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The Small Nuclear Ribonucleoprotein Polypeptide A (SNRPA) binds to the G-quadruplex of the BAG-1 5′UTR

François Bolduc , Marc-Antoine Turcotte , Jean-Pierre Perreault *

RNA Group/Groupe ARN, Département de biochimie et de génomique fonctionnelle, Pavillon de Recherche Appliquée au Cancer, Université de Sherbrooke, 3201, Jean-Mignault, Sherbrooke, Québec, J1E 4K8, Canada

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ABSTRACT

The protein "BCL-2-associated athanogene-1" (BAG-1), which exists in multiple isoforms, promotes cancer cell survival and is overexpressed in many different cancers. As a result, BAG-1-targeted therapy appears to be a promising strategy with which to treat cancer. It has previously been shown that the 5′UTR of the BAG-1 mRNA contains a guanine rich region that folds into a G-quadruplex structure which can modulate both its cap-dependent and its cap-independent translation. Accumulating data regarding G-quadruplex binding proteins suggest that these proteins can play a central role in gene expression. Consequently, the identification of the proteins that could potentially bind to the G-quadruplex of the BAG-1 mRNA was undertaken. Label-free RNA pulldown assays were performed using protein extracts from colorectal cancer cells and this leads to the detection of RNA G4 binding proteins by LC-MS/MS. The use of G-quadruplex containing RNA, as well as of a mutated version, ensured that the proteins identified were specific for the RNA G-quadruplex structure and not just general RNA binding proteins. Following confirmation of the interaction, the Small Nuclear Ribonucleoprotein Polypeptide A (SNRPA) was shown to bind directly to the BAG-1 mRNA through the G-quadruplex, and knock down experiments in colorectal cancer cells suggested that it can modulate the expression level of BAG-1.

1. Introduction

RNAs contain multiple *cis*- (ex. sequence and structural motifs) and *trans*- (ex. RNA binding proteins; RBPs) acting features that are involved in the regulation of gene expression. One interesting example is the RNA G4 (rG4) structure that involves the stacking of Hoogsteen hydrogen-bonded G-tetrads which, once stabilized by the chelation of monovalent metal ions such as potassium, form a stable four-stranded helical structure [1,2]. G-quadruplexes are highly abundant [3] and are involved in many post-transcriptional regulatory mechanisms including the regulation of translation, alternative splicing, polyadenylation, mRNA localization and microRNA binding and maturation [4,5]. The dysfunction of G4s suggests they may be involved in the pathogenesis of diseases such as neurological disorders and cancer [6].

The protein "BCL-2-associated athanogene-1" (BAG-1) is a multifunctional protein that is overexpressed in many cancers, and whose expression is correlated with both the pathologic grade and distant metastasis. Consequently, this protein is an interesting potential drug target for cancer treatment [7]. The BAG-1 mRNA is alternatively translated into three main isoforms: the long, BAG-1L (50 kDa); the medium, BAG-1M (46 kDa); and, the short, BAG-1S (36 kDa). These isoforms are translated from the same mRNA transcript via two mechanisms. The first mechanism is an alternative translational initiation called "leaky scanning" that occurs at the three alternative in-frame start codons that are present in the 5′UTR of the mRNA [8]. The second is an internal translational initiation mechanism which uses an internal ribosome entry site (IRES) secondary structure that is located upstream of the third start codon [9]. Recently, it was observed that the BAG-1 protein is overexpressed in colorectal cancer (CRC) tissue while the RNA level is decreased, suggesting some sort of a control at the translational level [10]. In the same report, two novel structural features found in the 5′UTR of the BAG-1 mRNA, an rG4 and a repressive upstream open reading frame (uORF) [10,11], were also described. For the first time it was demonstrated that an rG4 located in the 5′UTR acts at the same time on both cap-dependent and cap-independent translation. In fact, this rG4 acts like most of the 5′UTR rG4, by halting the ribosome, thereby repressing the cap-dependent translation. However, it also

* Corresponding author.
E-mail address: jean-pierre.perreault@usherbrooke.ca (J.-P. Perreault).

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modulates translation by maintaining the global 5′UTR secondary structure, which is essential for the IRES translational mechanism.

RNA G4 are commonly believed "to be events and not things" [12]. In other words, rG4s are folded transiently. In recent years, an increasing number of proteins with binding affinities for G4s have been identified [2]. In order to decipher and understand the complex expression of BAG-1, the identification and characterization of the G4 RNA binding proteins (G4 RBP) able to bind the G4 located in the 5′UTR of the BAG-1 mRNA was undertaken. One of the interesting candidates that appeared was the Small Nuclear Ribonucleoprotein Polypeptide A (SNRPA).

2. Materials and methods

The details of all protocols are available in the Supplementary File.

2.1. Plasmids

The four repeats of the S1m aptamer (S1mX4) were constructed by gene synthesis (GeneArt, Life Technologies) and were then cloned into the plasmid pMARQ, generating the plasmid pMARQ_S1mX4. The plasmids pMARQ_BAG1G4_S1m4X and pMARQ_BAG1G/Amut_S1m4X were created by PCR-amplifying the first portions of the 5′UTRs of BAG-1 and of a G/A mutated version contained in the psiCHECK-2 plasmid, respectively [10]. The PCR-fragments were digested with *Eco*RI and *Bam*HI and were inserted into the *Eco*RI and *Bam*HI restriction sites of the plasmid pMARQ_S1mX4 (Supplementary Fig. S1).

2.2. Label-free RNA affinity purification and LC-MS/MS analysis

RNAs were synthesized by *in vitro* transcription and were gel purified on 5% denaturing polyacrylamide gels as described previously [11]. Cellular extracts were prepared from 4 confluent 15-cm dishes of HCT-116 cells. The cell extracts were mixed with streptavidin-coated sepharose beads pre-bonded with chimeric RNAs, and the bound peptides were analyzed using an LC-MS/MS as illustrated in Fig. 1A. The proteins were then identified by MaxQuant analysis [13].

2.3. Western blot validation

The interactions of the potential RNA binding proteins (RBP) with the RNA were validated by Western blot using commercially available specific antibodies and both the WT and the G/A mutant sequences.

2.4. Gene knock down using siRNAs in HCT-116 cells

The reverse transfection of siRNAs was performed in 6-well plates using Lipofectamine RNAiMAX (Thermo Fisher) according to manufacturer's protocole. After the cells had recovered, total RNA extracts were prepared and the specific mRNAs were analyzed by RT-qPCR at the Laboratory of Functional Genomics of the Université de Sherbrooke (LGFUS). The proteins were monitored by western blotting.

2.5. Recombinant SNRPA purification and electrophoretic mobility shift assays (EMSA)

E. coli Rosetta-2 cells transformed with the plasmid pET42a_SNRPA-cHis (GeneScript) were used for the production of recombinant SNRPA that was subsequently purified using a Ni-NTA column. The purified SNRPA was used in electrophoretic mobility assays that were performed in the presence of either the short or the long 5′-labeled WT or G/A mutant BAG-1 5′UTR versions.

3. Results

3.1. Identification of the proteins that are able to bind to the G-quadruplex of the BAG-1 5′UTR

Label-free RNA affinity purification assays using a chimeric RNA composed of the first 83 nucleotides (nts) of the BAG-1 5′UTR, which contained either the wild type rG4 (WT) or a G/A mutated version fused to four repeats of the S1m aptamer, were performed. The S1m aptamer is a modified streptavidin-binding RNA aptamer [14]. *In-line* probing experiments were performed in order to confirm that the rG4 was correctly folded in the chimeric constructs. The results confirmed the efficient folding of the G4 in the presence of potassium, but not in the presence of lithium. An increased accessibility of the nucleotides located in the loops of the rG4 structure was clearly observed in the presence of potassium, but not lithium ions, a hallmark of rG4 folding. This accessibility was not encountered with the G/A mutant version, regardless the nature of the salt present (see Supplementary Fig. S2). Specifically, total protein extracts from HCT116 CRC cells were incubated with the chimeric RNA that had previously been bound to streptavidin-coated sepharose beads (Fig. 1A). Any non-specific interactions were washed away, and the bound proteins were then subjected to trypsin digestion. The resulting peptides were injected into an LC-MS/MS. The data were analyzed in order to determine which proteins are enriched in the WT samples relative to the G/A mutant samples. The experiment was performed in triplicate, and identified 37 potential candidates that were presented in at least two of the replicates and whose fold enrichment was >2.5 (Fig. 1B and see Supplementary Table S1 for a detailed list). Curiously, half of the proteins on this list are ones related to DNA (i.e. 10 DNA chromatin binding protein, 4 DNA helicases, etc.), and were not considered further since the objective was to find RBP. More interestingly, 3 RNA helicases, as well as 9 proteins associated with splicing and translation, were retrieved. In order to increase the confidence in these findings, western blotting experiments were performed using both the WT and the G/A mutant RNAs and antibodies specific for proteins known to bind RNA and for which antibodies were available from collaborators. Enrichment was observed in the WT sample for 6 proteins out of the 9 candidates tested (Fig. 1C).

3.2. Impact of the knock down of SNRPA on BAG-1 expression in HCT-116 cells

In order to identify candidates that can be associated with the modulation of the translation of BAG-1 mRNA, gene knock down experiments using specific siRNAs were performed. Two siRNAs targeting the expression of each of the 6 proteins observed to be enriched above were reverse transfected into HCT-116 cells. The expression of BAG-1 at the protein level (by western blots) as well as at the RNA level (by RT-qPCR) was monitored at both 48 and 72 h post-transfection. Consistent translational modulation of BAG-1 was observed only for the Small Nuclear Ribonucleoprotein Polypeptide A (SNRPA). Indeed, a knock down of the amount of SNRPA protein present of close to 90% at 48 h post-transfection, and close to 100% at 72 h, was observed (Fig. 2A). The intensities of the bands corresponding to the BAG-1 proteins were then measured and were discovered to be consistently increased in cells in which the expression of SNRPA was knocked down (Fig. 2B). The highest levels of expression were recorded for BAG-1S. Consistent increases in the expression of the other two isoforms (i.e. M and L) were also observed, although at lower levels. Concomitantly, level of BAG-1

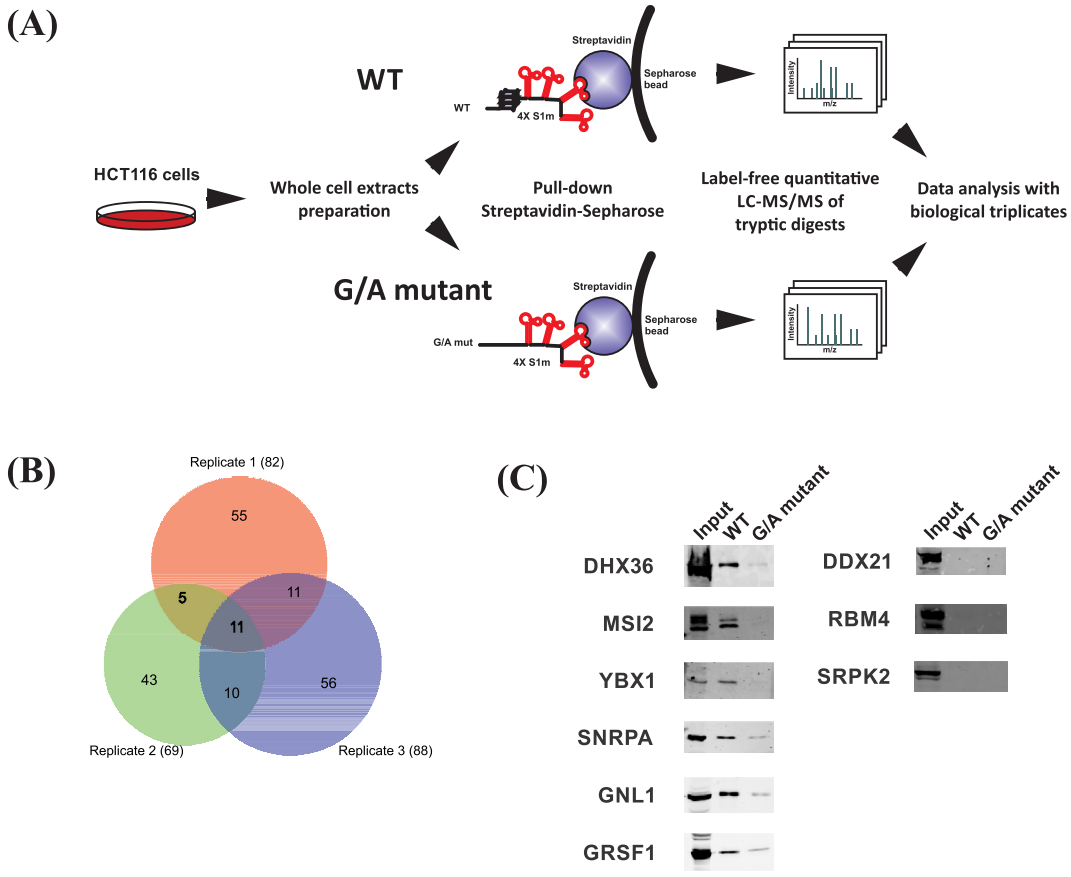

Fig. 1. Label-free RNA affinity purification and results from LC-MS/MS. (A) HCT-116 protein extracts were mixed with streptavidin-coated sepharose beads pre-bonded with chimeric RNAs. Following washes to remove any non-specific interactions, the tryptic digests were eluted and analyzed by LC-MS/MS. (B) Venn diagram generated from three RNA affinity purification assays. The number of proteins that pass the threshold (i.e. >2.5 fold enrichment, >5% coverage and a MaxQuant score >15) is indicated for each replicate. The different colors refer to the different replicates. (C) Western blots experiments used to validate the LC-MS/MS data for some of the RBP candidates.

RNA was not increased, which is characteristic of a translational regulation.

3.3. SNRPA can directly bind the G4 of the BAG-1 mRNA

In order to characterize the interaction between the rG4 of the BAG-1 mRNA and the SNRPA protein, the recombinant histidine-tagged SNRPA was purified on a nickel column and used in electrophoretic mobility shift assays (EMSA). The concentration of the 5′-labeled RNA was kept constant (~1 nM), while the protein concentration ranged from 0 to 45 μM. Two distinct RNA species of different lengths were tested (i.e. a 30 and an 83 nt versions). The folding of the G4 of both versions was confirmed for the WT of these RNAs using N-methyl mesoporphyrin (NMM) fluorescence assays. NMM is known to bind G4 structures with a parallel topology [15]. The ligand by itself emits a very low fluorescence, but upon binding to a parallel G4 its fluorescence can be increased from 2- to 10-folds (see Supplementary Fig. S3). Using the first 83 nts of the BAG-1 5′UTR, the SNRPA has a higher affinity for the WT as compared to the G/A mutant version (Fig. 2C). Dissociation constants (K_d) of 2.45 μM and 8.74 μM were calculated for the WT and the G/A mutant, respectively. The presence of several bands corresponding to the complex is most likely due to the fact that the SNRPA homodimerized, a characteristic that has been previously reported by others based on EMSA assays [16]. When using a shorter version (30 nts, i.e. without the surrounding nucleotides), the same conclusion was reached (K_d of 4.86 μM for the WT G4 and 20.00 μM for the G/A mutant sequence; Fig. 2D). The surrounding nucleotides seem to increase the affinity of SNRPA for the RNA by 2-folds. Moreover, only two bands of RNA/SNRPA complexes were observed, suggesting that there is less homodimerization with this smaller RNA species.

4. Discussion

The large scale identification of proteins binding RNA G4s is in its early stages and, despite the fact that some studies have already made it possible to highlight certain proteins of interest, this study reports, for the first time, SNRPA as a potential G4 RBP [17–22]. RNA affinity purification followed by LC-MS/MS analysis revealed the enrichment of 37 potential protein candidates that seem to have an increased affinity for the G4 located in the 5′UTR of the BAG-1 mRNA, as compared to an RNA in which the folding of the G4 has been abolished. Interestingly, although an RNA molecule was used as bait, many chromatin binding proteins ended up being detected. A high representation of members of the SWI/SNI family of proteins that display both helicase and ATPase activities (ex; SMARCC1, SMARCC2, SMARCD2 and SMACA4) was observed. These proteins are thought to regulate the transcription of certain genes by altering the chromatin structure. It is not impossible that these proteins may have dual functions and be able to bind RNA during the transcription of their target genes, which could explain their presence in this experiment. Also, it is important to mention that in total extracts the cellular compartments are broken down during their preparation, a

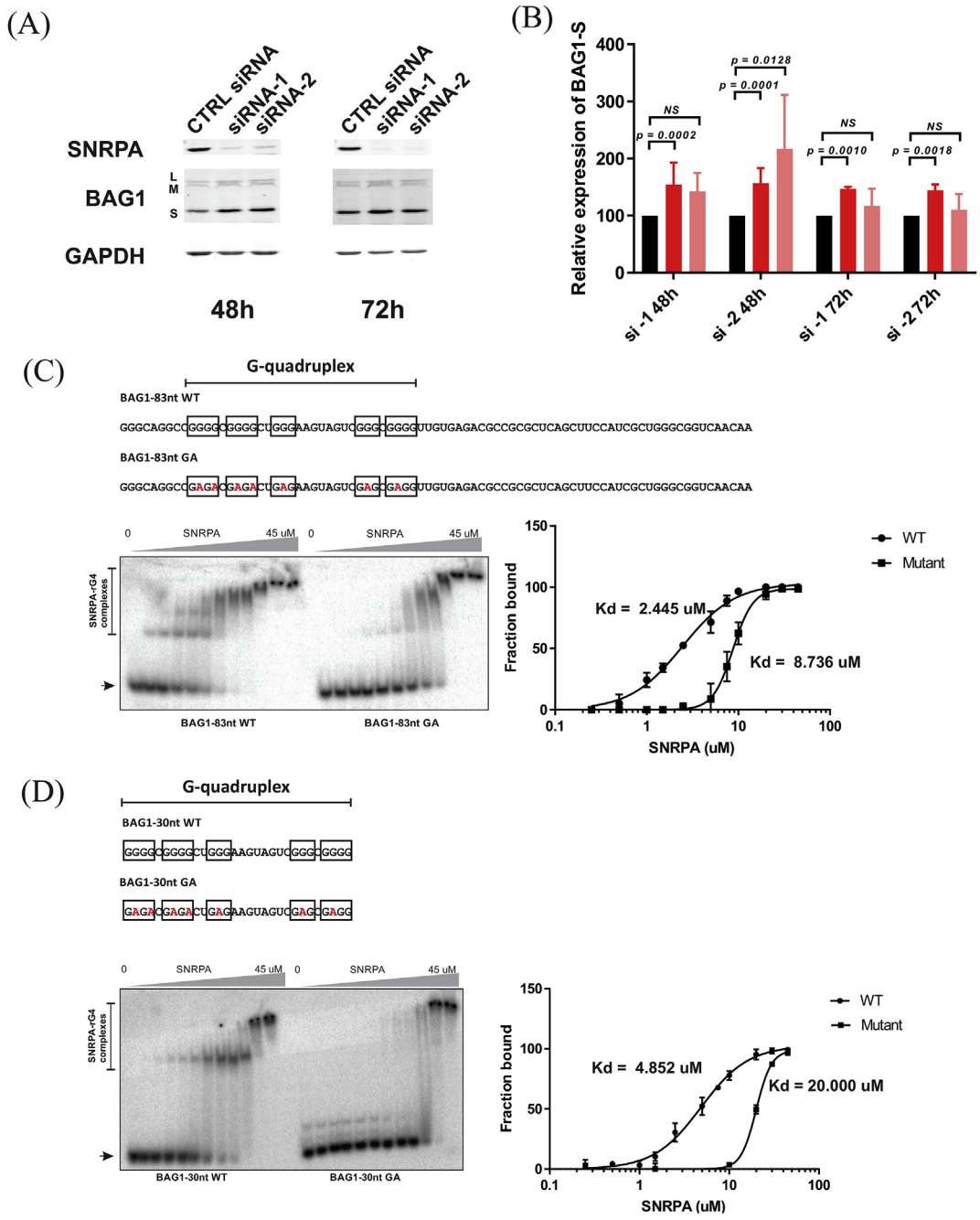

Fig. 2. Knock down of SNRPA in HCT-116 cells and EMSA. (A) Western blot using protein extracts from HCT-116 cells knocked down for the expression of SNRPA at both 48 and 72 h post-transfection of siRNAs. Two specific siRNAs (siRNA-1 and siRNA-2) and one control (CTRL) were used. The SNRPA and BAG-1 proteins were detected, as well as GAPDH which was used as a loading control. L, M and S refer to the large, medium and small BAG-1 isoforms, respectively. (B) Histograms of the relative expression of BAG-1 protein calculated from the western blots (red bars) and the mRNA (pink bars) as determined by RT-qPCR. The Black bars represent the expression levels from cells transfected with the control siRNA. The results presented are the means of three independent experiments, and each time two technical replicates were performed. The error bars indicate the standard deviations. P values were calculated by Dunnett's multiple comparisons test. (C) EMSA using increasing concentrations of purified SNRPA-his protein with a constant concentration of the first 83 nts of either the BAG-1 5′UTR WT or the G/A mutant version (1 nM). The arrow indicates the band corresponding to the free RNA, and the different complexes retained on the gel are identified on the left. The mutations of G to A are shown in red. (Right) Hill slope of two independent EMSA assays. For each concentration of SNRPA-his, the disappearance of unbound RNA was measured using the Image Studio Lite software. The error bars represent the standard deviation. (D) Identical to (C) except that the RNA used for EMSA was restricted to the G4 sequence.

fact which may have favored the detection of so many of these DNA binding proteins. More relevant was the finding of several RBP, including RNA helicases like DHX36 and DDX21 that are known to unfold RNA G4 [23,24], which increases the confidence in these candidates and validates the approach. That said, 6 out of the 9 candidates tested by Western blot were validated, including DHX36 (Fig. 1C). Among the RBP tested so far, some might be interesting candidates for the regulation of BAG-1 translation.

In order to explain the translational regulation which seems to be observed for BAG-1 in CRC cells, the potential role that these factors could have on this regulation was examined. Using a knock down approach for each candidate, a consistent dysregulation of

BAG-1 at the translation level was detected solely for SNRPA. Dysregulation was observed at different levels for other RBPs, but nothing compared to that observed for SNRPA. This protein of 282 amino acids (~34 kD) contains 2 RNA recognition motifs (RRM) and is well-known for its implication in splicing [25]. In fact, SNRPA, through its N-terminal RRM, can bind the loop part of the SL2 sequence AUUGCAC of the U1 RNA [16]. This sequence is not part of the BAG-1 5′UTR. Moreover, SNRPA expression has been shown to be higher in tumour tissues than in matched normal gastric mucosa tissues [26]. When SNRPA was knocked down in HCT-116 cells, an increase in BAG-1 protein expression (1.5-folds) was observed, while the RNA level remained relatively constant. Although mainly known as a splicing factor, SNRPA can shuttle between the nucleus and the cytoplasm [27]. That said, how this splicing factor can modulate the translation of BAG-1 remains an interesting question. Moreover, G4 are known to hinder translation by acting as a roadblock to the scanning of the ribosomes. In other words, they usually decrease translational efficiency. In the present case, the knockdown of SNRPA increases the expression of BAG-1 in HCT-116 cells, suggesting that SNRPA stabilized the G4 and decreased the expression level. As mentioned earlier, helicases like DHX36 and DDX21 are known to resolve G4 structures, and it is not impossible that such a protein can compete for binding the BAG-1 rG4. In this scenario the two would act together to fold/unfold this rG4 in order to modulate BAG-1 expression. Elucidation of this molecular mechanism might open the door to novel therapeutic approaches for CRC since the expression of nuclear BAG-1 is associated with a poorer prognosis and is potentially a useful predictive factor for distant metastasis [28]. Thus, disruption of the nuclear localised BAG-1 would sensitize cells to apoptosis and increase the efficacy of current therapeutic approaches. As shown in this report, the absence of SNRPA increases the expression of the three BAG-1 isoforms, although at different levels. The presence of SNRPA seems to be favorable regarding cancer treatment outcome, by maintaining lower levels of the BAG-1 isoforms.

5. Conclusion

In brief, the present study demonstrated that SNRPA binds directly to the BAG-1 mRNA through the G-quadruplex, and knock down experiments in colorectal cancer cells suggested that it can modulate the expression level of BAG-1.

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CRediT authorship contribution statement

François Bolduc: Formal analysis, Data curation, Writing - original draft. **Marc-Antoine Turcotte:** Writing - original draft. **Jean-Pierre Perreault:** Writing - original draft, Writing - review & editing.

Declaration of competing interest

Any of the authors has conflict of interest with the presented work.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biochi.2020.06.013.

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