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Destabilisation of Argonaute 2 generates a truncated protein: halfAgo2

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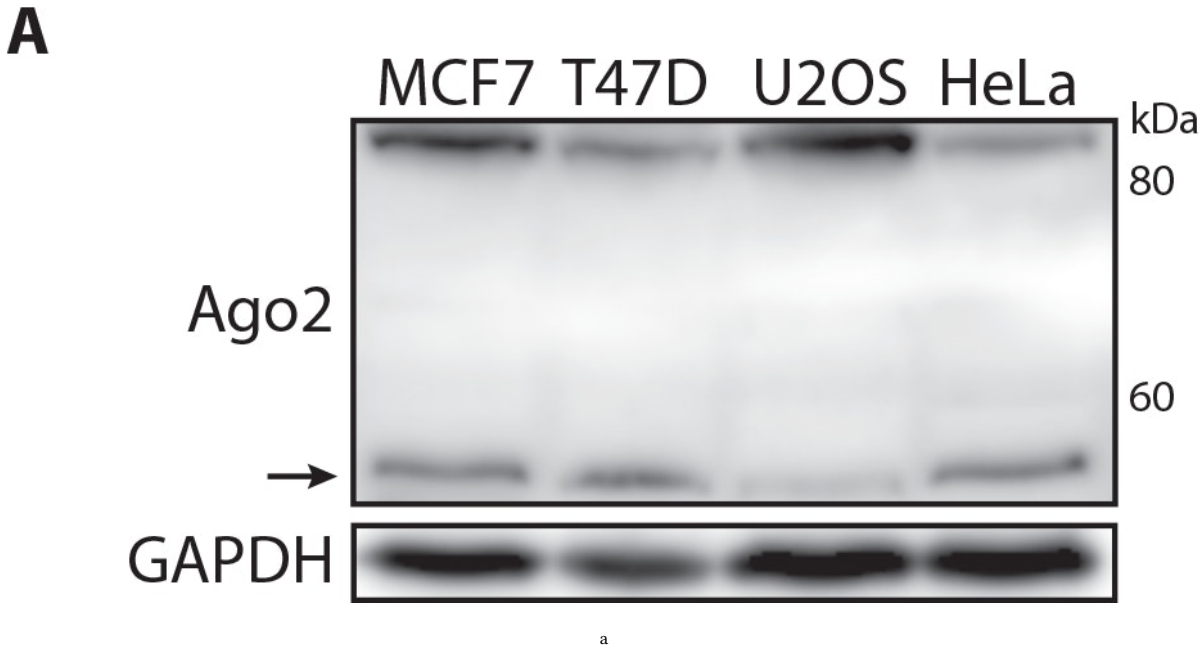
Abstract
The Argonaute 2 (Ago2) protein is an essential effector protein in miRNA-mediated mechanisms that regulate gene expression. Ago2 directly binds to the miRNA, forming the RISC. RISC function is critical to controlling key biological processes and when dysregulated can result in disease pathogenesis. Understanding Ago2 protein stability and turnover will further our understanding in how RISC function is regulated. In human cells, we discovered a previously unidentified ~55 kDa protein that is a truncated form of Ago2, that is formed from proteolytic cleavage of the full length Ago2 protein. Further experiments are needed to determine (i) the detailed mechanism that forms halfAgo2 (ii) the cellular or environmental triggers or stresses that initiate halfAgo2 production and (iii) if halfAgo2 has a potentially new role in gene regulation.

Introduction
miRNAs are endogenous small molecules that are essential regulators of human development. miRNAs function by targeting the post-transcriptional stages of gene expression, via several distinct mechanisms [1]. miRNA regulatory function depends on the miRNA directly binding to an Argonaute (Ago) protein, forming the RNA Induced Silencing Complex (RISC). In this complex the miRNA acts a guide, by binding to a complementary site within mRNA and bringing the RISC, and its associated regulatory proteins, to the target [2] [3].
RISC regulates key biological processes, therefore any disruption to RISC function can have severe consequences, and misregulation of RISC is implicated in the development of disease [4]. It is therefore important that the stability of RISC and accordingly its components, miRNA and Ago, are controlled. There are several known mechanisms that mediate miRNA stability and turnover, including homeostatic and feedback mechanisms that coordinate miRNA levels with Ago levels [5] [6] [7] [8]. However, we have only limited understanding into mechanisms that regulate the turnover of Ago proteins and the RISC complex.
There is emerging evidence that Ago function and stability is mediated by a variety of post-translational modifications of the protein, which occurs as a consequence of complex signalling pathways [9] [10] [11] [12] [13]. These modifications can alter protein function, stability, and localisation. Depending on the modification, these changes can be permanent or reversible. Therefore Ago levels are potentially highly dynamic and are responsive to internal and external stimuli.
While the 4 human Ago proteins (1–4) display some functional redundancy, Ago2 is the most abundant in commonly used human cell lines [14] and the most studied in miRNA regulation.

Objective
While analysing the protein expression of full-length (FL), endogenous, Ago2 (~85 kDa) in HeLa cell lysate, we observed that an Ago2-specific monoclonal antibody targeted to the N-terminus of the protein [15], also bound to a previously unidentified protein of approximately 55 kDa. The objective of this study was to investigate this 55 kDa protein, and to test our hypothesis that this protein is a truncated form of FL human Ago2.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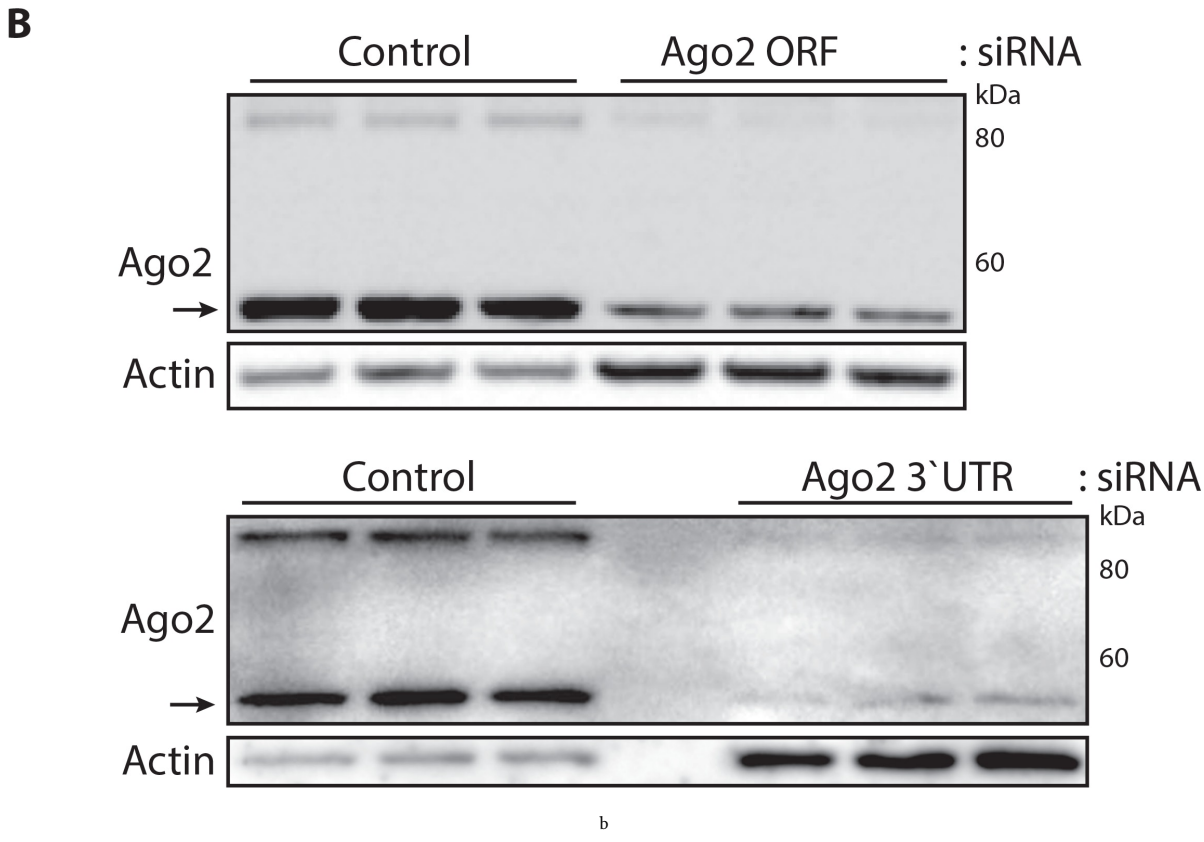
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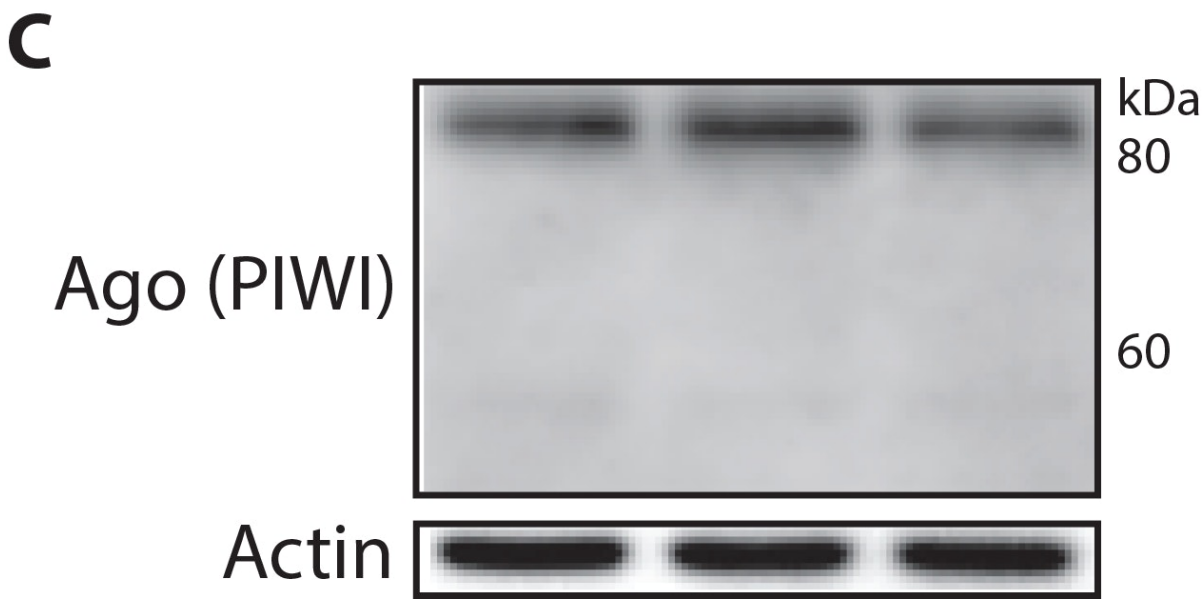
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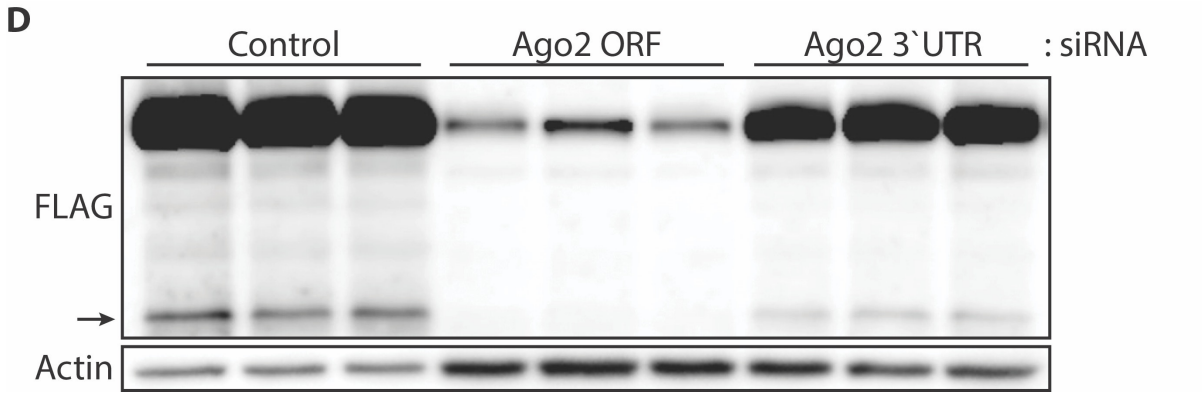
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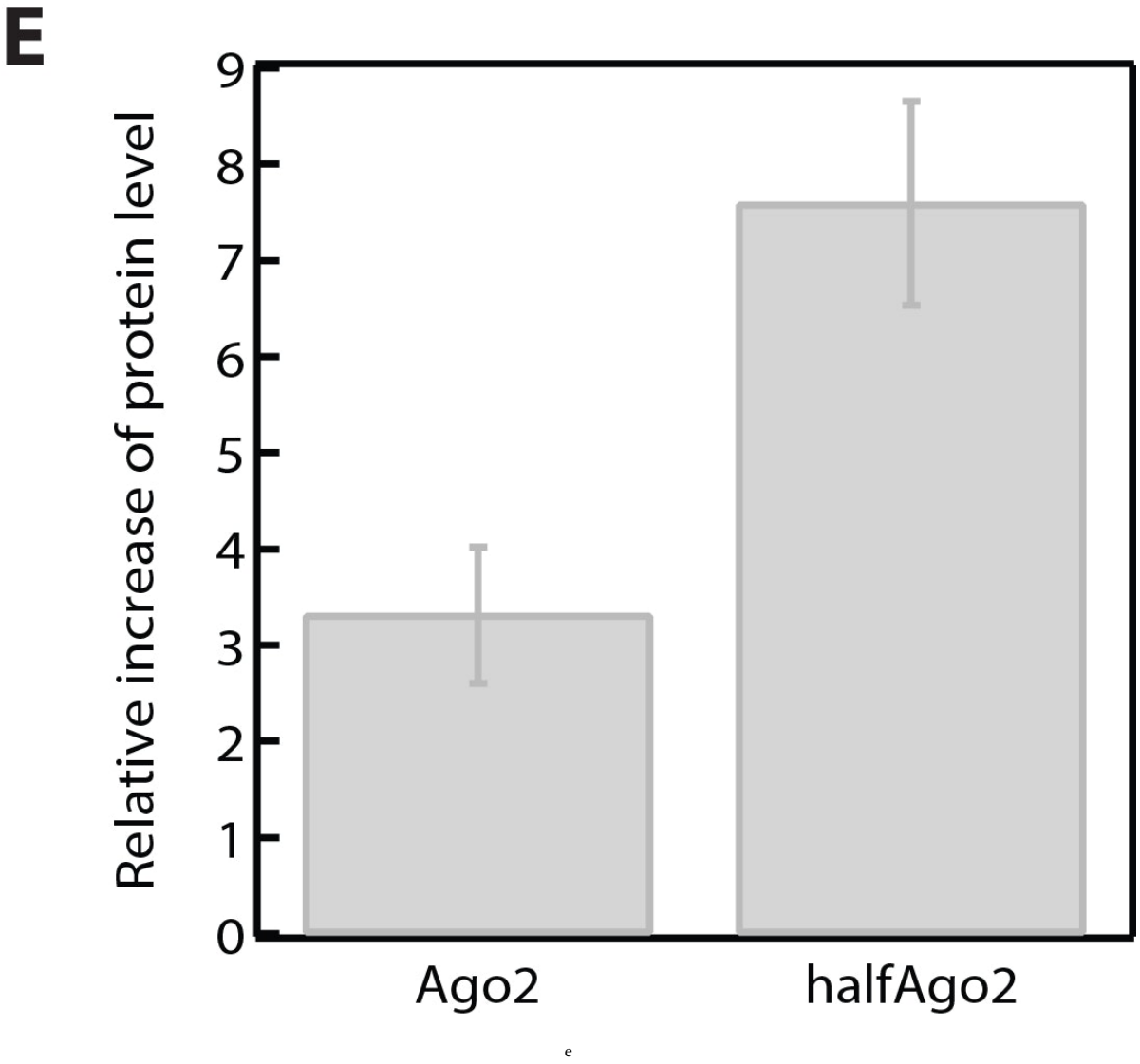

e

Figure Legend

Figure 1. Endogenous and recombinant Ago2 can form a stable, truncated protein: halfAgo2.

(A) Full length (FL) Ago2 (~85 kDa) and halfAgo2 (~55 kda; indicated by the arrow) are expressed in a variety of immortalized human cell lines.

(B) HeLa cells treated with siRNA that specifically targets the Ago2 open reading frame/coding sequence (Ago2 ORF) decreases expression of both FL Ago2 and halfAgo2. Using an alternate Ago2-specific siRNA that binds to the 3'UTR of the FL gene (Ago2 3'UTR) both FL Ago2 and halfAgo2 protein expression is similarly decreased when compared to control.

(C) halfAgo2 does not contain the PIWI domain. The expression of Ago proteins in HeLa lysate was tested with an antibody that specifically binds to the C-terminal PIWI domain. Only FL Ago proteins were visible.

(D) Transiently expressed N-terminally tagged FLAG::Ago2 also produced a stable, truncated N-terminal Ago2 product. The expression of FLAG::halfAgo2 is inhibited when treated with an siRNA that can target this plasmid by targeting the Ago2 ORF, but is unaffected when treated with an siRNA that cannot target the plasmid (siRNA targeting Ago2 3'UTR).

(E) FL Ago2 and halfAgo2 protein levels accumulate when the proteasome is inhibited by MG132 treatment in HeLa cells. Expression normalised to DMSO control (n=3).

Results & Discussion

We initially observed the unidentified 55 kDa protein, predicted to be a truncated Ago2 protein which includes the N-terminus of the FL Ago2 protein, in HeLa cells. To ensure that this phenomenon was not cell line specific, we tested several human immortalised cell lines, derived from a variety of tissues (breast, bone and cervix) (Fig. 1A, S1). We detected the 55 kDa band in the majority of all samples tested, with variable expression levels between cell lines. As other proteins tested in these lysates show no signs of degradation, we can also conclude that the 55 kDa protein is not due to general degradation of the cell lysate (Fig. S1). These data suggests the production of the 55 kDa protein is a regulated process, with a potential physiological function.

To eliminate the possibility that the 55 kDa band is a non-specific band cross-reacting with the N-terminal targeting Ago2 antibody, we wished to see if the expression of the 55 kDa band was dependent on FL Ago2 protein expression. We used an siRNA that specifically targets the open reading frame (ORF) of the Ago2 transcript, which will reduce FL Ago2 protein expression level. When FL Ago2 protein expression was knocked down, we observed that the 55 kDa protein expression levels also decreased when compared to the control samples (Fig. 1B). To confirm this result we repeated the Ago2 knockdown with an independent siRNA that targeted a different segment of the FL Ago2 transcript, the 3'UTR. This experiment produced similar results, with reduced protein expression of both the FL Ago2 and the 55 kDa protein, suggesting this effect is not due to off-target effects of the siRNAs used. These data indicate that FL Ago2 RNA production is required for the 55 kDa protein to be present. Furthermore, there are no identified alternatively spliced Ago2 isoforms that include the 3'UTR sequence of the FL Ago2 transcript. This suggests the truncated protein is not a product of alternative splicing.

Due to the size of this protein (~55 kDa) and that it contains the N-terminal domain of Ago2, we predicted that this truncated Ago2 does not contain the PIWI domain (required for target cleavage and binding the 5' end of mature miRNAs) present in the FL Ago2, as this domain is located near the C-terminus of the protein. To investigate this we used an antibody that targets the PIWI domain of human Ago proteins (Fig. 1C). This antibody only recognises FL Ago proteins, confirming that the 55 kDa truncated protein does not contain the PIWI domain, and therefore potentially functions independently of binding miRNAs. This data suggests that after FL Ago2 cleavage, the N-terminal containing polypeptide (halfAgo2) is more stable than the C-terminal containing product, suggesting halfAgo2 has an independent function. The N-terminal domain of Ago2 has an essential role in separating the strands of miRNA duplexes [16], while the adjacent PAZ domain can bind to the 3' end of RNA molecules [17]. These functional properties indicate that halfAgo2 could play a role in independent gene regulatory mechanisms.

To further investigate the origin of the 55 kDa protein, we transiently expressed a FLAG-tagged FL Ago2 gene expression plasmid into HeLa cells. The FLAG tag is situated at the N-terminus of the protein, and therefore the tag will be present in a truncated product derived from the exogenous FL Ago2 that includes the N-terminal domain. This plasmid contains the endogenous FL Ago2 ORF, but contains plasmid specific sequences upstream and downstream of the Ago2 coding sequence. When examining the lysate for FLAG-tagged proteins we also observed a FLAG-tagged truncated 55 kDa protein (Fig. 1D). When samples expressing the FLAG-Ago2 plasmid were also transfected with an siRNA that targets the Ago2 ORF, and could therefore target the FLAG-Ago2 transcript, expression of both the FLAG-tagged FL Ago2 and the smaller 55kDa protein was reduced. However, neither of their expression was altered when treated with the Ago2 3'UTR targeting siRNA, which exclusively targets endogenous Ago2 expression as it is incapable of binding to the FLAG-Ago2 transcript (Fig. 1D).

As the expression of the exogenous, FLAG-tagged Ago2 is derived from a cDNA sequence of the FL Ago2 transcript that has been inserted into an expression vector, the sequence does not contain introns. Therefore, FLAG-Ago2 expression is independent of splicing mechanisms. As FLAG-halfAgo2 expression is observed, this further suggests that this protein is not the result of alternative splicing, but may instead be formed from proteolytic cleavage of the FL Ago2 protein. We can conclude from this data that the 55 kDa Ago2 band is most likely derived from a post-translational mechanism that targets

the FL Ago2 protein. We will refer to this Ago2 protein as halfAgo2.
We investigated if, like FL Ago2, degradation of the 55 kDa Ago2 protein is mediated by the 26S proteasome [18]. We treated HeLa cells with the proteasome inhibitor MG132 and found that both FL Ago2 and the 55 kDa Ago2 protein levels accumulated upon proteasome inhibition, indicating that the turnover of both these proteins is proteasome dependent (Fig. 1E). However halfAgo2 accumulation was greater than FL Ago2, signifying that halfAgo2 is a less stable protein that FL Ago2. We confirmed that accumulation of Ago2 protein was via a post-transcriptional mechanism as Ago2 transcript levels remain unchanged in MG132 treated samples (Fig. S1).
There is precedent in the literature that alternate forms of key proteins of the small RNA pathway have distinct roles to the full length form. Dicer, which is responsible for key steps of small RNA biogenesis, is proteolytically cleaved to form a highly-expressed, stable fragment (sDCR-1) that functions independently of full length Dicer, but has distinct regulatory functions in both the RNAi and miRNA pathways [19]. Our data suggests that halfAgo2 is a stable truncated form of Ago2, and further studies are required to understand the function of halfAgo2 and the implications this has on Ago2 protein stability and RISC function.
Based on the size of the halfAgo2 protein, we predict halfAgo2 contains a functional PAZ domain, and therefore can potentially still bind small RNA molecules. This creates the possibility that halfAgo2 and FL Ago2 may be in competition for co-factor association and/or small RNA binding. Alternatively, halfAgo2 may bind to a unique set of RNA molecules forming a distinct regulatory complex. This possibility would expand the range of traditional Ago2 targets currently being investigated and may help to explain the number of predicted Ago2 targets, as identified by high-throughput immunoprecipitation and RNA-protein crosslinking experiments, which have no associated miRNA binding sites [20].
As factors that influence Ago2 and RISC stability are largely unknown, discovery of the novel mechanism behind halfAgo2 production and what regulates this mechanism, is essential to fully understand the role of Ago2 stability in regulation of gene expression in both healthy tissues. Additionally, this will provide critical insights into how the Ago2 and RISC complex responds to stress, and how they become dysregulated in disease pathogenesis.
As there is increasing evidence that Ago2 function, stability and turnover is regulated by post-translation modifications of the protein which are the result of a complex network of signalling pathways, it would be interesting to investigate if halfAgo2 production is a consequence of such a modification. If so, it suggests halfAgo2 formation is regulated in response to specific cellular or environmental triggers or stresses, as a mechanism to control RISC function in these particular situations.
halfAgo2 is a stable protein product which potentially has its own unique regulatory role. halfAgo2 may play a role in RISC formation or activity, or alternatively, is capable of functioning independently from the miRNA pathway. Elucidating the production and function of halfAgo2 will further our understanding of Ago2 stability and therefore miRNA expression and function. This can impact the development of future therapeutics which act to overexpress or inhibit miRNA function.

Conclusions

Our results indicate that unloaded human Ago2 can form a stable protein product, of approximately 55 kDa, called halfAgo2. We propose that halfAgo2 is produced from the post-transcriptional proteolytic cleavage of Ago2. We predict that halfAgo2 is formed as a consequence of a mechanism which controls Ago2 stability and turnover.

Limitations

Due to the nature of this study and the current tools available we are unable to isolate endogenous halfAgo2. This is because Ago family members are highly conserved. Therefore, the development of Ago2 specific antibodies depends on a unique N-terminal domain only present in Ago2. However, the N-terminal domain is also present in halfAgo2. It is therefore impossible to separate full length Ago2 from halfAgo2 via immunopre-

cipitation, or other antibody based methods. It will therefore be necessary to accurately map what is encoded within the halfAgo2 protein, to better understand its potential functions and so halfAgo2 constructs can be generated. Alternatively, halfAgo2 can be purified via size exclusion chromatography.

We propose that halfAgo2 is formed as a consequence of a proteolytic cleavage mechanism which controls Ago2 stability and turnover. There is mounting evidence that Ago2 stability is regulated by post-translational modifications (PTMs) of the protein. As many sites of PTMs have been predicted within the FL Ago2 protein, only a few have known functional consequences [9]. Therefore it would be interesting to investigate if the formation of halfAgo2 was precipitated by such a modification. And if so, identify the signalling pathways involved in triggering this mechanism, and the specific stresses or environmental triggers required to prompt such a signalling cascade, as there is evidence that PTMs can control RISC activity and miRNA regulation efficiency in a signal dependent manner [11].

Additional Information

Methods and Supplementary Material

Please see https://sciencematters.io/articles/201811000001.

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Ethics Statement

Not Applicable.

Citations

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