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Histone and RNA-binding protein interaction creates crosstalk network for regulation of alternative splicing

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ABSTRACT

Alternative splicing is an essential process in eukaryotes, as it increases the complexity of gene expression by generating multiple proteins from a single pre-mRNA. However, information on the regulatory mechanisms for alternative splicing is lacking, because splicing occurs over a short period via the transient interactions of proteins within functional complexes of the spliceosome. Here, we investigated in detail the molecular mechanisms connecting alternative splicing with epigenetic mechanisms. We identified interactions between histone proteins and splicing factors such as Rbfox2, Rbfox3, and splicing factor proline and glutamine rich protein (SFPQ) by *in vivo* crosslinking and immunoprecipitation. Furthermore, we confirmed that splicing factors were bound to specific modified residues of histone proteins. Additionally, changes in histone methylation due to histone methyltransferase inhibitor treatment notably affected alternative splicing in selected genes. Therefore, we suggested that there may be crosstalk mechanisms connecting histone modifications and RNA-binding proteins that increase the local concentration of RNA-binding proteins in alternative exon loci of nucleosomes by binding specific modified histone proteins, leading to alternative splicing. This crosstalk mechanism may play a major role in epigenetic processes such as histone modification and the regulation of alternative splicing.

1. Introduction

Alternative pre-mRNA splicing (AS) is a key regulatory mechanism for generating functional proteome diversity from the limited repertoire of the genome. AS plays an important role in cellular differentiation and organismal development in response to cell- and tissue-specific regulatory mechanisms; the mis-regulation of AS can lead to various genetic diseases in humans, including progeria, myotonic dystrophy, frontotemporal dementia, cystic fibrosis, and cancer [1]. Recently, based on high-throughput sequencing techniques, it was estimated that over 95% of pre-mRNAs in humans undergo AS [2]. However, despite efforts to fully understand the regulation of AS, current knowledge is limited and generally restricted to splicing enhancers and silencers or *cis*-acting RNA elements [3].

Recent studies have found that histone modification acts as a key regulator of AS. Nucleosomes, which are composed of ~147 bp DNA wrapped around an octamer of histone proteins, are more abundant in included (Alternative exons) AEs than in excluded ones [4]. Furthermore, many histone modifications are present at a high density in exon nucleosomes; H3K36me3, H3K4me3, and H3K27me2 are especially elevated in these nucleosomes [1]. Trichostatin A, a histone deacetylase (HDAC) inhibitor that increases the acetylation level of histone proteins, has been demonstrated to alter the splicing patterns of approximately 700 genes [5]. However, although these results showed that histone modification is associated with the regulation of AS, they did not determine the mechanisms controlling AS site selection. Therefore, a chromatin-splicing adaptor model has emerged, which proposes that direct physical crosstalk between chromatin and the spliceosome occurs via a specific adaptor molecule. Genes regulated by the polypyrimidine tract-binding protein (PTB) splicing factor were discovered to be enriched for H3K36me3 in alternatively spliced regions. MRG15 also specifically binds to H3K36me3, indicating that a high density of H3K36me3 in the AE region attracts MRG15 as an adaptor protein, which interacts with PTB to recruit the splicing machinery to the nascent RNA. Other examples of combinations of specific histone modifications, chromatin-binding proteins, and

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splicing factors exist, including H3K4me3/CHD1/U2 snRNP, H3acetyl/Gcn5/U2 snRNP, and H3K9me3/HP1α/hnRNPs [1]. Although this chromatin-splicing adaptor model explains the regulation of AS in terms of histone modification and splice site selection, the presence of adaptor proteins seems to be more important than distinct histone modifications.

Although many studies have focused on the mechanisms of AS regulation, it remains difficult to describe how AS is regulated owing to the great complexity of AS regulation. Here, we aimed to demonstrate a novel mechanism of AS regulation through crosstalk mechanisms controlled by interactions between histone modifications and RNA-binding proteins.

2. Materials and methods

2.1. Cell culture, plasmid, transfection, and chemicals

HeLa cells, a human cervical cancer line, were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and 1% penicillin-streptomycin at 37 °C in a humid 5% CO_2 atmosphere. The construction of plasmids containing N-terminal myc-tagged Rbfox2 and Rbfox3 using the pCS3+MT vector was performed as described previously [6,7]. Myc-tagged SFPQ and NonO in the pCS3+MT vector were obtained from Open Biosystems (Thermo Fisher Scientific). Cells were transfected with the above plasmids by electroporation using the Amaxa Nucleofector (Lonza, Basel, Switzerland). The histone methyltransferase inhibitors DZNep, EPZ004777, and BIX-01338 were purchased from Sigma Aldrich (St. Louis, MO, USA).

2.2. Intracellular crosslinking

HeLa cells were trypsinized and washed twice in phosphate-buffered saline (PBS) to remove amine-containing components from the media, as they would quench the crosslinking reaction. The cells were then resuspended in PBS. Disuccinimidyl suberate (DSS) (Thermo Fisher Scientific) was prepared immediately in dimethyl sulfoxide (DMSO) before use. DSS was added to the cell suspension at a final concentration of 5 mM and incubated for 30 min at room temperature. The reaction was stopped by adding a quenching solution containing 20 mM Tris and incubating for 15 min at room temperature. Cells were then washed three times in PBS.

2.3. Nuclear protein extraction and immunoprecipitation for mass spectrometry

To prepare the nuclear protein extraction, cells crosslinked with DSS were resuspended in hypotonic buffer using a Nuclear extract kit (Active Motif, Carlsbad, CA, USA). After incubating for 15 min on ice, the suspension was centrifuged at 14,000 × *g* and 4 °C for 30 s. The pelleted nuclei were lysed in radioimmunoprecipitation assay buffer (Sigma Aldrich) containing a complete protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland). The lysate was sonicated fifteen times for 1 s each at six amplitude microns power setting using the Soniprep 150 (MSE, London, UK). To interrupt DNA- or RNA-mediated protein interactions, the lysate was incubated with DNase and RNase-A/T1 mix (Thermo Fisher Scientific) at 37 °C for 5 min. After centrifugation at 14,000 × *g* and 4 °C for 10 min, the supernatants were subjected to immunoprecipitation with anti-myc antibody (Thermo Fisher Scientific) and anti-histone H3 antibody (Abcam, Cambridge, UK) or control IgG. Immune complexes were isolated through incubation with Dynabeads protein G (Thermo Fisher Scientific). Immunoblotting was performed on 1% of the immunoprecipitated samples to confirm successful crosslinking of myc-tagged Rbfox2 or Rbfox3 with histone H3. The remaining 99% of the immunoprecipitated samples were subjected to SDS-PAGE. After Coomassie blue staining, the protein bands in the gels were digested with trypsin. The recovered peptides were analyzed using Applied Biosystems 4700 matrix-assisted laser desorption/ionization time-of-flight/time-of flight analysis (MALDI-TOF/TOF) to acquire tandem mass spectrometry (MS/MS) spectra. A compiled protein database was searched for the peptide sequence.

2.4. Immunoblot analysis

HeLa cells were extracted using M-PER protein extraction reagent (Thermo Fisher Scientific) supplemented with complete protease inhibitor cocktail (Roche Diagnostics). For immunoblot analysis, the SDS-denatured and reduced protein samples were separated on a 4–12% polyacrylamide NuPAGE Bis-Tris gel (Thermo Fisher Scientific) and transferred onto a nitrocellulose membrane. Immunoblotting was performed as described previously [8]. The primary antibodies used in this study were mouse monoclonal anti-myc (Thermo Fisher Scientific), rabbit polyclonal anti-histone H3, rabbit polyclonal anti-histone H3K79me2, rabbit polyclonal anti-histone H3K9me3, mouse monoclonal anti-histone H3K27me3 (Abcam), and mouse monoclonal anti-GAPDH (Meridian Life Science, Memphis, TN, USA).

2.5. Modified histone peptide array

To screen for Rbfox2, Rbfox3, SFPQ, and NonO interactions with the modified histone tail, a modified histone peptide array kit (Active Motif) was used. Myc-tagged Rbfox2, Rbfox3, SFPQ, and NonO were synthesized using a transcription/translation (TNT)-Coupled Reticulocyte Lysate system (Promega, Madison, WI, USA). The modified histone peptide array was incubated with blocking buffer AM2, included in the kit, for 2 h at room temperature. Then, the histone peptide array was washed three times for 5 min with 5 ml wash buffer. Recombinant Rbfox2, Rbfox3, SFPQ, or NonO protein were added to 3 ml protein binding buffer (20 mM HEPES, 100 mM KCl, 1 mM EDTA, 0.1 mM DTT, 10% glycerol, pH 7.5), followed by incubation with the histone peptide array at 4 °C for 3 h. After washing three times with wash buffer, the histone peptide array was incubated with anti-myc-HRP antibody (Thermo Fisher Scientific) in 3 ml blocking buffer at 4 °C for 2 h. Binding of proteins to modified histone peptides was detected using the SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific).

2.6. RNA preparation and RT-PCR

Total RNA was extracted from HeLa cells using an RNeasy Mini Kit (Qiagen, Hilden, Germany) and reverse transcribed using the SuperScript III First-Strand System (Thermo Fisher Scientific) with random hexamers. PCR was performed using FastStart PCR Master Premix (Roche Diagnostics). The following PCR primers were used: 5′-GAGCAAGGCAGCATAGCACT-3′ and 5′-CCAACATGCCTTTACGCTTC-3′ for SPTAN1, 5′-GATCCTTCACGCAGGCTTTA-3′ and 5′-TCGGCTTTTTGGATTTCTTG-3′ for BRD9, 5′-TGCCAAAAGATGAAGACATTC-3′ and 5′-CCAGGGATAGCAGATTGGTG-3′ for ARHGAP21, and 5′-TGCTGTGTCTGTGACAGTGG-3′ and 5′-TGCATCTTTTCCTCTTCTGGA-3′ for EPHA5. The PCR products were separated using a 1.8% agarose gel electrophoresis. The inclusion of AEs was determined by calculating the intensity of each band using ImageJ.

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