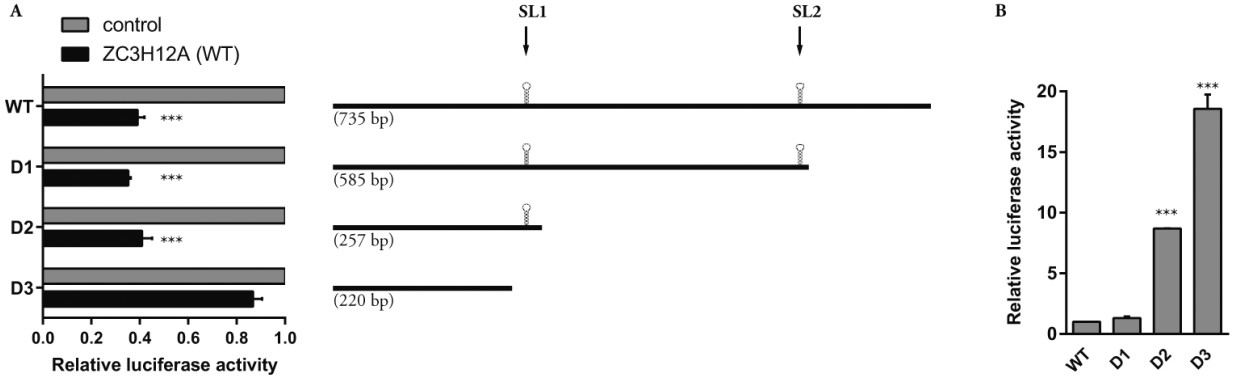


**Figure 4. Fragment between nucleotides 220–257 within the 3'UTR of ZC3H12A mRNA is crucial for its regulation by the ZC3H12A protein.**
**(A)** Truncation studies using naturally occurring restriction sites within the human *ZC3H12A* 3'UTR reveal that fragment spanning nucleotides 220 and 257 is indispensable for ZC3H12A-dependent regulation. HeLa cells were co-transfected with wild-type pLucZC3H12A-3'UTR, pLuc-ZC3H12A-3'UTRD1, pLuc-ZC3H12A-3'UTRD2 or pLuc-ZC3H12A-3'UTRD3 (constructs schematically depicted next to the graph) and ZC3H12A expression vector or empty control vector, and 24 hrs later firefly and *Renilla* luciferase activity in cell lysates were measured. Luciferase data are presented as relative luciferase activity (firefly/*Renilla*) and normalized to 1.0 for the control samples **(B)** Comparison of the non-normalized relative luciferase values for control samples of the truncation variants presented in (**A**) reveals that the fragment between nucleotides 220–257 within the 3'UTR of *ZC3H12A* transcript is crucial for the post-transcriptional mRNA regulation. Plots show mean results from three independent experiments ± S.D., $^{***}p<0.001$. Two-way ANOVA with Bonferroni *post-hoc* test **(A)** or two-tailed *t*-test **(B)** was used.

shown to be important for ZC3H12A-dependent regulation in the murine system. However, Iwasaki *et al.* (2011) used a different approach for studying the influence of murine Zc3h12a on self-mRNA. Instead of deletion of the structure, they created substitution mutations within the stem of the structure which, supposedly by inhibition of the stem-loop formation, impeded the response to ZC3H12A over-expression. Whether this approach would result in a loss of response of *ZC3H12A* 3'UTR to ZC3H12A needs more detailed studies. However, our data (data not shown) indicate that also in the murine *Zc3h12a* 3'UTR the deletion of the stem-loop does not abrogate the response to human ZC3H12A over-expression. The answer why there are discrepancies between results presented in this study and the ones presented by Iwasaki *et al.* (2011) is as yet unknown. One of the possibilities is that ZC3H12A binding to substrates does not solely rely on the stem-loop but also on some other sequences/structures in its vicinity. These may not change in deletion constructs but may be disrupted by introducing substitutions which can potentially cause the RNA to fold differently. The other possibility is that the substrate specificity of human and murine ZC3H12A proteins is not exactly the same in terms of the stem-loop structure. What is more, the RNA *in silico* folding does not represent the "in-cell" conditions where the presence of RNA binding proteins, divalent ions etc. may influence the folding and the final RNA structure. Nonetheless, this study shows that defining a transcript instability elements is cumbersome and extreme care must be taken during experimental design and setup.

The authors declare no competing interests.


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M.W. and J.K. conceived the project, designed and performed the experiments, and analyzed the data. The manuscript was written by M.W., J.K. and A.K. All authors read and approved the manuscript.

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