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POINT OF VIEW

A game of tag: MAPS catches up on RNA interactomes

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ABSTRACT

In the last few decades, small regulatory RNA (sRNA) molecules emerged as key regulators in every kingdom of life. Resolving the full targetome of sRNAs has however remained a challenge. To address this, we used an *in vivo* tagging MS2-affinity purification protocol coupled with RNA sequencing technology, namely MAPS, to assemble full bacterial small RNAs targetomes. The impressive potential of MAPS has been supported by a number of reports. Here, we concisely overview RNA-tagging history that preceded the development of the MAPS assay and expose the range of possible uses of this technology.

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The first description of protein tags by Munro and Pelham in 1984 had an immense impact on research.[1] Since this seminal work, biologists have been able to pull-down a specific protein of interest and characterize its interacting partners. Following the report of Munro and Pelham, many tags (e.g. Arg-Tag,[2] FLAG-Tag[3] or His-Tag[4]) were added to the list of powerful tools available to study protein biology. Over the years, it became clear that the field of RNA biology also needed a similar system. A tagging system specific for RNA would certainly help describe the function of regulatory RNAs such as micro-RNAs or bacterial small RNAs (sRNAs) that have been established as major modulators of gene expression. Hence, it is imperative to understand how these molecules fulfill their functions and to identify any necessary interacting protein or RNA partners.

Artificial RNA tags were first developed and used for the pull-down of specific RNAs. Bachler *et. al.* (1999) synthesized artificial RNA tags that bound specifically and strongly to the antibiotic streptomycin.[5] Named Strepto-Tag, this RNA is structured as a long stem-loop in which nucleotides mainly present in 2 bulges are responsible for the interaction with the antibiotic (Fig. 1A).[6] After tagging an RNA of interest, the hybrid RNA is incubated with crude cell extracts to allow *in vitro* RNA-protein complex assembly. Then, samples are passed through a sepharose column on which streptomycin has been immobilized. Elution of complexes is carried out by the addition of free streptomycin. It is noteworthy to mention that free streptomycin will bind the RNA tag and therefore be present in output samples which could interfere with subsequent analysis. In the same vein, Srisawat and Engelke (2001) also developed RNA tags (termed aptamers) with high specificity for streptavidin.[7] Similar to the Strepto-Tag, the structure of the streptavidin tag presents itself as a stem-loop with a bulge. In this case, the nucleotides in the loop, in the bulge and in the stem play a role in the binding to streptavidin[7] (Fig. 1B). Srisawat and Engelke demonstrated that the tagged RNA could be expressed *in vivo* and purified either in its native form or in complex with proteins. Elution is then carried out by addition of free biotin, which breaks the bond between the tag and the affinity column. A downside of this technique is that avidin needs to be added to cell extracts to sequester cellular biotin or biotinylated proteins and prevent them from binding the streptavidin-agarose chromatography column. Streptavidin aptamers have since then been used and improved by many research groups. For example, Leppek and Stoecklin (2013) modified one of the original aptamer by creating perfect complementarity between the basal and terminal stem, and by increasing stem length[8] (Fig. 1C). This modification increased the binding to streptavidin efficiency by 3 to 4-fold.[8]

In the early 2000s, an alternative system was described, exploiting a naturally strong RNA-protein interaction.[9] This system is founded on the property of the bacteriophage MS2 coat protein to interact with high specificity with a short RNA, namely the MS2 stem-loop aptamer (for reviews, see refs.[10,11,12]). During viral infection of *Escherichia coli* by the MS2 bacteriophage, the MS2 coat protein acts as a threshold signal, dictating its own binding to the MS2 RNA.[13,14] This interaction is crucial for induction of the assembly step of the phage's life cycle, accurate packaging of phage RNA and massive production of functional bacteriophages. In 1998, Bertrand *et al.* described a fluorescence technique exploiting the MS2 protein-MS2 RNA interaction allowing tracking of a specific mRNA in living cells.[15,16] Later, the Vogel group developed an *in vivo* method to pull-down specific sRNAs and identify their protein partners.[17,18] After production of a MS2-tagged sRNA, cell lysates are applied to an amylose resin on which a MBP-MS2 protein (Maltose Binding Protein fused with the MS2 coat protein) has been immobilized. Following elution by addition of a competitor for binding to the MBP, samples were analyzed by liquid chromatography tandem mass

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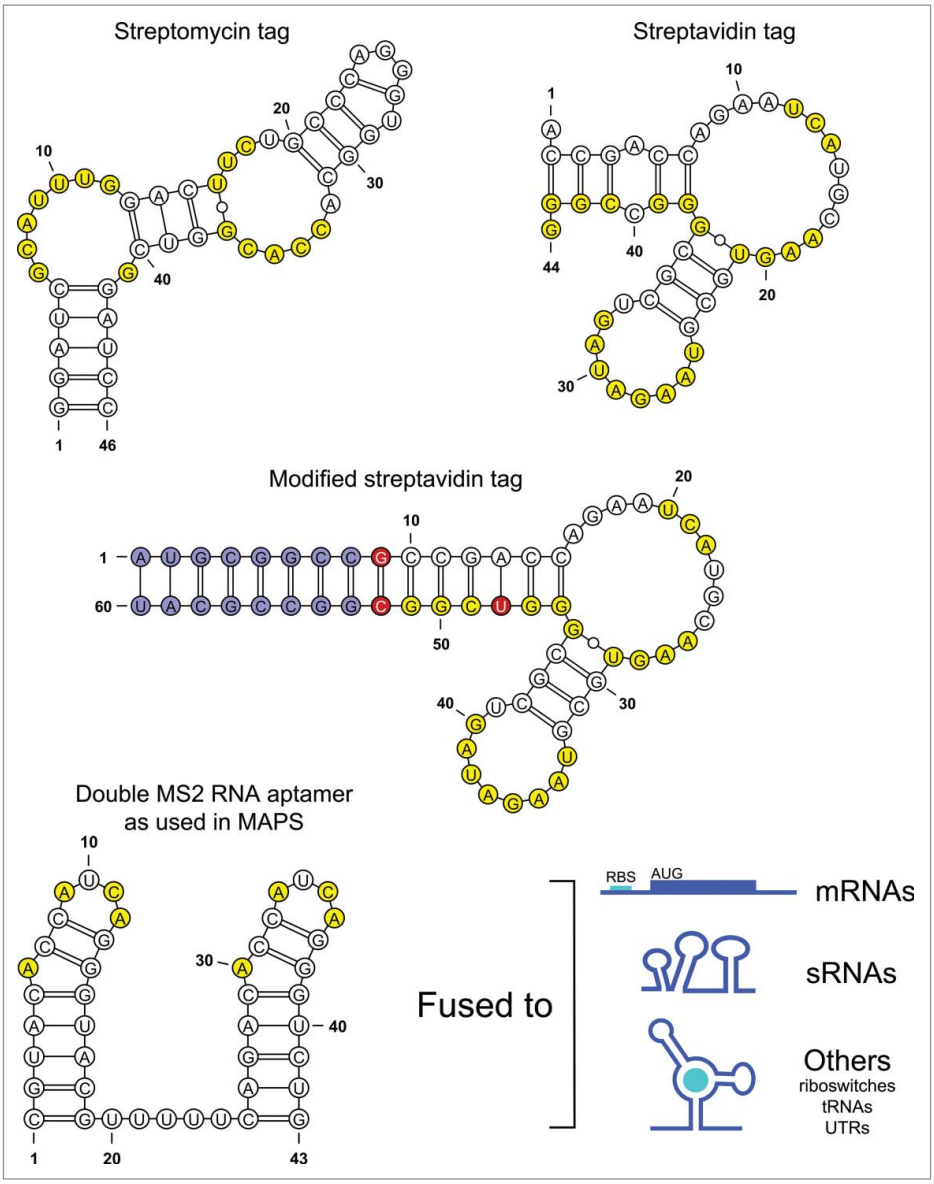

Figure 1. Overview of RNA tags. Nucleotides implicated in the interaction with specific partners are highlighted in yellow. (A) Structure of the Strepto-Tag. (B) Main region of a class I streptavidin aptamer. (C) Structure of a modified class I streptavidin aptamer. Nucleotides highlighted in red are mutations of the original sequence. Nucleotides in blue have been added to the original sequence. (D) Structure of the double MS2 aptamer as used in MAPS. The tag can ben fused to a wide range of RNA molecules to perform MAPS assays.

spectrometry (LC-MS/MS). This technique was revolutionary as it was performed *in vivo* and didn't require addition of external proteins or molecules to achieve adequate purification.[17,18] Notably, this method allowed purification and identification of a protein found in most sRNA-protein complexes, the RNA chaperone Hfq. The protein Hfq is known to interact with sRNAs to stabilize them *in vivo* and to facilitate sRNA-mRNA complexes formation.[19,20] An opposite *in vivo* high-throughput technology able to identify ribonucleic partners of specific proteins also exists and was first introduced in 2010, the RIP-seq.[21] Two major steps are required to complete a RIP-seq experiment. First, a native co-immunoprecipitation of a protein complexed to its RNA partners is performed. Then, following output collecting, samples are analyzed by RNA sequencing. Both these high-throughput sequencing technologies allow identification of RNA partners of either a specific RNA or protein, respectively.

Shortly after, our group used MS2 pull-down assays to purify specific sRNAs in order to analyze RNA-RNA interactions. We used a double MS2 RNA aptamer as a tag (Fig. 1D) and expressed the MS2-sRNA constructs *in vivo*. These experiments allowed us to demonstrate an unexpected regulatory mechanism of sRNAs. Using a MS2-mRNA (MS2-*sdhC*) construct to perform affinity purification, Desnoyers and Massé (2012) demonstrated that the interaction of Spot42 sRNA with *sdhC* mRNA serves as a recruitment platform for Hfq protein.[22] Indeed, Spot42 brings Hfq near the translational initiation region (TIR) of *sdhC* transcript, where the protein competes with the 30S ribosomal subunit to inhibit translation. It was the first example of Hfq being the primary actor in sRNA-mediated genetic regulation. In particular, Desnoyers and Massé (2012)

used MS2 pull-down assays with *sdhC* mRNA bearing different mutations at either Spot42 pairing site or Hfq binding site. Through these experiments, they were able to show *in vivo* that those mutations impaired interactions with their respective partners, confirming MS2 pull-down assay as a powerful technique to study RNA interactions *in vivo*.

Next, we wanted to expand and adapt the MS2 pull-down assays to characterize the full targetomes of bacterial sRNAs. These short functional RNAs greatly vary in structure and in mechanism of action.[23,24] To achieve genetic regulation, sRNAs often imperfectly base-pair with a set of given mRNAs,[25] resulting in various outcomes (repression of translation, mRNA degradation or translation enhancement).[23] A single sRNA can even adopt different mechanisms of action depending on the mRNA targeted.[25,26] All these characteristics prevent straightforward *in silico* target prediction. Usually, available bioinformatic programs yield a large pool of false positives and true targets may not present the most intuitive base-pairing pattern.[27] To solve this problem, we took advantage of high-throughput RNA sequencing technology (RNA sequencing, RNAseq) combined with MS2-based sRNA pull-down assays. We called this new *in vivo* tool MAPS for MS2-affinity purification coupled with RNA sequencing (schematic representation in Fig. S1 of Lalaouna *et. al.*, 2015).[28] After tagging a sRNA with the MS2 aptamer, its regulatory activity is verified on known target mRNAs by Northern blot analysis. Following this validation step, the MS2-sRNA construct is expressed *in vivo* and then purified by affinity chromatography. Samples are submitted to RNAseq and results are compared to a control experiment (untagged sRNA MAPS).

As reported in Lalaouna *et. al.* (2015),[28] we applied MAPS to RyhB and RybB, 2 well characterized sRNAs as a proof of principle. Notably, this study allowed us to identify numerous known target mRNAs of both sRNAs, independently from their mode of action (negative or positive regulation). Interestingly, we also identified new targets of RyhB and RybB. In the case of RyhB, 2 targets were identified. The first one is *erpA* mRNA, which was previously shown to be a target during the course of our study.[29] The second is the *grxD* mRNA that is negatively regulated by RyhB. In the case of the other sRNA, RybB, we were able to demonstrate that *yifE* mRNA is stabilized following expression of RybB, validating *yifE* as a positive target. Many other putative targets identified in this study are still awaiting validation. Once these putative targets are validated, we expect to get the full targetome of these sRNAs without discrimination of any targets or technical limitations.

Surprisingly, the identification of new sRNA targets was not the only valuable information revealed by MAPS. In fact, MAPS datasets allowed us to identify a new role for bacterial tRNA-derived RNA fragments (tRFs). Functional tRFs have been reported in the literature and have been gaining interest in the last few years as they seem to be implicated in the regulation of various metabolisms (for reviews, see refs.[30-32]). The tRF we identified that interacts with RyhB and RybB corresponds to the 3′ external transcribed spacer of *glyW-cysT-leuZ* pre-tRNA (3′ETS^{leuZ}). Our data indicated that the 3′ETS^{leuZ} acts as a sRNA sponge, which prevents sRNA transcriptional noise in non-inducing conditions.[28] Indeed, MAPS data allowed us to demonstrate that the 3′ETS^{leuZ} interacts *in vivo* with RyhB and RybB sRNA. Then, further experiments conceded evidence that 3′ETS^{leuZ} acts as a concentration threshold setter, buffering a certain amount of sRNA corresponding to their transcriptional noise.

After these results were obtained by MAPS, we applied the same method to other sRNAs. Results obtained for the sRNA DsrA were particularly exciting.[26] DsrA is a well-known effector of positive and negative expression of *rpoS* and *hns* mRNA, respectively.[33-36] As expected, we first validated that both mRNA targets were co-purified with MS2-DsrA. Surprisingly, MAPS unveiled a new atypical target of DsrA sRNA, the *rbsD* mRNA. In the case of DsrA-*rbsD* interaction base-pairing occurs far downstream the TIR, in the open-reading frame (ORF), which is in sharp contrast to most sRNAs that base-pair near the TIR.[37] In 1998, Lease *et. al.* presented *in silico* predicted base-pairing between DsrA and *rbsD* at the same location.[36] However since then, no report was able to confirm *rbsD* as a true target of DsrA. DsrA MAPS data prompted us to re-investigate this RNA-RNA interaction further, leading to the confirmation that *rbsD* was indeed negatively regulated by DsrA, as the sRNA induces mRNA decay following base-pairing.[26]

Recently, we also applied MAPS to tRNA-derived RNA fragments in order to determine their binding partners. Deriving from pre-tRNA transcript, tRF proved to be a challenge in accomplishing MAPS experiment due to their specific maturation processes. The 3′ETS^{leuZ} was the first tRF candidate to be analyzed by MAPS, which confirmed the *in vivo* interaction between 3′ETS^{leuZ} and 2 sRNAs (RyhB and RybB) (unpublished data). Likewise, short RNA molecules (33 nt) corresponding to the internal transcribed spacers (ITS) of *metZ-metW-metV* pre-tRNA polycistronic transcript were also successfully tagged and analyzed by MAPS,[28,38] leading to the identification of 2 sRNAs (RybB and MicF) interacting with both ITS. Interactions were confirmed by Northern blot analysis after affinity purification.[28] While it is still unclear what is the cellular role of the ITS of *metZ-metW-metV* pre-tRNA, these data suggest a more widespread phenomenon than previously thought.

It is now clear that MAPS is a powerful tool to study interaction partners of various types of RNA molecules whether these partners are mRNA, sRNAs or tRFs (Fig. 1D). Its unique output data set led us to a breakthrough in the bacterial tRFs field, in addition to helping us identify new targets of the well characterized sRNAs RyhB, RybB and DsrA. As sRNAs, tRFs or mRNAs, other type of RNA molecules such as rRNAs and even riboswitches can be tagged with the MS2 aptamer. Therefore, it is not difficult to imagine the diverse application of MAPS.

Disclosure of potential conflicts of interest

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