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10-million-years AGO

Argonaute on chromatin in yeast and human, a conserved mode of action?

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Whereas in yeast the function and mode of action of nuclear RNAi are well documented, mammalian nuclear RNAi is a matter of debates. Several papers support a role for nuclear Argonaute in alternative splicing. However, the molecular mechanism remains elusive. Here, we discuss the human nuclear RNAi mechanism in light of what is known of the yeast process.

Keywords: nuclear RNAi, splicing, mammals, AGO, AS lincRNA

Abbreviations: AGO, Argonaute; AS lincRNA, antisense long non-coding intronic RNA; CD44, cluster of differentiation 44; hnRNPs, heterogeneous nuclear ribonucleoproteins; KMT, lysine methyltransferase; PMA, phorbol-12-myristate-13-acetate; RNAi, RNA interference; PTGS, posttranscriptional gene silencing; RITS, RNA-induced initiation of transcriptional gene silencing; RNAP II, RNA polymerase II; snRNPs, small nuclear ribonucleoproteins; TGS, transcriptional gene silencing

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RNA interference in the cytoplasm (or posttranscriptional gene silencing, PTGS) is a widely used natural mechanism for posttranscriptional gene regulation.[1] It involves a ribonucleoproteic complex termed RISC (RNA-induced silencing complex) in which the main effector molecule is the Argonaute (AGO) protein.[2] The RNA component is a short RNA sequence (in the range of 20 nucleotides) processed from longer double-stranded sequences by an endoRNase termed DICER.[3] The small RNA guides the complex toward long mRNA targets containing sequences that are fully or partly complementary to the small RNA. Depending on the degree of complementarity, AGO cleaves the mRNA target or else induces translation blockade and/or accelerated degradation of the target mRNA.[4]

Cytoplasmic mRNA processing is not the only mode of gene regulation used by RNA interference, and a nuclear component termed transcriptional gene silencing (TGS), also operates. TGS has been documented in several species. In plants, TGS involves a specific nuclear complex with small RNAs of specific size and processed by a dedicated DICER, and a specific member of the AGO protein family.[5] It induces target DNA methylation and silencing via a mechanism involving an RNA-dependent RNA polymerase.[6] Similarly, in the fission yeast *Schizosaccharomyces pombe*, chromatin is modified via a dedicated nuclear AGO-containing complex termed RITS (for RNA-induced initiation of transcriptional gene silencing). The mechanism involves a dedicated member of the yeast DICER family and an RNA-dependent RNA polymerase. In the case of the yeast, however, it is not DNA but histone H3 which is methylated.[7] This mechanism is used to silence repeated sequences and transposons during S phase.[8] Nuclear RNA interference has also been described in *C. elegans*.[9]

In mammals, the existence of an RNA-dependent RNA polymerase remains to be clearly demonstrated even though in vitro RNA polymerase II (RNAP II) itself seems to have RNA-dependent RNA polymerase activity.[10] Thus, as the RNA-dependent RNA polymerase is a key component of TGS in plants and in *S. pombe,* the mechanism of TGS in mammals has to be different from the one operating in these organisms.

Both in flies and mammals, a small RNA-based mechanism operates in germ cells to silence transposons and/or repeated sequences. This mechanism was first shown in Drosophila germ cells, where the PIWI proteins, a dedicated subgroup of AGO proteins bound to specific

small RNAs (piRNAs), participate in transposon and repeated-sequence silencing by inducing DNA methylation.[11] The biosynthesis of piRNAs is independent of DICER, and results from a primary mechanism followed by an amplifying mechanism, the "Ping-Pong" cycle.[12] A similar Ping-Pong mechanism is operating in mouse and human germ cells.[13]

In somatic cells, piRNAs, PIWI proteins and the Ping-Pong mechanism are generally absent, and the existence of TGS in these cells is still debated. A TGS-like effect of exogenous siRNAs in human somatic cells has been reported by several groups.[14-16] In these papers, ectopic short double-stranded RNAs, homologous to gene promoter sequences, suppressed promoter activity, but this mechanism remains to be demonstrated at the endogenous level. It is clear, however, that a proportion of AGO proteins, at least of AGO1 and AGO2, is located in the nucleus of human cells: AGO proteins shuttle between the nucleus and the cytoplasm, a process which seems to be required for micro RNA-dependent gene regulation.[17] The physiological function of nuclear AGO in mammalian somatic cells, however, is not completely clarified.

A first cue was brought by Allo et al. in the Kornblihtt group, who showed that exogenous intronic siRNAs influence the alternative splicing of the closest upstream exon in the *Fibronectin* gene.[18] These authors also showed that the mechanism requires two members of the mammalian AGO protein family, AGO1 and AGO2, and is accompanied by local deposition of heterochromatin histone marks (H3K27me3 and H3K9me2) close to the siRNA target site. Moreover, siRNAs are able to decrease RNAP II velocity on a reporter minigene. This data supports a model in which the active strand of the siRNA guides AGO to the nascent mRNA and induces chromatin condensation, which would reduce the speed of RNAP II, thereby facilitating the inclusion of alternative exons.[19] In a similar mechanism, transcription elongation was blocked by siRNAs in C. *elegans*.[20] Allo et al. also demonstrated that AGO1 or DICER depletion impacts on the alternative splicing of cancer-related genes.[18]

We have addressed the question of nuclear AGO function in human somatic cells by identifying chromatin-associated AGO protein partners, using biochemical purification by the TAP-TAG procedure. We found that AGO1 and AGO2 interact with each other and are associated with chromatin proteins such as HP1γ and the histone methyltransferase (KMT) Suv39H1. More surprisingly, the AGO complexes also contained several splicing proteins, and, in particular components of the U2 and U5 snRNP complexes, hnRNPs and SR proteins,[21] suggesting a role of this complex in alternative splicing. This hypothesis was supported by AGO loss-of-function assays showing that AGO1 and AGO2 were both required for proper splicing of a wide variety of genes, and for PMA (phorbol-12-myristate-13-acetate)-induced alternative (variant) exon integration in CD44 mRNA. Consistent with this data, depletion of AGO1 or AGO2 also reduced the local deposition of histone marks (H3K9 trimethylation) accompanying variant exon integration, as well as HP1γ, U5 snRNPs and RNAP II accumulation at variant exons. ChIP assays demonstrated the physical presence of AGO1 and AGO2 proteins throughout the *CD44* locus, with a peak at a position corresponding to the peaks of PMA-induced H3K9 trimethylation and RNAP II accumulation. Finally, CD44 alternative splicing was also dependent on DICER, suggesting the involvement of small RNAs. Taken together, these results support the Kornblihtt's model in which AGO protein binds to the nascent mRNA through an antisense small RNA targeting specific positions, and induces the deposition of histone marks and the slow down of RNAP II.

The mechanism through which AGO influences histone mark deposition, which is an essential issue, still remains elusive. In the yeast *S. pombe*, which has been the best-studied model so far, it appears that AGO helps recruiting the KMT Clr4 in a completely indirect manner.[8] The Martienssen lab indeed showed that histone mark deposition at heterochromatic loci occurs only during S-phase, and that the histone methyl-transferase is not recruited by AGO, but rather by the leading strand DNA polymerase. AGO facilitates histone methylation by helping resolving collision conflicts between DNA polymerase and the RNA polymerases that transcribe the loci during S-phase. In this model, AGO induces the release of RNAP II, thereby allowing replication to resume, which is accompanied by histone methylation. In the absence of AGO, DNA polymerase is stalled. The conflict is resolved via homologous recombination in the absence of the KMT, and thus without histone modification. The precise molecular mechanism trough which AGO helps releasing RNA polymerase is unclear, but the hypothesis is that AGO, guided by antisense sequences, targets nascent transcripts. Although AGO modes of action are likely to be different in yeast and in mammals, as there is no clear evidence for a dedicated RNA dependent RNA polymerase activity in mammals, it is reasonable to propose that the basic mechanism is conserved and similar in the two organisms.

In mammalian cells, our deep sequencing of small RNAs associated to chromatin-AGO complexes revealed a majority of sense sequences with regard to transcribed genes with only a small proportion of antisense.[21] Analysis of AGO-associated *CD44* sequences confirmed the absence of antisense and the association of sense sequences located throughout the *CD44* locus. Since AGO proteins are bound to nascent CD44 mRNA,[21] CD44 small sense sequences may represent degradation products of nascent CD44 mRNA. Whatever their origin, they may guide AGO proteins toward target RNAs that would, thus, be antisense to CD44 RNA. As a matter of fact, a natural long noncoding intronic RNA (AS lincRNA) has recently been annotated in *CD44*, as well as in a number of loci in mammalian genomes.[22] This AS lincRNA remarkably coincides with the peak of AGO recruitment, histone deposition and RNAP II accumulation,[21] consistent with the idea that the AS lincRNA is involved in AGO-dependent regulation of *CD44* alternative splicing. In support of this hypothesis, a link between antisense transcription and alternative splicing has been described *in silico*.[23] How the AS lincRNA impacts on CD44 splicing is still a matter of conjecture. A possible mechanism would be that the antisense-transcribing RNAP II

molecules are colliding with the RNAP II molecules forward transcribing the *CD44* locus. The collision would slow down sense RNA transcribing units and initiate histone mark deposition. The AS lincRNA is located ideally to perform this function: being downstream of variant exons, its transcription would slow down the sense mRNA transcribing RNAP II molecules right after variant exon transcription and before the inclusion of the next constant exons in the nascent mRNA, thus preventing the rapid inclusion of constant exons and facilitating the inclusion of variant exons. This mechanism would be used to initiate the process as a primary response to PMA. The AS lincRNA transcription would thus be at the origin of the process, upstream of RNAP II slowdown. Following this initial step, histone modifications would take over for the maintenance of variant exon inclusion.

The exact step at which AGO is required and its mode of action remain elusive in this model. By analogy to the yeast system, in which AGO helps resolving a conflict between an RNA polymerase and DNA polymerase, a tempting hypothesis would be that AGO helps resolving a conflict between the two colliding RNA polymerases. In the yeast model, the chromatin modifier is brought to silenced loci by the DNA polymerase. In mammalian cells, there is no identified carrier for chromatin modifiers other than AGO itself, as KMTs, HDAC and HP1γ molecules are found in association – albeit loose – with AGO. The exact mechanism of AGO mode of action in mammals thus remains to be explored.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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