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BS69/ZMYND11 Reads and Connects Histone H3.3 Lysine 36 Trimethylation Decorated Chromatin to Regulated Pre-mRNA Processing

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RG, LJZ, HC, FFJ, FL, SRM, HW, ZTW carried out essentially all the experiments described in this manuscript. JWP and YX were responsible for the bioinformatics analysis of the RNA-seq data. FFJ carried out part of IR and ES analyses. PYY was responsible for proteomics analyses while RTL, WQX and FZW carried out bioinfomatics analyses of the ChIP-seq data. HC carried out the MST experiments; HW performed BS69 ChIP-seq; ZTW expressed recombinant proteins from insect cells; SRM, JSQ, JYH and XDF contributed to RNA splicing regulation experimentations and discussion. XBS provided discussion and unpublished information on BS69 chromatin binding. FL, GYJS and YS directed all the experiments; YS conceived the project and co-wrote the manuscript with RG, FL and YX with input from GYJS, XDF and XBS.

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Yang Shi is a co-fonder of Constellation Pharmaceuticals, Inc. and a member of its scientific advisory board.

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Supplemental Information

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SUMMARY

BS69 (aka ZMYND11) contains tandemly arranged PHD, BROMO and PWWP domains, which are chromatin recognition modalities. Here we show that BS69 selectively recognizes histone variant H3.3 lysine 36 trimethylation (H3.3K36me3) via its chromatin-binding domains. We further identify BS69 association with RNA splicing regulators including the U5 snRNP components of the spliceosome such as EFTUD2. Remarkably, RNA-seq shows that BS69 mainly regulates intron retention (IR), which is the least well-understood RNA alternative splicing event in mammalian cells. Biochemical and genetic experiments demonstrate that BS69 promotes IR by antagonizing EFTUD2 through physical interactions. We further show that regulation of IR by BS69 also depends on its binding to H3K36me3-decorated chromatin. Taken together, our study identifies an H3.3K36me3-specific reader and a regulator of IR, and reveals a novel and unexpected role of BS69 in connecting histone H3.3K36me3 to regulated RNA splicing, providing significant new insights into chromatin regulation of pre-mRNA processing.

INTRODUCTION

Chromatin structure is organized by nucleosomes, each of which consists of an octamer composed of four core histones (H2A, H2B, H3 and H4) and ~147 bp DNA, which wraps around the octamer. The N-terminal tails of histones are extensively modified post-translationally, including phosphorylation, acetylation and methylation, which contribute to the modulation of gene expression. For instance, histone H3K4 trimethylation marks promoters that are either active or poised for transcription while H3K36 trimethylation is often associated with gene bodies and has been shown to suppress spurious gene transcription and promote transcriptional elongation (Carrozza et al., 2005; Li et al., 2002). More recently, H3K36 trimethylation has also been suggested to regulate RNA splicing (Luco et al., 2010; Pradeepa et al., 2012), but by and large regulation of RNA splicing by chromatin modifications remains poorly understood. RNA splicing is involved in the expression of most human genes, playing key roles in differentiation, cell cycle progression, and development. Mis-regulation of RNA splicing is frequently associated with various human diseases. Emerging evidence suggests that splicing is often co-transcriptional, thus splicing regulation is believed to be dependent not only on the core splicing factors binding to their pre-mRNA target sites but also on transcription-associated features such as local chromatin structure (Bentley, 2014). Out of the five known alternative splicing events (exon skipping, mutually exclusive exons, alternative donor site, alternative acceptor site and intron retention), intron retention (IR) is the least well understood. Gene transcripts whose introns are retained are subject to RNA nonsense-mediated decay (NMD) therefore increased IR is often correlated with reduced level of expression. Recent evidence suggests

that IR may be deployed as a potential mechanism for coordinated regulation of gene expression during differentiation and tumorigenesis (Wong et al., 2013; Yap et al., 2012). However the molecular mechanism by which IR is regulated is essentially unknown.

It's generally accepted that once histone modification patterns are laid down, they are recognized by various so called "reader' proteins, which help to execute the transcriptional and possibly RNA splicing programs. Perhaps not surprisingly, cells have developed a complex network of proteins that carry distinct protein motifs dedicated to recognizing histone modifications, including acetylation and methylation on canonical histones such as histone H3.1 and H3.2 (Ruthenburg et al., 2007; Taverna et al., 2007). The variant histone H3.3 differs from H3.1 by only five amino acids, but unlike H3.1 and H3.2, H3.3 is incorporated into chromatin in a DNA replication independent manner (Tagami et al., 2004), and is localized to active genes as well as telomeric regions (Goldberg et al., 2010; Jin and Felsenfeld, 2006; Li et al., 2002; Mito et al., 2005; Wong et al., 2009). Recently, H3.3 has gained significant attention owing to the identification of recurrent H3.3 point mutations in pediatric brain tumors (Behjati et al., 2013; Lewis et al., 2013; Schwartzentruber et al., 2012; Wu et al., 2012). But it remains unclear whether there are specific readers for H3.3 and if so what roles these readers may play.

Here we report the identification of BS69/ZMYND11, originally identified as an interacting protein of adenoviral E1A proteins (Hateboer et al., 1995), an H3.3-specific reader and a regulator of IR. BS69 possesses multiple histone reader modalities, including a plant homeodomain (PHD), a bromodomian and a PWWP domain, suggesting a potential role in recognizing histone modification(s). We demonstrate that BS69 functions as an H3.3-specific reader; preferentially recognizing H3.3K36me3 over H3.1K36me3 in vitro through its "reader" modules, in particular the PWWP domain. We show that BS69 localizes mainly to gene bodies in a manner that is dependent on the H3K36 trimethyltransferase, SETD2 (Edmunds et al., 2008), consistent with the in vitro binding data. Importantly, our biochemical and proteomics analyses revealed an association of BS69 with RNA spliceosome machinery, including the U5 snRNP such as EFTUD2, which is critical for spliceosome activation (Bartels et al., 2002). As predicated by the biochemical results, genome-wide RNA profiling analyses revealed hundreds of altered splicing events as a result of BS69 knockdown. Among them, IR is the main event that appears to be impacted by the BS69 loss, indicating that BS69 is a regulator of IR. ChIP-seq data show a statistically significant correlation between BS69 occupancy and IR regulation, suggesting a direct regulation. Further genetic rescue experiments demonstrate that binding of BS69 to EFTUD2 as well as H3K36me3 are necessary for BS69 to antagonize the activity of EFTUD2 and to promote IR. Our investigation thus discovered BS69 as a novel H3.3 specific reader, which regulates IR by targeting a specific step during spliceosome activation, connecting BS69-mediated IR regulation to H3.3K36me3-decorated chromatin.

RESULTS

The Genome-wide Distribution of BS69 Resembles That of H3K36me3

To better understand the function of BS69 during transcription, we sought to first identify BS69 genomic locations. ChIP-seq of endogenous BS69 yielded 18,406 potential BS69

binding events (covering 8,080 genes) compared with input, which appear to be enriched at both promoters (p-value < 2.2e-16, binomial test) and gene bodies (p-value < 2.2e-16, binomial test) relative to the distribution of random peaks in HeLa cells (Figure 1A and Figure S1A). Consistently, analysis of the genomic distributions of BS69 across an average Refseq gene also shows a high density of BS69 at promoters and gene bodies (Figure 1B), indicating that BS69 binds both promoters and gene bodies. Further bioinformatics analysis showed that BS69 binding to gene body represents the majority of BS69 genomic distribution events (73.3%, 13,492/18,406). Taken together, these findings suggest that BS69 mainly localizes to gene bodies but it may also play a role at promoters.

Exons are known to be preferentially marked with H3K36me3 relative to introns (Kolasinska-Zwierz et al., 2009). The ChIP-seq profiles of BS69 and H3K36me3 display similarities at gene bodies and exons (Figure 1B and 1C) but also differences at promoters (Figure 1B). Indeed, 91% (7,332/8,080) of the BS69-bound genes are also marked by H3K36me3 (Figure S1B), while only 31% (17457/56223) of all genes are marked by H3K36me3 in our ChIP-seq analyses. Representatives of such an overlap between H3K36me3 and BS69 binding are shown over three BS69 target genes (taken from the ChIP-seq data) (Figure 1D). This was confirmed by ChIP-qPCR, which showed BS69 gene body enrichment similar to that of H3K36me3 (Figure S1C). These findings suggest that BS69 binding to gene bodies may be dependent on H3K36me3. To address this possibility, we carried out ChIP analysis upon knockdown of SETD2, the main methyltransferase that mediates H3K36 trimethylation in mammalian cells (Edmunds et al., 2008; Kolasinska-Zwierz et al., 2009; Strahl et al., 2002). Knockdown of SETD2 not only reduced H3K36me3 signals, as expected, but also reduced BS69 gene body (not promoter) occupancy on a number of BS69 target genes (Figure 1E, F). As an additional control, knockdown of SETD2 did not affect BS69 expression (Figure S1D). These findings support the notion that BS69 gene body association is dependent on H3K36 trimethylation mediated by SETD2. However, H3K36me3 is known to primarily mark gene bodies, thus the mechanism by which BS69 is recruited to promoter remains unclear but may involve sequence-specific DNA binding factors, such as E2F6 reported previously (Velasco et al., 2006).

BS69 Preferentially Binds H3.3K36me3 In Vitro

BS69 carries three, tandemly arranged, putative chromatin modification recognition modalities (readers) including PHD, BROMO and PWWP domains (Figure 2A). The PWWP domains of BRPF1, DNMT3A, MSH6, and NPAC all have recently been shown to recognize H3K36me3 (Dhayalan et al., 2010; Li et al., 2013; Vezzoli et al., 2010), raising the possibility that BS69 dependency on SETD2 for gene body association may be accomplished by direct recognition of H3K36me3 through its PWWP domain. To address this possibility, we carried out in vitro binding assays using recombinant BS69 carrying all three putative reader modalities ($BS69_{50-401}$) and various histone H3.1 and H3.3 peptides. We found that $BS69_{50-401}$ (as well as full length BS69, Figure S2A) does not bind unmodified N-terminal tails of H2A, H2B, H3 or H4 (Figure 2B). Interestingly, it seems to preferentially bind H3.3 when lysine (K) 36 is tri-methylated (H3.3K36me3) (not mono- or dimethylated) but bind H3.1K36me3 much more poorly, if at all (Figure 2B and Figure S2A). As additional specificity controls, we show that $BS69_{50-401}$ failed to bind H3 peptides

methylated on H3K4, H3K9 and H3K79 as well as H4K20 methylated substrates (Figure 2B). These findings suggest that BS69 mainly recognizes H3K36 trimethylation on H3.3 in vitro. To explore this further, we established a HeLa cell line stably expressing a FLAG-tagged H3.3 and carried out ChIP-seq of FLAG tagged H3.3 as well as H3K36me3. Consistently, a comparison of the genome-wide distributions of H3.3, H3K36me3 and BS69 showed strong correlation, especially at TES (transcription termination site) regions (Figure S2B). By sequential ChIP assay, we have further confirmed that BS69 and H3K36me3 likely co-occupy regions in the gene bodies of BS69 target genes (Figure 2C).

The PWWP Domain is Critical for BS69 Binding to H3.3K36me3

Previous studies have identified amino acids critical for the PWWP domain of MSH6 and BRPF1 to bind H3K36me3 (Li et al., 2013; Vezzoli et al., 2010), which are conserved in the PWWP domain of BS69 (Figure S2C). Importantly, mutations of these amino acids, F293 and W294, to alanines abolished binding of $BS69_{50-401}$ to H3.3K36 trimethylated peptide as judged both by in vitro pull-down assays (Figure 2D), as well as by MST (Wienken et al., 2010) (Microscale Thermophoresis). Specifically, the Kd value for wildtype $BS69_{50-401}$ binding to H3.3K36me3 is 50.5 μM (Figure 2E), while in contrast, the Kd value for the interaction between the $BS69_{50-401}$ PWWP point mutant and the H3.3K36me3 histone peptide was significantly higher (Figure 2F, Kd=277 μM), supporting the pulldown result that the PWWP point mutant has a significantly reduced ability to bind H3.3K36me3. Consistently, the Kd values for wildtype $BS69_{50-401}$ binding to H3.1K36me3, H3.1K36me0 and H3.3K36me0 were undetectable (Figure S2D). These results suggest that BS69 preferentially binds H3.3K36me3 in a manner that is dependent on the PWWP domain. In addition to histone peptides, full length BS69 also binds nucleosome purified from HeLaS cells (Figure S2E, top panel). Consistently, we found chromatin association of BS69 to the gene bodies of 7 target genes in vivo is essentially abrogated when its PWWP domain is deleted (Figure 2A, 2G) (BS69 ΔPWWP was expressed at a similar level and localized to the nucleus, Figure S2F and data not shown). Taken together, these findings indicate that BS69 directly binds H3.3K36me3 and associates with chromatin in a PWWP domain-dependent manner. Interestingly, deletion of the PHD or the Bromo domain also resulted in a loss of BS69 association with its target genes (assayed by ChIP-qPCR of their binding to the same 7 BS69 target genes) (Figure S2G), suggesting that in addition to PWWP, the PHD and Bromo domains of BS69 may also participate in the recognition of H3.3K36me3 and BS69 chromatin association in vivo. Of course, we cannot exclude the possibility that deletions could have resulted in improper folding of the mutant proteins.

Phosphorylation at Serine 31 Disrupts the Binding of BS69 to H3.3K36me3

The exquisite specificity of BS69 binding to H3.3K36me3 was unexpected given the fact that the entire H3.3 differs from the canonical H3.1 by only five amino acids (Hake and Allis, 2006). Furthermore, the only difference between the H3.3 and H3.1 histone peptides used in our binding assays is the amino acid at position 31 where H3.3 has a serine (S) and H3.1 contains an alanine (A) residue. We speculated that S31 must be playing an important role in this highly specific interaction between H3.3 and BS69 and therefore modifications such as phosphorylation may impact BS69 binding to H3.3K36me3. Indeed, the H3.3K36me3 peptide with S31 phosphorylation (H3.3S31PK36me3) was significantly

compromised in its ability to bind BS69 (Figure 2H and Figure S2H, Kd ~ 1.5mM). Interestingly, phosphorylation of H3.3S31 has been found to be enriched during mitosis at telomere ends in mES cells and pericentric heterochromatin after differentiation (Hake et al., 2005; Wong et al., 2009), suggesting that BS69 binding H3.3 may be subject to regulation in vivo, and may play a role in these specific biological processes.

Chromatin-Bound BS69 Associates with Multiple Components of Spliceosome

H3K36 trimethylation has recently been demonstrated to play a role in regulating transcriptional elongation and alternative splicing (Li et al., 2002; Luco et al., 2010; Pradeepa et al., 2012; Schaft et al., 2003) but the underlying mechanisms remain incompletely understood. To investigate a potential role of BS69 in these processes, we established a HeLa cell line expressing FLAG-HA-tagged BS69 and purified the tagged BS69 by sequential immunoprecipitation using FLAG and HA antibodies, respectively. The BS69 purified from soluble nuclear fraction appears to mainly associate with factors involved in transcriptional regulation, including DNA binding transcription factors and cofactors, as well as chromatin regulators, such as E2F6, MLL, NSD1, KDM3B and BRG1, suggesting that BS69 may directly regulate transcription by working with these proteins (Table S1). In addition to purifying BS69 from the soluble nuclear fraction, we also purified chromatin-bound BS69 by dissociating it from the nuclear pellet with micrococcal nuclease (MNase). Interestingly, the chromatin-bound BS69 mainly associates with a very different set of proteins that are involved in regulating RNA splicing (Figure 3A). The presence of many of these proteins in the purified BS69 from chromatin fraction can be further confirmed by Western blot analysis (Figure S3A). These BS69 associated proteins can be grouped into at least two classes, i.e., the U5 snRNP proteins (EFTUD2, PRPF8, and SNRNP200) and Serine and Arginine-rich (SR) proteins (SRSF1, PNN). Reciprocal immunoprecipitation using an EFTUD2 antibody, incubating with FLAG immunoprecipants purified from HeLa cells stably expressing FLAG-HA-tagged BS69, not only brought down EFTUD2 and tagged BS69, but also PRPF8 and SNRNP200, suggesting that these proteins are likely to be in the same complex (Figure 3B). We further demonstrated by co-immunoprecipitation that endogenous BS69 also interacts with EFTUD2 (Figure 3C).

BS69 Directly Binds EFTUD2, a component of the U5 snRNP

We next wished to determine which splicing regulators directly bind BS69. Using recombinant splicing factors purified from insect Sf9 cells, we only detected interactions of SRSF1 and EFTUD2 with BS69 in vitro (Figure S3B, left panels). We further demonstrated that EFTUD2, but not SRSF1, directly binds BS69 in a RNA and DNA independent manner (Figure S3B, right panels). Using various deletion mutants, we identified that the C-terminal region of BS69 (aa 401-602 (B2)) is important to mediate this interaction (Figure 3D and 3E, compare B1 and B2 with BS69 FL). Further analyses narrowed the interacting region to amino acids 556-562 whose removal from the full length BS69 significantly reduced BS69 interaction with EFTUD2 (Figure 3D, F, compare B6 with BS69 FL), suggesting that the region encompassing aa 556-562 is necessary for BS69 interaction with EFTUD2.

RNA splicing is a multi-step process involving many splicing factors and U1, U2, U4, U5 and U6 snRNPs (Wahl et al., 2009). EFTUD2 is involved in unwinding U4/U6 RNA during

spliceosome activation, an essential step for spliceosome activation (Bartels et al., 2002). The fact that BS69 interacts with EFTUD2 and other splicing factors predicts that BS69 may also interact with the spliceosomal snRNPs. We immunoprecipitated FLAG-tagged BS69 and then interrogated the presence of the spliceosomal U1, U2, U4, U5, and U6 snRNAs by Northern blotting. As shown in Figure 3G, tagged BS69 co-immunoprecipitated U4, U5, and U6 snRNA (compare lane 4 with 3). We next used real-time PCR to quantify each of the co-precipitated snRNA and found that BS69 mainly associates with U4 snRNA, but also to a lesser extent with U5 and U6 (Figure 3H). In contrast, EFTUD2 antibody mainly co-precipitated U5 snRNA (Figure S3C). Collectively these findings support a potential role for BS69 in regulating RNA splicing.

BS69 Primarily Promotes Intron Retention

To determine whether BS69 regulates splicing, we performed RNA-seq using mRNA samples isolated from two independent BS69 shRNA treated HeLa cells (Table S2). MATS (Multivariate Analysis of Transcript Splicing) program detected a number of differential alternative splicing (AS) events upon BS69 knockdown. As shown in Figure 4A, we identified 217 significant AS events in BS69 shRNA-1 versus control, and 391 significant AS events in BS69 shRNA-2 versus control (FDR<10%). The higher number of events detected in the BS69 shRNA-2-treated sample is likely to be due to better knockdown efficiency of BS69 shRNA-2 (Figure S4A and S4B). Importantly, we find a high degree of data overlap between the two independent RNA-seq experiments (with two independent BS69 shRNAs), i.e., a total of 89 AS events passed the genome-wide significance cutoff (FDR<10%) in both RNA-seq experiments (Figure 4A). Among the 89 AS events regulated by BS69, we identified 61 intron retention (IR) and 16 exon skipping (ES) events. Given that the overall frequency of IR is the lowest among the different types of AS events in mammalian genes (Sakabe and de Souza, 2007), it is striking that more than 2/3 of the AS events regulated by BS69 are IR events.

The analysis described above on BS69-regulated IR events was done for ~6,000 documented IR events (Ensembl transcript database). To perform a truly genome-wide, unbiased search for more BS69-regulated IR events, we expanded our analysis to ~92,000 introns, which are human introns without partial overlap with exons of sense or antisense transcripts, in order to avoid confounding signals from overlapping transcripts. This new analysis identified 590 and 1,027 BS69-regulated IR events, respectively, from the same two RNA-seq experiments (Figure 4A, numbers are in parenthesis). Importantly, a total of 248 BS69-regulated IR events are shared by both RNA-seq experiments, which are distributed among 219 genes (after correction for genome-wide multiple testing, FDR<10%) (Figure 4A). It should be noted that RNA-seq detection of differential alternative splicing is known to have substantial false negatives especially for moderately or lowly expressed genes(Dittmar et al., 2012), as we need high sequencing coverage for the events of interest to call statistically significant changes in splicing levels. Here we adopted a conservative criterion to define BS69-regulated IR events, by requiring that the events were called significant in both shRNA experiments, and this likely would underestimate the impact of BS69 on IR regulation. To evaluate this possibility further, we examined the concordance of the two shRNA experiments (Figure S4C). As expected, the 248 IR events called significant in both shRNA

experiments showed a strong correlation in their shRNA-induced changes in IR levels (Pearson correlation r=0.96). Interestingly, targets called significant in only one of the two shRNA experiments also showed a strong correlation between the two shRNA experiments (n=342, r=0.70; and n=779, r=0.79, respectively; see Figure S4C), which were further supported by additional RT-qPCR confirmation (Figure S4D). These findings suggest that the actual number of IR events regulated by BS69 is likely to be significant higher than 248. Strikingly, among the 248 BS69-regulated IR events, 238 (96.0%) had decreased levels of IR upon BS69 knockdown, suggesting that BS69 functions primarily to promote retention of specific introns.

BS69-Regulated IR Events are Significantly Enriched for BS69 Binding

Further analysis showed that the 248 BS69-regulated IR events are significantly enriched for BS69 binding (38.3%) as compared to BS69-independent IR events (28.9%) (Fisher's Exact Test $P=5.4e^{-3}$) (Figure S4E). Binding of BS69 to ten of these genes (whose IR events are regulated by BS69) is further demonstrated by ChIP-qPCR (Figure S4F), supporting a direct involvement of BS69 in their IR regulation. As the sensitivity of ChIP-seq may be limited, we employed ChIP-qPCR to determine whether some BS69 binding signals at its regulated IR events may have been missed as a result. Indeed, we were able to find BS69 binding at 5 out of 6 randomly selected BS69-regulated IR events that are not detected by ChIP-seq (Figure S4G, Left panel), while no BS69 binding was detected at another set of 6 randomly selected, BS69-independent IR sites (Figure S4G, Right panel). These results suggest that ChIP-seq may have only detected strong BS69 binding events, and as a result we are underestimating the actual BS69 binding events as well as the correlation of BS69 binding with BS69 regulated IR events.

In addition to identifying BS69 enrichment at its regulated IR, about 62.1% of BS69 regulated IR introns are also H3.3 enriched (assessed by ectopic expressing H3.3-FLAG) as opposed to the control (53%) (Fisher's Exact Test $p=1.2e^{-2}$) (Figure S4H), and 88.7% BS69 regulated IR introns are decorated by H3K36 trimethylation as to the control (79.9%) (Fisher's Exact Test $p=1.2e^{-3}$, Figure S4I). Consistent with the idea that BS69 is recruited to specific genomic locations to regulate alternative splicing events by binding H3.3K36me3 via its PWWP domain, the average ChIP-seq densities of BS69, H3K36me3 and H3.3 at the 248 BS69 regulated introns are significantly higher than those at the control BS69-independent set (Figure S4J) further supporting the notion that BS69 regulates IR events directly at genomic regions that are enriched for trimethylated histone H3.3K36.

We next asked whether there are specific features associated with BS69-regulated IR events that are directly bound by BS69, we compared the 95 BS69-bound IR events that respond to BS69 depletion with the 272 BS69-bound IR events that do not. Our bioinformatics analyses found that BS69-bound IR introns that respond to BS69 depletion are significantly longer (median length 1,025bp vs 548bp, P= 0.0003 by two-sided Wilcoxon test) and have significantly stronger 5′ splice sites (median 5′ splice site score 8.94 vs 8.05, P= 0.004 by two-sided Wilcoxon test, calculated by MAXENT)(Yeo and Burge, 2004). In contrast, we did not observe any other unique features at the 3′ splice sites and any significant reported motifs for RNA-binding proteins and splicing factors in the first 250bp and the last 250bp of

the introns (Anderson et al., 2012; Ray et al., 2013). How do such features contribute to BS69 regulation of IR remains an interesting question for future investigation.

BS69 Knockdown Results in an Increase of the Steady-State mRNA Level of Its Target Genes Containing BS69-regulated Introns

Intron-retaining transcripts often contain a premature stop codon, which triggers nonsense mediated decay pathway (NMD) (Braunschweig et al., 2013; Lejeune and Maquat, 2005). As shown by the heatmaps (Figure 4B and Figure S4K), most of the genes that contain BS69-regulated introns showed an increase in their steady-state mRNA levels upon knockdown of BS69. This was further confirmed by ChIP-qPCR of six selected genes (Figure 4C, top panel). Consistently, the BS69-regulated RNA transcripts by and large showed an increased level in the cytoplasm and a reduced level in the nucleus, respectively (Figure 4C, middle and lower panels), suggesting that the unspliced RNAs are retained/degraded in the nucleus while the spliced RNAs are exported to the cytoplasm, resulting in an overall increase in gene expression. This finding supports the notion that BS69 regulates IR and further suggests that BS69 may coordinately regulate a select group of genes by regulating their IR. Importantly, among different types of RNA alternative splicing, including ES and alternative splice site usage, IR is the least well understood, and therefore identification of BS69 as a regulator that primarily controls IR represents an important step towards understanding the molecular mechanisms of IR regulation. Given the vast number of introns in the genome, the finding that BS69 impacts a limited number of introns indicates a highly specific role of BS69 in regulated RNA splicing.

BS69 Antagonizes EFTUD2 in IR regulation Dependent on Its Physical Interaction with EFTUD2

To confirm RNA-seq findings, we carried out RT-qPCR and RT-PCR assays for BS69-regulated alternative splicing events, including 14 selected from the original 61 IR events identified from the ~6,000 introns and an additional 4 from the expanded analysis (248 IR events), as well as 4 ES events, respectively. As shown in Figure S5A–C, these AS events are readily detectable upon BS69 knockdown. The regulation of IR by BS69 is not limited to HeLa cells as we have observed the same impact of BS69 on IR events in a number of other cell lines, including the human lung cancer cell line A549 and human lung fibroblast HFL-1 (Figure S5D). To rule out potential shRNA off-target effects, we carried out rescue experiments by adding back RNAi-resistant BS69. Indeed, wildtype BS69 readily rescued these AS events (Figure 5A, B), indicating that the observed change in splicing is due to the knockdown of BS69. Importantly, reintroduction of an EFTUD2-interaction defective mutant of BS69 ($BS69_{\Delta 556\text{-}562aa}$), which was expressed at a comparable level to that of wildtype BS69, localized in the nucleus (Figure S5E and S5F) and retained the ability of H3.3K36me3 binding (Figure 5C), failed to do so (Figure 5A, B), suggesting that EFTUD2 interaction is critical for BS69 to regulate RNA splicing.

We next examined whether these splicing events were also regulated by EFTUD2. Strikingly, knockdown of EFTUD2 had an opposite effect to that of BS69 knockdown on either the IR or ES events. Specifically, we examined the same 14 IR events and found that loss of EFTUD2 resulted in an increase in IR as opposed to a decrease when BS69 was

knocked down (Figure 5D, Figure S5G). Similarly, we also examined the same 4 ES events and found that knockdown of EFTUD2 likewise had the opposite effects (Figure 5E, Figure S5H). These findings suggest that BS69 and EFTUD2 may function antagonistically in regulating RNA splicing and raises the possibility that BS69 may promote IR by suppressing the core splicing machinery.

SETD2 Knockdown affects BS69-Dependent AS Events Similar to That of BS69 Knockdown

As mentioned earlier, H3K36 trimethylation has been suggested to play a role in RNA splicing but exactly how it regulates splicing remains incompletely understood. Since BS69 binds its target genes in a SETD2-dependent manner, which mediates H3K36 trimethylation, we asked whether the same AS events regulated by BS69 are similarly regulated by SETD2. In our analyses, the same set of 14 IR events (Figure 6A and Figure S6A) and 2 ES events (Figure 6B) regulated by BS69 showed a similar regulation (12 out of 14 IR events) by SETD2 knockdown. Furthermore, SETD2 knockdown also reduced H3K36me3 levels, as expected, as well as BS69 binding to these genes (Figure S6B, and S6C). These findings suggest that BS69 may be an important downstream player in mediating the function of H3K36me3 to regulate RNA splicing. To investigate this possibility further, we carried out genetic rescue using the wildtype BS69 and a PWWP point mutant ($BS69_{FW}$) defective in binding to H3K36me3 but expressed at a comparable level to that of the wildtype BS69 and localized to the nucleus (Figure S6D and 6E). As shown in Figure 6C and 6D, while wildtype BS69 restored the normal splicing patterns of both the IR and ES events, the $BS69_{FW}$ mutant failed to do so, indicating that the ability of BS69 to regulate RNA splicing is also dependent on its binding to chromatin via H3K36me3.

DISCUSSION

We have shown that BS69 regulation of RNA splicing occurs in a SETD2-dependent manner, which mediates H3K36 trimethylation. The specificity that BS69 exhibits towards H3.3K36me3 is quite remarkable as the entire H3.3 and H3.1 differ only by five amino acids with only one amino acid difference (amino acid 31) in the vicinity of K36. Amino acid 31 is a serine in H3.3 and alanine in H3.1, suggesting that phosphorylation may play a role in regulating BS69 recognition of H3.3K36me3. Indeed our result shows that S31 phosphorylation significantly impedes BS69 binding to H3.3K36me3 in vitro. Given that BS69 mainly binds H3.3K36me3 in vitro and that the ability of BS69 to regulate splicing is dependent on its ability to bind H3K36me3, our findings strongly suggest a role for BS69 downstream of H3.3K36 trimethylation, thus linking K36 trimethylation of the histone variant H3.3 to RNA splicing regulation. Although binding of BS69 to H3.1K36me3 in vitro was barely detectable, we cannot formally exclude the possibility that in vivo, BS69 may bind H3.1H36me3 in some genomic regions and therefore the effects of BS69 on RNA splicing could also be in part mediated by H3.1K36me3. Importantly, our genome wide ChIP-seq location analysis of BS69 and H3.3 showed that not all H3.3 are bound by BS69, predicting the existence of additional H3.3K36me3-specific readers. Furthermore, not all BS69 directly bound targets displayed alterations in IR regulation in response to BS69 knockdown, suggesting that additional unknown factors may influence the ability of BS69 to

regulate IR. Our study further identified an antagonistic relationship between BS69 and the core RNA splicing machinery, supporting the model whereby BS69 promotes IR by antagonizing the activity of core splicing machinery through physical interactions with snRNPs such as EFTUD2. Interestingly, while physically associated with the U5 snRNA protein EFTUD2, unexpectedly, BS69 mainly interacts with U4 snRNA, suggesting that BS69 may suppress splicing by sequestering U4 snRNA, whose release is a prerequisite for spliceosome activation (Wahl et al., 2009).

A very recent study by Wen et al (Wen et al., 2014) reported binding of BS69/ZMYND11 to H3.3K36me3. In that study, the authors generated and analyzed the co-crystal structure of BS69 reader domains bound to H3.3K36me3 and identified critical contacts that involve both the PWWP and Bromo domains, as well as S31, thus providing atomic level understanding of the interaction between BS69 and H3.3K36me3. Wen et al also reported that loss of BS69 impacts transcriptional elongation, consistent with the previously proposed model that elongation rates regulates alternative splicing (Wen et al., 2014). We compared our BS69-regulated IR event genes (both BS69-bound and unbound IR genes) with the BS69 elongation-regulated genes (268 up-regulated genes) but found no significant overlap. Since these two studies were conducted in two different cell lines, HeLa (this study) and U2OS (Wen), it remains to be determined whether there is a functional relationship between BS69-regulated elongation versus IR or other RNA alternative splicing events. However, our genetic rescue result demonstrating that the direct interaction of BS69 with EFTUD2 is critical for BS69 to regulate IR favors the model that BS69 directly participates in IR regulation.

Another recent study investigated the mechanism by which H3K36me3 regulates RNA splicing and identified MRG15, which binds H3.1K36me3 via its chromodomain and recruits the Polypyrimidine Tract Binding protein (PTB) to regulate alternative splicing of the human fibroblast growth factor receptor 2 gene, thus making the first connection between chromatin and regulated RNA splicing (Luco et al., 2010). Interestingly, a separate study found that PTB coordinates neuronal gene expression through regulation of IR events specific to a subset of neuronal genes (Yap et al., 2012). However, whether this regulation is connected to chromatin via MRG15 remains unclear. Another reported connection between H3K36me3 and alternative splicing is the PSIP1 short isoform (p52) (Pradeepa et al., 2012). Similar to our findings, PSIP1 (p52) was reported to be associated with a large number of splicing factors, including SR proteins, U5 snRNP proteins, hnRNP proteins and DEAD/H box helicases. Knockdown of PSIP1 (p52) resulted in 95 alternative exon usage events from a survey of 40,443 exons in 7,715 genes with one or more predicted alternative transcripts. However, whether PSIP1 (p52) regulates IR has not been investigated. Thus, our findings identify for the first time a novel regulator that controls IR, mediated primarily by K36 trimethylated histone variant H3.3. Collectively, these findings suggest that MRG15, PSIP1 (p52) and BS69 may represent separate pathways that link chromatin to RNA splicing regulation.

In yeast, regulated IR has been shown to be important for coordinated expression of meiotic and ribosomal protein genes (Averbeck et al., 2005; Cremona et al., 2011; Moldon et al., 2008; Munding et al., 2010; Parenteau et al., 2011). However, the biological function of

regulated IR in mammals is unclear at the present time. Interestingly, recent studies showed that regulated IR helps to coordinate gene expression during neuronal differentiation and granulopoiesis suggesting a possible role for IR in differentiation (Wong et al., 2013; Yap et al., 2012). Alterations in IR and ES have also been identified as major RNA splicing events in breast cancer (Eswaran et al., 2013), suggesting that altered IR and ES events may also contribute to tumorigenesis. In this context, it's interesting to note that recent studies identified histone H3.3 point mutations, which either directly (K36M) or indirectly (G34R/V) abolished H3.3K36 trimethylation, as driver mutations in a number of different tumors (Behjati et al., 2013; Lewis et al., 2013; Schwartzentruber et al., 2012; Wu et al., 2012). Furthermore, SETD2 and H3K36me3 have also been reported to be frequently mutated and lost in renal cell carcinoma and pediatric high-grade glioma (Duns et al., 2010; Fontebasso et al., 2013). Additionally, our data that H3.3S31 phosphorylation impedes BS69 binding suggests the importance of S31 in regulating BS69 function, and raises the question of whether S31 may play a role in tumorigenesis and is also subject to mutational events in cancers. The ongoing efforts of deep sequencing of cancer tissues and identifying the signaling pathway that regulates H3.3S31 phosphorylation will provide insight into these issues. We speculate that BS69 may participate in tumorigenesis through regulation of RNA splicing events such as IR and ES and that an impaired RNA splicing regulation as a result of inactivation of the SETD2-H3K36me3-BS69 axis and/or the H3.3S31 signaling pathway may contribute to tumorigenesis of various types of cancers.

METHODS

The Microscale Thermophoresis (MST) analysis

The determination of the binding capacity of BS69 to histone peptides was performed by microscale thermophoresis (MST) according to manufacturer's instruction with 20% LED and 20% MST power (Wienken et al., 2010) (Nano Temper, Monolith NT.115). Fluorescently labeled $BS69_{50\text{-}401}$ or $BS69_{50\text{-}401}$ mutant were used as tracer. The final concentrations of peptides ranged from 122 nM to 2 mM.

BS69 protein complex purification and reciprocal immunoprecipitation

Tandem affinity purification was performed as described previously (Ogawa et al., 2002) except that HeLaS nuclear extract was treated with MNase at 37°C for 3 min. For reciprocal immunoprecipitation in Figure 2b, FLAG-purified BS69 complex was precipitated using either anti-EFTUD2 or IgG antibody. The immunoprecipitants were analyzed by Western blotting using corresponding antibodies.

snRNA Association analyses

HeLa cells transfected with FLAG-BS69 plasmid grown in 150 cm plates were covered with ice-cold PBS buffer and subjected to UV-irradiation (150 mJ/cm^2) for crosslinking. Immunoprecipitation was performed using anti-FLAG beads. After washing, RNA was extracted with TRIzol, and analyzed by qRT-PCR and northern blot.

Chromatin immunoprecipitation (ChIP) and ChIP-seq

ChIP assays were carried out as previously described (Lan et al., 2007). ChIP-Seq was performed according to Illunima's protocol. The FASTQ data were mapped to the human genome (hg19) using Bowtie, and significant enrichments were identified by MACS2.0 using Broad Peak mode with Q-value = 0.05 as a cutoff to call peaks from the aligned results (Zhang et al., 2008). Gene lists bound by BS69 or H3K36me3 were generated based on Ensembl version 65 gene annotation.

RNA-seq and bioinformatics analysis

HeLa cells were infected with lentiviruses carrying either control or BS69 shRNA. After puromycin selection for 7 days, RNA samples were prepared using TRIZOL and subjected to 100bp × 2 non-strand-specific paired-end RNA-seq by Genome Center, WuXi App Tec. We used MATS(Shen et al., 2012) (http://rnaseq-mats.sourceforge.net/; version 3.0.7) to identify BS69-regulated differential alternative splicing events corresponding to all five types of alternative splicing patterns.

Splicing Assay

Total RNA was subjected to gDNA eraser treatment for 5 min, followed by reverse transcription using random primers for each sample (Takara, PrimeScript™ RT reagent Kit with gDNA Eraser). For intron retention analyses, the cDNA samples were subjected to qPCR analyses with primers designed for the retained introns (In) and one if the adjacent exons (All), the IR ratios (Inclusive %) were calculated as $100\% * 2^{[Ct_{(All)} - Ct_{(In)}]}$. For exon skipping analyses, the same cDNA samples were subjected to PCR amplification with primers corresponding to the flanking exons of the alternatively spliced exons and the PCR products were analyzed by TBE gel electrophoresis and quantitated by Kodak Image Station 4000R, the exon skipping ratios (Inclusive %) were calculated as $100\% * Intensity_{(Inclusive)} / [Intensity_{(Inclusive)} + Intensity_{(Exclusive)}]$.

Figure 1. BS69 shows a similar genomic distribution pattern as H3K36me3. Related to Figure 1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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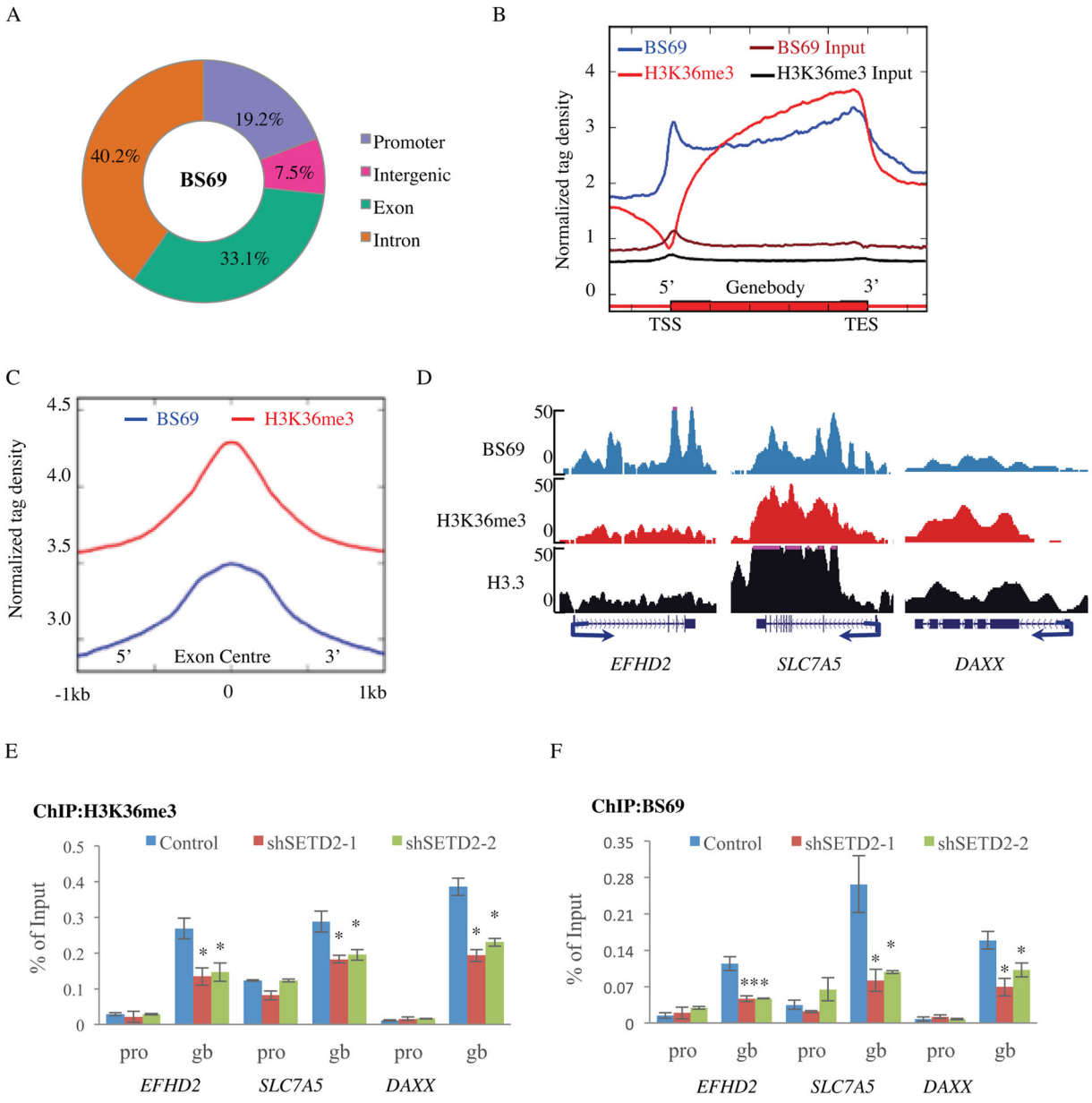

Figure 1. BS69 shows a similar genomic distribution pattern as H3K36me3

(A) Genomic distribution of BS69 peaks (18,406) in HeLa cells. **(B)** Normalized tag density of BS69 and H3K36me3 along the transcription unit. Each gene body is represented from 0% (TSS) to 100% (Jorgensen et al.) on the X-axis. Normalized Tag density is plotted from 30% upstream of TSS to 30% downstream of TES. **(C)** Averaged exonic occupancies of BS69 and H3K36me3. The centers of exons are aligned at position 0 with 1kb of both upstream and downstream sequences included in the analysis. **(D)** Representatives of BS69, H3K36me3 and H3.3-FLAG ChIP-seq peaks in HeLa cells. Arrows denote TSS and transcription orientation. **(E)** and **(F)** Occupancies of H3K36me3 and BS69 at selected gene bodies (gb), but not promoters (pro), are reduced in SETD2 KD HeLa cells. Data are

represented as mean ± SEM from 3 biological replicates (E, F), *P<0.05, **P<0.01. See also Figure S1.

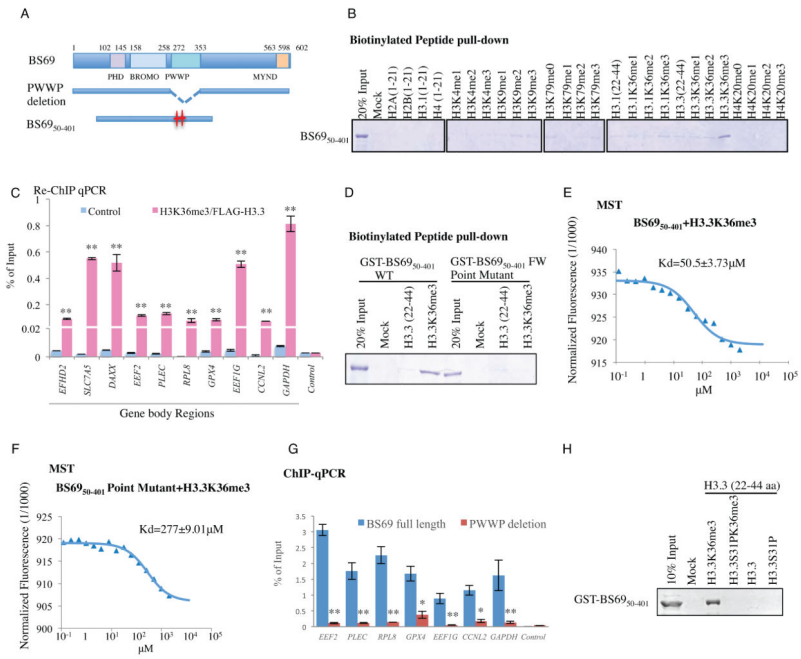

Figure 2. BS69 binds H3.3K36me3 in vitro via its PWWP domain

(A) Schematic diagrams of human BS69 and mutants carrying PWWP deletion and point mutations denoted by red stars. **(B)** Recombinant GST-BS69$_{50-401}$ specifically recognizes H3.3K36me3 peptide in the biotinylated histone peptide pull-down assays. **(C)** Sequential ChIP using H3K36me3 and anti-FLAG antibodies in HeLa cells overexpressing H3.3-FLAG shows co-occupancy of H3K36me3 and H3.3-FLAG at indicated locales of BS69 target genes. **(D)** GST-BS69$_{50-401}$ FW point mutant (F293A, W294A) loses the ability to bind H3.3K36me3 in vitro. **(E)** and **(F)** MicroScale Thermophoresis analysis determined the Kd of GST-BS69$_{50-401}$ WT (E) interaction with H3.3K36me3 to be 50 μM. The Kd value of the interaction between the BS69 PWWP point mutant and H3.3K36me3 (F) was determined to be 277 μM. **(G)** HA-ChIP and q-PCR demonstrated that the ectopically expressed WT BS69 but not the BS69 PWWP deletion mutant binds the BS69 targets (identified through BS69 ChIP). **(H)** The binding of GST-BS69$_{50-401}$ to H3.3K36me3 peptide is abolished by phosphorylation at position S31. Data are represented as mean ± SEM from 3 biological replicates (C,G), *P<0.05, **P<0.01. See also Figure S2.

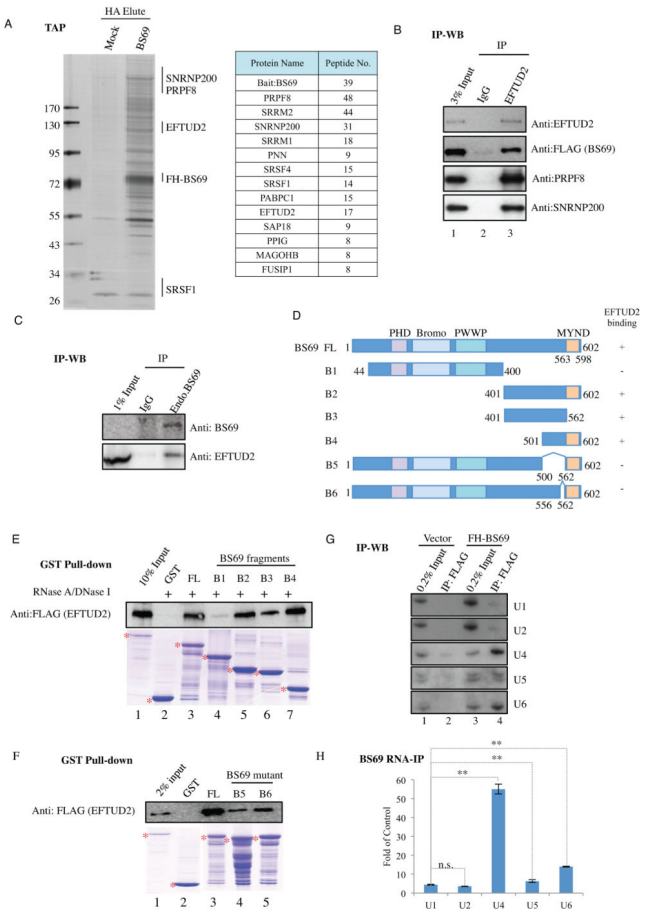

Figure 3. BS69 interacts with splicing factors

(A) Left panel: TAP purified FLAG-HA-BS69 protein complex was resolved by gradient SDS-PAGE and visualized by silver staining. The positions of the tagged BS69 (FH-BS69) as well as some of the RNA splicing factors are indicated on the right. Right panel: polypeptides identified by tandem mass spectrometry. **(B)** Reciprocal immunoprecipitation by EFTUD2 antibody incubated with FLAG immunoprecipitants purified from HeLa cells stably expressing FLAG-HA-BS69 brought down FLAG-HA-BS69 as well PRPF8 and SNRNP200. **(C)** Interaction between endogenous BS69 and EFTUD2 was confirmed by co-immunoprecipitation using HeLa nuclear extract. **(D)** Schematic representation of full length and various truncated forms of BS69. Amino acid positions are indicated. **(E)** and **(F)** Interactions between EFTUD2 and full length and various truncated forms of BS69 were determined by GST pull-down assays. Recombinant BS69 proteins and FLAG-EFTUD2 were purified from *E. coli* and insect cells, respectively. FL: full length. **(G)** and **(H)**, Interactions between FLAG-BS69 and snRNAs were determined by Northern blotting (G) and RT-qPCR (H). Data are represented as mean ± SEM from 3 biological replicates, **P*<0.05, ***P*<0.01; n.s., not significant. See also Figure S3, Table S1.

A

Type of AS events	BS69sh1 vs. control	BS69sh2 vs. control	Overlap
Exon Skipping (ES)	69	107	16
Mutually exclusive exon (MXE)	13	11	2
Alternative 5' splice site (A5SS)	11	17	6
Alternative 3' splice site (A3SS)	13	31	4
Intron retention(IR)	111 (590)	225 (1027)	61 (248)

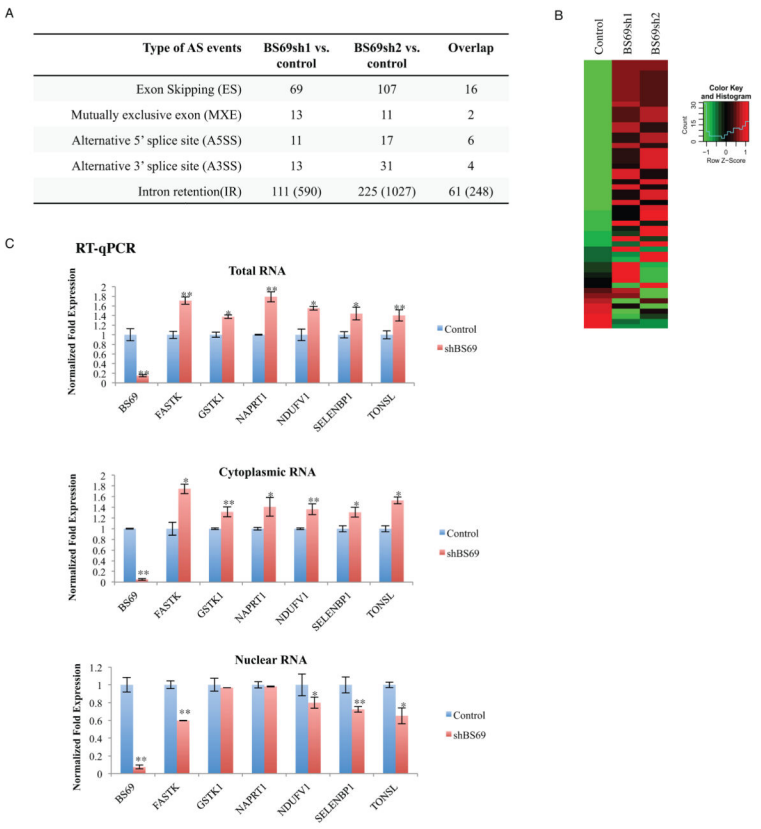

Figure 4. BS69 mainly regulates intro retention

(A) Summary of altered splicing (AS) events in HeLa cells upon BS69 KD detected by RNA-seq from three biological replicates. Two independent BS69 shRNAs were used. **(B)** Heatmap comparison of the mRNA levels of genes whose IR events are regulated by BS69 in the presence and absence of BS69. **(C)** RT-qPCR analyses of mRNA levels of six BS69-regulated IR genes in different cellular compartments. Data are represented as mean ± SEM from 3 biological replicates, **P*<0.05, ***P*<0.01; n.s., not significant. See also Figure S4, Table S2.

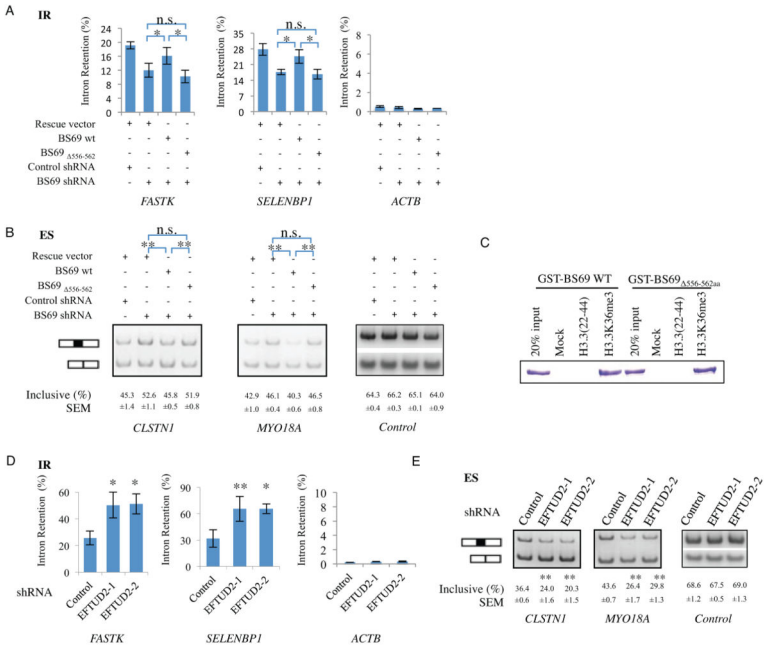

Figure 5. BS69 regulates intron retention and exon skipping events by antagonizing EFTUD2 through physical interaction

(A) and (B) Wild type but not EFTUD2 interaction defective mutant (Δ556-562) rescued the alteration of IR (Intron Retention, panel A) and ES (Exon Skipping, panel B) caused by BS69 knockdown. **(C)** EFTUD2-interaction defective mutant of BS69 ($BS69_{\Delta 556\text{-}562aa}$) retains H3.3K36me3 binding ability at a comparable level as that of wildtype in histone peptide pull-down assay in vitro. **(D)** and **(E)** Knockdown of EFTUD2 caused an increase in IR (D) and an altered ratio of ES (panel E) of target genes in HeLa cells. Data are represented as mean ± SEM from 3 biological replicates (A, B, D, E), **P*<0.05, ***P*<0.01; n.s., not significant. See also Figure S5.

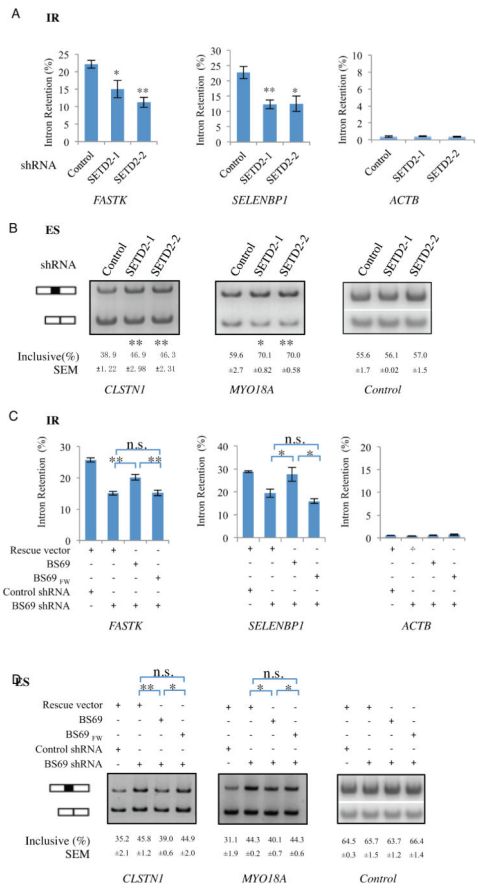

Figure 6. Binding H3K36me3 is important for BS69 to regulate alternative splicing

(A) and **(B)** Knockdown of SETD2 by two independent shRNAs in HeLa cells caused a decrease in IR (A) and an altered ratio of ES (B) of BS69 target genes. **(C)** and **(D)** Wild type but not the PWWP FW point mutant rescued the alteration of IR (C) and ES (D) caused by BS69 knockdown. Data are represented mean ± SEM from 3 biological replicates, **P*<0.05, ***P*<0.01; n.s., not significant. See also Figure S6.