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研究課題名（和文）Study on molecular mechanism of anti-tumor activity by splicing modulation

研究課題名（英文）Study on molecular mechanism of anti-tumor activity by splicing modulation

研究代表者
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交付決定額（研究期間全体）：（直接経費）　　3,300,000円

研究成果の概要（和文）：Spliceostatin A（SSA）によるスプライシング変調は、mTORC1の不活性化によるグローバルな翻訳プロセスを阻害します。 RNAシーケンシングを使用したシステム全体の分析（トランスクリプトーム）、リボソームプロファイリング（トランスラトーム）およびBONCAT質量分析（プロテオーム）スプライシング阻害により、本質的に無秩序な領域を提供する保持されたイントロンからのかなりの数のエクソン-イントロンキメラタンパク質 コンデンセートが発生しやすい特性。 BONCATと生化学的実験により、凝集しやすいイントロンタンパク質の生成が確認されました。

研究成果の学術的意義や社会的意義
SSA induce proteotoxicity from intron-derived truncated proteins which activates JNK inhibiting mTORC1 pathways, essential pathway in cancer cell survival. Hence, SSA ultimately attenuates global protein synthesis, this mechanism of action may not be exclusive but is vital one found from this study.

研究成果の概要（英文）：Splicing modulation by Spliceostatin A (SSA) inhibit the global translation process by mTORC1 inactivation. System-wide analysis using RNA sequencing (transcriptome), ribosome profiling (translatome) and BONCAT mass spectrometry (Proteome)upon splicing inhibition produced the substantial number of exon-intron chimeric proteins from the retained introns which have the intrinsically disordered regions providing condensate-prone properties. The BONCAT and the biochemical experiments verified the production of aggregation-prone intron proteins causing cellular proteotoxic stress in tumor cell. activation of JNK pathway to disintegrate the mTORC1 components inactivating its function to reduce the mTORC1 mediated translation.
mTORC1 signaling pathway being one of the key molecular pathway upregulated in the number of cancers, and hence SSA seems to plays an important anti-tumor mechanism via mTORC1 inhibition through the proteotoxic stress from the truncated chimeric-intron proteins.

研究分野：Chemical Biology

キーワード：spliceostatin A　splicing modulation　proteotoxic stress　aggregation

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様 式 Ｃ－１９、Ｆ－１９－１、Ｚ－１９（共通）

１．研究開始当初の背景

<u>Scientific background:</u> In eukaryotes, splicing of introns, the intervening sequences between exons are an essential step during mRNA processing. Hence, splicing perturbation is associated with pronounced changes in gene expression and metabolism in normal and tumor cells. Different splicing modulator compounds have been discovered from natural products such as spliceostatin A (SSA), the stable derivative of FR901464 from *Pseudomonas* sp. represents a well-characterized anti-tumor compound which targets the SF3Bs subcomplex of the U2 snRNP (Chhipi-Shrestha et al. 2021) (Figure 1). Strikingly, those compounds show potent anti-tumor activity and, indeed, several of them have also been commenced into the clinical trials for the treatment of some solid tumors and leukemias. Despite the pharmaceutical application, the precise mechanism of the anti-tumor effect by splicing modulator compounds is still unclear.

２．研究の目的

<u>Purpose:</u> The overall rationale for how splicing modulators suppress tumor growth remains unclear. Hence, the purpose of this research is to unravel the molecular mechanism for the anti-tumor activity of SSA. For the same, the role of functional chimeric protein, and pathways signaling-axis for anti-tumor activity during splicing modulation should be investigated.

３．研究の方法

<u>Research method and approaches:</u> Primarily, to understand the comprehensive changes system wide in transcriptome, translatome and proteome upon splicing modulation, I have performed simultaneous RNA sequencing, ribosome profiling and BONCAT mass spectrometry respectively in the HeLa S3 cell lysate after SSA (the splicing modulator described above) treatment (Chhipi-Shrestha et al. 2022) (Figure 1). Ribosome profiling is more recent and advance technique having the power to survey the active translation change genome-wide based on a deep sequencing of the ribosome-protected mRNA fragments from RNase which provides the best overview of translation dynamics at a subcodon resolution. The system-wide results were analyzed and the candidates were studied for their molecular properties bioinformatically and developed a reporter assays for the biochemical validation experiments. Global genome-wide changes, differential gene expression analysis and pathway analysis were investigated.

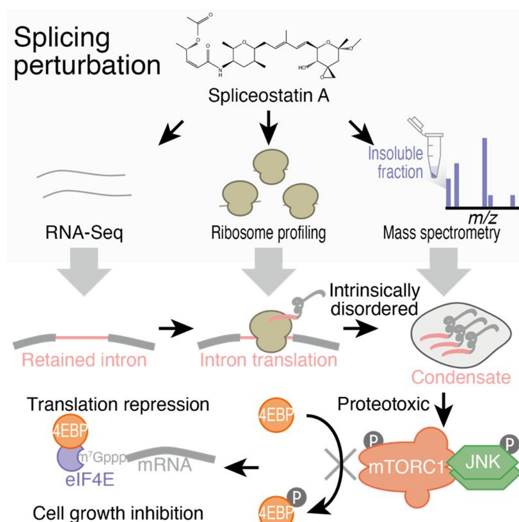

Figure 1. Summary of findings

４．研究成果

Research Results

(1) SSA induces widespread intron retention and intron translation

RNA-Seq data depicted substantial number of retained introns upon splicing modulation by SSA i.e. 5920 introns among which ribosome profiling interestingly demonstrated 1078 of them were translated until the first premature termination codon (PTC) with discrete 3-nt periodicity (Figure 2A, 2B). The transcripts may not be targeted to non-sense mediated decay (NMD). This could be explained by the possibility that

overproduction of target transcripts simply overwhelms the cellular NMD capacity. Alternatively, SSA may lead to PCPA in the middle of introns (Yoshimoto R, Chhipi-Shrestha et al., 2021). This would result in shorter isoforms without downstream exonexon junctions and EJCs and thus escape NMD. These two scenarios are not mutually exclusive.

Further analysis of the products of intron translation were performed by a proteome approach by enriching for newly synthesized protein by bio-orthogonal noncanonical amino acid tagging (BONCAT). This technique is based on metabolic labeling of newly synthesized proteins by the noncanonical amino acid homopropargylglycine (HPG), which allows cycloaddition of azidebiotin by "click chemistry," enrichment of the biotinylated proteins by streptavidin beads, and subsequent detection of denovo-synthesized proteins by mass spectrometry. BONCAT revealed a substantial number of stable chimeric proteins (n = 238) (Figure 2C). In all, SSA treatment leads to the stable production of chimeric proteins containing both exon- and intron-derived sequences.

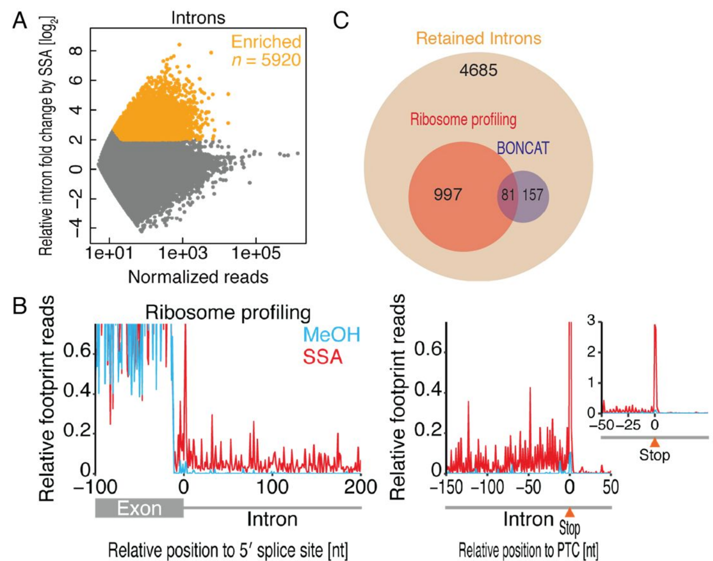

Figure 2. (A) MA plot highlighting retained introns upon SSA (orange). (B) Meta analysis of ribosome footprints centred on splice site (left), centred on PTC (right). (C) Intersection of intron candidates observed in RNA-Seq, Ribo-seq and BONCAT.

(2) Characteristics of intron-translated transcripts and proteins

The majority of the translated introns (71%) were derived from the first intron.In contrast to fulllength proteins, truncated peptides showed significantly higher levels of IDRs and positively charged (Figure 3A). The amino acids serine (S), proline (P), glycine (G), and arginine (R) were highly overrepresented in translated intron regions therefore producing stretches of low complexity in the resulting proteins (Figure 3B).

(3) A subset of intron-derived peptides is condensation-prone

The enrichment of IDRs in intron-derived peptides motivated to test the condensation propensity of the chimeric proteins. So, BONCAT proteomic analysis of the cellular soluble and insoluble fractions were performed. chimeric peptides detected in the pellet of SSA-treated cells appeared more prevalent than in the supernatant. Many remarkable chimeric candidates that are intron translated as depicted by ribosome profiling have been observed enriched in the pellet fraction in BONCAT experiments such as ferritin heavy chain1(FTH1).. It could also be validated well by biochemical experiments such as western blotting, microscopy and recombinant protein study.

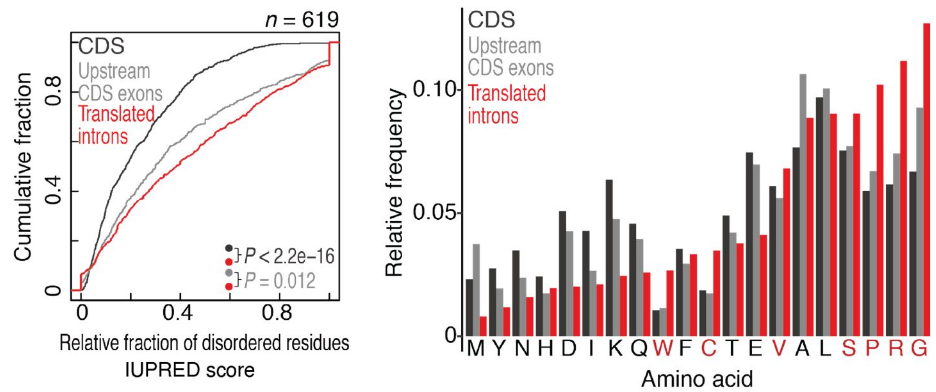

Figure 3. (A) Cumulative distribution of relative disorderness . (B) Relative frequency of amino acids present.

(4) Condensation-prone intron-derived peptides are proteotoxic

Since the truncated proteins forming condensates/aggregates formed from the improperly spliced transcripts, I hypothesized that the cells will have the proteotoxic stress response upon SSA treatment. Investigating for the same as sensed by the multifaceted JNK kinase, proteotoxic stress was evidenced increased by its hyperphosphorylation. To generalize the effect, we used other splicing modulator, Pladienolide B and target protein SF3B1 knockdown both of these treatment condition recapitulates the SSA`s phenotype of proteotoxic stress response. The results are also supported by the use of other proteotoxic stress reporters such as unstable DMFluc-EGFP (R188Q-R261Q double mutant [DM]), which requires chaperone surveillance to fold, fused to GFP to assess a possible imbalance in proteostasis (Gupta et al., 2011). SSA treatment or exogenous intron protein expression leads to the Fluc-DM-EGFP reporter aggregation corroborating the proteotoxic stress induction. Global system-wide protein aggregation was depicted by filter trap assay which were validated by top enriched chimericintron protein candidates such as FTH1*.

(5) Proteotoxic stress response by JNK inhibit global translation inhibition by mTORC1 inhibition

Investigating ribosome profiling and RNA-Seq data, we found that splicing inhibition led to a intensive decrease of translation (4-fold) that could be explained by transcript abundance decrease (2-fold) (Figure 4A). Hence, translation regulation under splicing regulation may be present. Further estimating the translation efficiency change by taking the ration of ribosome footprint change to the transcript change, differential changes across the transcripts were observed. KEGG pathway analysis and GO analysis depicted important pathways were affected such as cytosolic ribosome and mTORC signaling pathway (Figure 4B). mTORC1 has been evidenced to be deactivated upon sensing proteotoxic stress via JNK (Figure 4C). mTORC1 phosphorylation of several translational key regulators, such as eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4EBP1) and ribosomal protein S6 kinase beta-1

(S6K1). Upon splicing inhibition by SSA, dephosphorylation of these mTORC1 substrate proteins were observed dependent on JNK kinase (Figure 4D, 4E). The results were substantiated by other splicing modulators, pladienolideB and SF3B1 target knockdown generalizing the phenomenon of mTORC1 mediated translation repression upon splicing modulation. Our results indicate that toxic, condensate-prone proteins generated by splicing perturbation lead to mTORC1-dependent translational repression via JNK activation (Figure 1). In this study, comprehensive proteome and translatome analysis gave a global overview of introndrived peptides and their function. We demonstrated that treatment with the splicing modulator SSA generates chimeric proteins with intrinsically disordered and condensation-prone properties. The proteotoxic condensates of peptides from intron translation lead to mTORC1 inhibition and subsequent global repression of translation. Since hyperactivated mTORC1 is a hallmark of cancer and its inhibition is a well-characterized option for tumor treatment, our results provide insight into the mechanisms underlying the antitumor activity of splicing modulators._

Furthermore, with regards to investigate such phenomenon in Acute Myeloid Leukemia (AML) cells having number of splice factor mutation and efficient anti-cancer activity by splicing modulators such as SSA, I have performed RNA-Sequencing and ribosome profiling. There seems to present higher transcriptional and post-transcriptional regulation due to long rna reads accumulated in RNA-Seq library preparation regardless of stringent fragmentation condition (data not shown). Hence, comprehensive investigation in

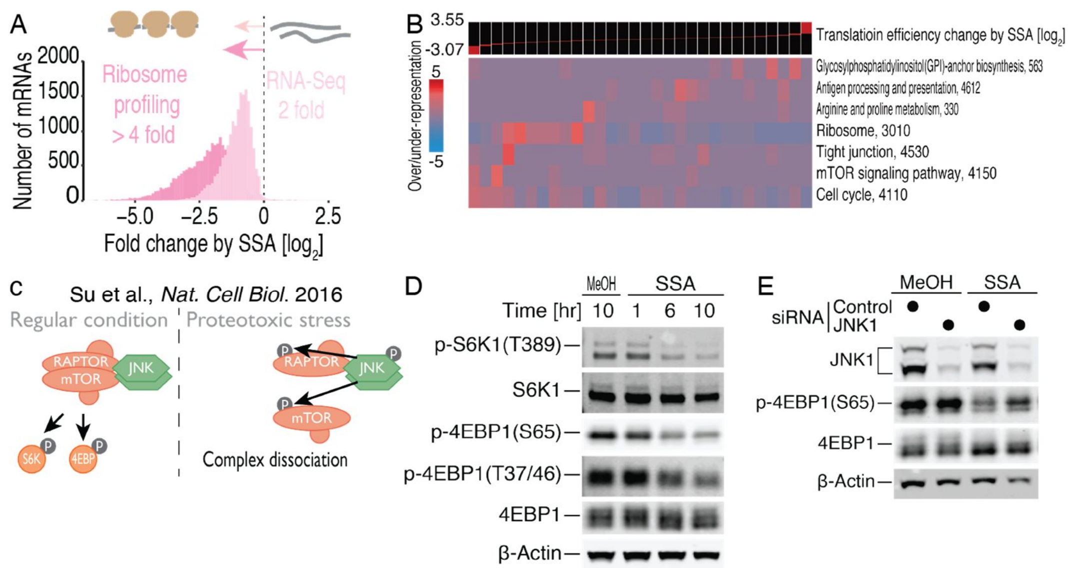

Figure 4. (A) Histograms showing absolute change in RNA-Seq reads and ribosomal footprints under SSA treatment. (B) KEGG pathway analysis based on the differential change in translation efficiency. (C) Schematic of proteotoxic stress-mediated mTORC1 inhibition via activated JNK. (D) Western blot

leukemic cell upon splicing modulation is preferable and is ongoing. Also, investigation of candidate intron proteins and their fates are under study for further significant inferences under splicing modulation.

5．主な発表論文等

〔雑誌論文〕　計4件（うち査読付論文　1件／うち国際共著　2件／うちオープンアクセス　0件）

1．著者名	4．巻
Shrestha Jagat Krishna Chhipi、Schneider-Poetsch Tilman、Suzuki Takehiro、Mito Mari、Khan Khalid、Dohmae Naoshi、Iwasaki Shintaro、Yoshida Minoru	NA
2．論文標題	5．発行年
Splicing modulators elicit global translational repression by condensate-prone proteins translated from introns	2020年
3．雑誌名	6．最初と最後の頁
bioRxiv	NA
掲載論文のDOI（デジタルオブジェクト識別子）	査読の有無
10.1101/2020.11.23.393835	無
オープンアクセス	国際共著
オープンアクセスではない、又はオープンアクセスが困難	該当する

1．著者名	4．巻
Yoshimoto Rei、Chhipi-Shrestha Jagat K.、Schneider-Poetsch Tilman、Furuno Masaaki、Burroughs A. Maxwell、Noma Shohei、Suzuki Harukazu、Hayashizaki Yoshihide、Mayeda Akila、Nakagawa Shinichi、Kaida Daisuke、Iwasaki Shintaro、Yoshida Minoru	NA
2．論文標題	5．発行年
Spliceostatin A interaction with SF3B limits U1 snRNP availability and causes premature cleavage and polyadenylation	2021年
3．雑誌名	6．最初と最後の頁
Cell Chemical Biology	In Press
掲載論文のDOI（デジタルオブジェクト識別子）	査読の有無
10.1016/j.chembiol.2021.03.002	有
オープンアクセス	国際共著
オープンアクセスではない、又はオープンアクセスが困難	該当する

1．著者名	4．巻
Chhipi-Shrestha Jagat K.、Schneider-Poetsch Tilman、Suzuki Takehiro、Mito Mari、Khan Khalid、Dohmae Naoshi、Iwasaki Shintaro、Yoshida Minoru	29
2．論文標題	5．発行年
Splicing modulators elicit global translational repression by condensate-prone proteins translated from introns	2022年
3．雑誌名	6．最初と最後の頁
Cell Chemical Biology	259～275.e10
掲載論文のDOI（デジタルオブジェクト識別子）	査読の有無
10.1016/j.chembiol.2021.07.015	無
オープンアクセス	国際共著
オープンアクセスではない、又はオープンアクセスが困難	－

1．著者名	4．巻
Schneider-Poetsch Tilman、Chhipi-Shrestha Jagat Krishna、Yoshida Minoru	74
2．論文標題	5．発行年
Splicing modulators: on the way from nature to clinic	2021年
3．雑誌名	6．最初と最後の頁
The Journal of Antibiotics	603～616
掲載論文のDOI（デジタルオブジェクト識別子）	査読の有無
10.1038/s41429-021-00450-1	無
オープンアクセス	国際共著
オープンアクセスではない、又はオープンアクセスが困難	－

〔学会発表〕　計1件（うち招待講演　1件／うち国際学会　0件）

1．発表者名 Jagat Krishna Chhipi Shrestha
2．発表標題 Splicing modulators elicit global translational repression by condensate-prone proteins translated from introns
3．学会等名 The 43rd Annual Meeting of the Molecular Biology Society of Japan, MBSJ2020 online.（招待講演）
4．発表年 2020年

〔図書〕　計0件

〔産業財産権〕

〔その他〕

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6．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考

7．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

8．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関