Cyclin-dependent kinase 1 (CDK1) and CDK2 have opposing roles in regulating interactions of splicing factor 3B1 with chromatin

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Abstract:
Splicing factor 3B1 (SF3B1) is a core splicing protein that stabilizes the interaction between the U2 snRNA and the branch point in the mRNA target during splicing. SF3B1 is heavily phosphorylated at its N terminus and a substrate of cyclin-dependent kinases (CDKs). Although SF3B1 phosphorylation coincides with splicing catalysis, the functional significance of SF3B1 phosphorylation is largely undefined. Here, we show that SF3B1 phosphorylation follows a dynamic pattern during cell cycle progression that depends on CDK activity. SF3B1 is known to interact with chromatin, and we found that SF3B1 maximally interacts with nucleosomes during G1/S and that this interaction requires CDK2 activity. In contrast, SF3B1 disassociated from nucleosomes at G2/M, coinciding with a peak in CDK1-mediated SF3B1 phosphorylation. Thus, CDK1 and CDK2 appear to have opposing roles in regulating SF3B1–nucleosome interactions. Importantly, these interactions were modified by the presence and phosphorylation status of linker histone H1, particularly the H1.4 isoform. Performing genome-wide analysis of SF3B1–chromatin binding in synchronized cells, we observed that SF3B1 preferentially bound exons. Differences in SF3B1 chromatin binding to specific sites, however, did not correlate with changes in RNA splicing, suggesting that the SF3B1–nucleosome interaction does not determine cell cycle–dependent changes to mRNA splicing. Our results define a cell cycle stage–specific interaction between SF3B1 and nucleosomes that is mediated by histone H1 and depends on SF3B1 phosphorylation. Importantly, this interaction does not seem to be related to SF3B1’s splicing function and, rather, points toward its potential role as a chromatin modifier.

Introduction:
SF3B1, also known as SAP155, is a core component of the U2 small nuclear ribonucleoprotein complex (snRNP) that is essential for pre-mRNA splicing. The U2 snRNP interacts with the branch point (BP)
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adenosine during splicing catalysis, and SF3B1 is known to stabilize the interaction between the U2 snRNA and the BP adenosine (1,2). Whole genome sequencing has identified recurrent SF3B1 mutations in multiple neoplastic processes. SF3B1 hotspot mutations are most commonly found in myelodysplastic syndrome (MDS), specifically in the refractory anemia with ring sideroblasts (RS) subtype, a clonal hematopoietic disorder characterized by anemia and characteristic morphologic atypia with immature erythroid progenitors containing peri-nuclear, iron laden mitochondria (3,4). SF3B1 mutations are also found in uveal melanoma, chronic lymphocytic leukemia (CLL) and breast cancer, albeit at lower frequencies (3-7). While much attention has been focused on understanding the role of SF3B1 hotspot mutations in disease pathogenesis (8-12), many questions regarding the function and regulation of SF3B1 during splicing, with particular relevance to the development of therapeutic strategies for the treatment of SF3B1-related disorders, remain unanswered.

In addition to its role during splicing catalysis, SF3B1 interacts with chromatin via chromatin remodeling proteins (13,14) and may co-regulate certain histone modifications (15). A large body of evidence suggests that RNA splicing occurs co-transcriptionally and that components of the spliceosome machinery interact with chromatin during transcription (16,17). Kfir et al. recently demonstrated that SF3B1 interacts with nucleosomes near exons in an RNA-independent manner. Using genome-wide occupancy data for SF3B1 in combination with splicing analyses and knock-down approaches, the authors contend that SF3B1 occupancy of chromatin determines splicing outcomes (18).

SF3B1 has long been known to be a substrate protein for cyclin-dependent kinases (CDKs), though current understanding of the function of CDK-dependent phosphorylation is relatively sparse in light of the number of phosphosites potentially utilized by these kinases (Table 1). Most known CDK sites reside within the N-terminus domain of SF3B1 outside the HEAT motif-containing region, whereas relatively few reside in the C-terminal portion. SF3B1 associates with cyclin E and both CDK1 and CDK2 are known to phosphorylate SF3B1 (19,20). SF3B1 is also phosphorylated by DYRK1A, which contributes to the regulation of pre-mRNA splicing (21,22). Phosphorylation at specific amino acid residues within the SF3B1 N-terminus are known to be important in mediating its interaction with other nuclear proteins, including NIPP1 (20), and phosphorylation of SF3B1 is temporally associated with active splicing (23,24). The presence of phosphorylated SF3B1 in spliceosomes during active splicing is particularly interesting given evidence suggesting that splicing is coordinated with cell cycle progression and follows a cell cycle stage-specific program (25). Given that CDKs are also known to interact with chromatin and phosphorylate various chromatin-associated proteins (26,27), we hypothesized that phosphorylation of SF3B1 during cell cycle progression regulates its interaction with nucleosomes and in turn could influence cell cycle stage-specific splicing of exons and introns in proximity to SF3B1-bound nucleosomes.

In this manuscript, we report that SF3B1 phosphorylation is dynamic during cell cycle progression and dependent on CDK activity. The cell cycle-dependent phosphorylation of SF3B1 differentially regulates its interaction with nucleosomes during G1/S and G2/M, with CDK2 and CDK1 playing opposing roles in regulating the interaction. Data from in vitro binding studies demonstrated that the interaction between SF3B1 and mono-nucleosomes is dependent on the presence and phosphorylation status of linker histone H1. Using chromatin immunoprecipitation followed by high-throughput sequencing (ChIP) and RNA sequencing (RNA-seq) of synchronized cells, we mapped regions of the genome of cell cycle stage-specific occupancy of SF3B1 within genes and determined the extent to which these
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correlated with changes in splicing. Our findings in toto provide new insights into the role of phosphorylation of SF3B1 by CDKs and suggest that splicing-independent functions may be regulated by cell cycle-dependent SF3B1-chromatin binding.

Results:

SF3B1 phosphorylation is dynamic during cell cycle progression.
SF3B1 contains numerous serine or threonine residues juxtaposed to a proline at the +1 position, representing potential sites for phosphorylation by proline-directed kinases, including cyclin-CDKs, glycogen synthase kinase 3 (GSK3), and mitogen-activated protein kinases (MAPK). Although several mass spectrometry (MS) studies have demonstrated SF3B1 phosphorylation sites clustering within the N-terminal domain of the protein (Table 1), the functional significance of SF3B1 phosphorylation is largely undefined. SF3B1 phosphorylation is coupled with splicing catalysis (23); specifically, phosphorylation of threonine 313, a cyclin E-CDK2 substrate, is associated with active splicing (24). Cyclin E-CDK2-mediated phosphorylation of SF3B1 at threonines 244, 248 and 313 has been shown to mediate interaction with NIPP1, a protein phosphatase that localizes within the nucleus (20). In order to understand first whether SF3B1 phosphorylation changes during cell cycle progression, we used mitotic arrest to synchronize two human cell lines, HeLa and K562, and measured SF3B1 expression and serine/threonine phosphorylation (using anti-phosphoserine/threonine-proline (pSer/Thr-Pro) and anti-phospho-threonine 313 (pSF3B1) (24) antibodies by immunoblot. We found a highly dynamic pattern of SF3B1 phosphorylation through cell cycle progression, wherein SF3B1 is phosphorylated at low levels in G1, declining further at G1/S, but is highly phosphorylated at G2/M (Figures 1A and 1B). Based on previous MS data that demonstrated CDK-mediated phosphorylation of SF3B1, we hypothesized that the increased phosphorylation of SF3B1 in G2/M was dependent on CDK1 activity. We further hypothesized that the subsequent decrease in SF3B1 phosphorylation following mitotic exit was mediated by phosphatases active during re-entry into G1. To test these hypotheses, we first treated HeLa and K562 cells arrested in G2/M with Purvalanol-A, a specific CDK1 (CDC2) inhibitor (28,29). Brief pharmacologic inhibition of CDK1 (at 0.5 and 4 hours) completely blocks detectable SF3B1 phosphorylation (Figures 1C and 1D). Inhibition of phosphatase activity in G1/S cells using okadaic acid (OA) treatment that inhibits both PP2A and PP1 phosphatases (30) results in increased SF3B1 phosphorylation (Figure 1E). Together, these results demonstrate that SF3B1 phosphorylation is dynamic during the course of cell cycle progression, peaking at G2/M in a CDK-dependent manner and then decreasing due to phosphatase activity as cells progress through G1/S.

SF3B1-nucleosome interactions are dynamic during cell cycle progression.
Work by several groups has demonstrated that SF3B1 interacts with chromatin or chromatin-associated proteins (13,14). Of note, Kfir et al. (18) showed that SF3B1 associates with mono-nucleosomes near exons and positively influences splicing of these occupied exons. Moreover, splicing is known to be coordinated with cell cycle progression, such that the expression and splicing of specific transcripts are regulated in a stage-specific manner (25). In light of these data and our finding of dynamic phosphorylation of SF3B1, we hypothesized that changes to phosphorylation of SF3B1 during cell cycle progression influences its association with nucleosomes. To test this, we first prepared whole cell lysates and
mono-nucleosome-enriched fractions from mitotically arrested and synchronized HeLa and K562 cells. Mono-nucleosome fractions containing nuclear proteins and chromatin were prepared by isolating nuclei and digesting them with micrococcal nuclease (MNase), such that the resulting DNA fragments are approximately 150 base pair (bp) size (length of DNA wrapped around a single nucleosome is 147 bp) (Figure 2A inset). We compared SF3B1 protein abundance in nucleosome-enriched versus whole cell lysates of synchronized cells. In nucleosome-enriched lysates, SF3B1 levels are significantly reduced in G2/M, when SF3B1 phosphorylation peaks (Figure 1), and increased in G1 and G1/S. However, SF3B1 protein abundance remained unaltered between G1, G1/S and G2/M in whole cell lysates (Figures 2A-2B), supporting our hypothesis that cell cycle dependent phosphorylation influences SF3B1 association with nucleosomes.

Next, we asked whether the change in chromatin association was specific to SF3B1 or if other spliceosome proteins also exhibit cell cycle-dependent chromatin association. We hence determined the total abundance and nucleosome association of SF3B2, a component of the U2 snRNP (similar to SF3B1), and also for U1-70k (or snRNP70), a subunit of a distinct snRNP (U1). Both SF3B2 and U1-70k followed the same cell cycle-dependent chromatin association as SF3B1 (Figure 2C). Next, to confirm that this cell cycle-related change in splicing factor abundance within chromatin-enriched lysates was directly linked to a change in interaction with nucleosomes, we examined the association of SF3B1, SF3B2 and U1-70k with the core nucleosome protein, histone H3, in synchronized cells. As expected, SF3B1, SF3B2 and U1-70k did not associate with histone H3 in G2/M cells but showed increased histone association in G1/S (Figure 2D-2E). Treatment of G1/S HeLa cells with OA resulted in decreased SF3B1-nucleosome interaction (Figure 2F). Since OA treatment did not alter cell cycle distribution (not shown), this result suggests that the dynamics of SF3B1 phosphorylation and not merely the progression of cells through G1/S regulate SF3B1-nucleosome interactions.

Splicing is a co-transcriptional process, and hence it is possible that the observed cell cycle-dependent interaction between spliceosome proteins and chromatin are dependent on nascent, transcribed RNA or snRNA within spliceosome. To address this, we tested the effect of RNAse on the interaction of the spliceosome proteins with histone H3 and chromatin. While RNAse-A treatment significantly diminished the interaction between histone H3 and SF3B2 and U1-70k in G1/S-phase, the SF3B1 interaction with nucleosomes remained intact, consistent with an RNA-independent mode of association during G1/S (Figure 2G), consistent with previous reports (18).

Taken together, our data demonstrate that SF3B1 interactions with nucleosomes are dynamic during cell cycle progression. SF3B1 interacts with nucleosomes during G1 and G1/S stages where it demonstrates lower level phosphorylation, whereas its interaction with nucleosomes is greatly diminished during G2/M when CDK1 activity peaks, suggesting that SF3B1 phosphorylation during G2/M directly contributes to its dissociation from nucleosomes. Importantly, unlike other spliceosomal proteins we tested (both U2 snRNP components and non-U2 components), this cell cycle-dependent interaction of SF3B1 with histone is RNA-independent and hence likely not solely due to transcription.

**SF3B1-chromatin interactions are dependent on CDK activity.**

We next hypothesized that dynamic SF3B1-nucleosome interactions are dependent on CDK-dependent phosphorylation. We tested this hypothesis by pharmacologic inhibition of CDK1 activity in G2/M and of CDK2 activity in G1/S. Treatment of G2/M cells with Purvalanol-A restored the interaction between SF3B1 and nucleosomes (Figure 3A). In contrast, inhibition of CDK2 activity in G1/S by treatment with Roscovitine (31)
results in diminished SF3B1-nucleosome interaction (Figures 3B and 3D), suggesting that CDK1 and CDK2 play opposing roles in regulating SF3B1-nucleosome interactions during cell cycle progression. Another kinase, DYRK1A, is also active during G1 and is known to phosphorylate SF3B1 at threonine 434 (21). We hence evaluated the role of DYRK1A in regulating SF3B1-nucleosome interactions. Inhibition of DYRK1A by treatment with a selective inhibitor, Harmine (32), did not affect SF3B1-nucleosome interaction, while the inhibitor did elicit an increase in cyclin D2 protein, which is normally destabilized in response to DYRK1A-mediated phosphorylation (33) (Figures 3C-3D). These data suggest that CDK1 and CDK2 play a selective role in regulating SF3B1-nucleosome interactions during cell cycle progression.

Previous studies have implicated specific threonine residues within the N-terminal region of SF3B1 in mediating its interaction with other proteins, including NIPP1 (20). While our data from pharmacologic inhibition of kinase activity addressed the role of overall phosphorylation, the role of phosphorylation of SF3B1 at specific amino acid residues in regulating its interaction with nucleosomes is unclear. Mass spectrometry (MS) has identified CDK-dependent phosphorylation of SF3B1 at multiple sites in addition to those CDK sites previously described as regulating the NIPP1 interaction, including threonines 142, 211, 257, 261, 426 and 434, all of which have +1 prolines (34). We hence mutated all 6 of these MS-identified threonine residues (Table 1) (21,34). On probing the SF3B1-nucleosome interactions in K562 cells expressing wild-type (WT) or the compound phospho-mutant SF3B1 (6A), we found partially decreased nucleosome interaction of 6A suggesting that aggregate phosphorylation of SF3B1's N-terminus is required for regulation of SF3B1-nucleosome interactions (Figure 3E). Consistent with this result we found only a modest decrease in cyclin E-CDK2 mediated phosphorylation of 6A when compared to WT (Supplementary Figure 1), likely reflective of the large number of N-terminal SF3B1 phosphosites contributing to overall phosphorylation of the protein. Indeed, single point mutations in SF3B1 phosphosites showed no alteration in nucleosome interactions, whereas a more extensive mutant containing 25 alanine substitutions within predicted S-P/T-P phosphosites was not expressed sufficiently in cells to study (data not shown).

**SF3B1-chromatin interactions in vitro depend on linker histone H1 and phosphorylation of both SF3B1 and H1.**

A large number of nucleosome binding proteins influence chromatin organization. SF3B1 is part of the SF3B complex, which in turn forms part of a large multi-protein complex, the U2 snRNP that is also known to contain chromatin-associated proteins (35). It is thus unclear whether the interaction between SF3B1 and nucleosomes is direct or depends on other proteins known to complex with nucleosomes. To help determine which of these scenarios is more likely in vivo, we reconstituted SF3B1-nucleosome binding in vitro. Given that the majority of serine/threonine residues identified as potential substrates for CDKs in SF3B1 are located within an N-terminal domain that excludes the HEAT-containing domain (Table 1), we subcloned this fragment (1-500 aa or SF3B11-500) for use in these studies. Examining previously published mass spectrometry-based SF3B1 interactome datasets, we noted that histone H1 is part of the complex that contains SF3B1 and other nucleosome proteins (18,36). H1 is involved in chromatin organization during cell cycle progression and is a known CDK substrate during cell cycle progression (37). Using co-immunoprecipitation, we first confirmed that SF3B1, histone H1 and histone H3 are in complex in vivo (Figure 4A). The SF3B11-500 with N-terminal GST tag was then expressed in *E.coli* and isolated for in vitro binding assays (Figure 4B). We confirmed that SF3B11-500 could be phosphorylated by purified cyclin E-CDK2 in vitro (Figure 4C). Using an in vitro binding assay in which
SF3B1^{1-500} is first phosphorylated in vitro using purified cyclin E-CDK2 and then incubated with purified HeLa mononucleosomes in the presence or absence of purified calf thymus histone H1, also phosphorylated in vitro by cyclin E-CDK2, we found phosphorylation of both histone H1 and SF3B1^{1-500} is required for robust binding of the latter to purified mono-nucleosomes. However, prolonged SF3B1^{1-500} incubation with cyclin E-CDK2 in vitro led to reduced binding (Figure 4D, upper panel), suggesting that lower amounts of SF3B1 phosphorylation are permissive to chromatin interactions, whereas hyper-phosphorylated SF3B1 impedes the interaction.

Histone H1 has multiple isoforms: Histones H1.1, H1.2, H1.3, H1.4 and H1.5 are ubiquitously expressed in a cell cycle-dependent manner, while histone H1.0 and the H1.X isoforms are expressed mainly in differentiated cells independent of cell cycle (37). Among the ubiquitous isoforms, H1.4 undergoes phosphorylation during S-phase and mitosis. H1.4 phosphorylation has been detected at serine residues 172 and 187 in interphase cells, and additional phosphorylations at serine 27 and threonines 18, 146 and 154 have been detected in mitotic cells (38). Importantly, H1.4 is known to maintain S-phase progression, as selective depletion of it leads to a decrease in cell cycling and S-phase (39). Also, with affinity purification and MS, H1.4 was recently identified as a specific interacting partner of SF3B1 (36). We thus hypothesized that H1.4 promotes SF3B1-nucleosome interactions during G1/S. To test this, we performed separate in vitro binding assays of SF3B1-nucleosome interactions using either native calf thymus histone H1 isoform mix, purified human histone H1.4 or histone H1.0. As shown in Figure 4E (upper panel), we found that the presence of purified histone H1.4 most enhances the interaction between SF3B1^{1-500} and mono-nucleosomes compared to the other linker histone preparations. This result suggests that the dynamics of linker histone isoform interactions with mono-nucleosomes during cell cycle progression contribute to the regulation of SF3B1-chromatin interactions.

Moreover, we found that phosphorylation of H1.4 by CDK2 results in the most robust SF3B1-mononucleosome interaction in vitro (Figure 4E). Notably, H1 in complex with H3 undergoes an increase in phosphorylation from G1/S to G2/M in vivo (Figure 4F), suggesting that while H1.4 phosphorylation promotes SF3B1^{1-500} interaction with mono-nucleosomes in vitro, high levels of phosphorylation on both SF3B1 and H1, evident during G2/M, diminish SF3B1-nucleosome interactions. In this way, phosphorylation of both SF3B1 and linker histone H1 may enable switch-like regulation of U2-chromatin interaction during cell cycle progression, by permitting histone binding when both are phosphorylated at lower levels (e.g. during G1/S) and disfavoring binding when they are hyper-phosphorylated during progression to G2/M.

Integrated analysis of SF3B1 genome occupancy and cell cycle-dependent splicing.

Our biochemical data demonstrate that SF3B1-nucleosome interactions are regulated by CDK-dependent phosphorylation of SF3B1 and linker histone H1. To understand the functional significance of this SF3B1-nucleosome interaction (and specifically how this interaction relates to stage-specific splicing programs), we performed SF3B1 chromatin immunoprecipitation followed by sequencing (ChIP-Seq) and paired-end RNA sequencing (RNA-Seq) using G1/S and G2/M HeLa cells. Mono-nucleosome-enriched fractions from synchronized HeLa cells (G1/S and G2/M, in biological duplicates) were immunoprecipitated with antibodies against SF3B1. Illumina compatible libraries were prepared from DNA fragments that immunoprecipitated with SF3B1, sequenced and analyzed per informatics pipeline detailed in Materials and Methods. Input libraries were prepared prior to IP. Control libraries (nonspecific antibody control and input) were also prepared. Resulting reads were pre-processed and mapped to the hg19
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Genome assembly using STAR Aligner. Correlation coefficients of biological replicate samples were confirmed prior to further downstream analysis (Supplemental Figure 2). Peak calls and comparative analysis were performed using HOMER (40).

G1/S cells had higher specific SF3B1-binding peaks relative to G2/M (7386 vs. 4096) (Supplementary Files 1 and 2) in comparison to input controls. We also found that in G1/S cells, SF3B1 peaks are over-represented within exons, compared to G2/M cells, in which SF3B1 was found to occupy primarily intergenic sites (Figure 5A). Similar to a previous observation by Kfir et al. (18), the density of peaks in exons when normalized to proportional length in human genome was significantly higher in exons than those in introns or intergenic areas in G1/S, but not G2/M cells. (Figure 5B). A modest enrichment of exonic reads was noted relative to surrounding intronic regions in both G1/S and G2M (Supplementary Figure 3). G1/S and G2/M peaks were largely distinct, with only 167 (1.45%) peaks overlapping between the two datasets (Supplementary File 3). G1/S-specific peaks also showed a distribution skewed towards intragenic regions, within both exons and introns (Figure 5C), consistent with our observations for total peaks in G1/S. When analyzed for DNA motifs, both G1/S and G2/M datasets showed highly significant enrichment of non-overlapping DNA motifs (Supplementary Figure 4). In summary, our ChIP-Seq results confirm our biochemical data showing increased affinity of SF3B1 for chromatin during G1/S compared to G2/M. Importantly, the increased binding in G1/S is primarily in transcribed intragenic areas (including transcription start and end sites).

Next, paired-end RNA-Seq was performed using matched samples from synchronized HeLa cells that were used for the ChIP-seq analysis. cDNA libraries were prepared from polyA enriched total RNA and paired end sequencing was performed (75 bp, average depth of approx.122 million). Reads were aligned to hg19 using STAR aligner and first analyzed for changes in gene expression by Cufflinks (41). In line with previous reports, significant differences were found in gene expression profiles of G1/S and G2/M (1248 differentially expressed genes enriched in cell cycle and cell death/apoptosis pathways; Supplementary Files 4 and 5). We then used the rMATS (42) algorithm to identify alternative splicing events in G1/S and G2/M samples. A total of 48,105 alternative splicing events (distributed across 5 different types - A3SS: alternative 3' splice site; A5SS: alternative 5' splice site; RI: retained intron; MXE: mutually exclusive exon; SE: skipped exon) were detected between G1/S and G2M, of which 2340 met statistical cut-off (Supplementary File 6 and Figure 5D, FDR < 0.05 and delta PSI > 5%). Our results are in agreement with previous reports that suggested specific changes to RNA transcriptome linked to change in cell cycle (25).

To test our hypothesis that SF3B1-chromatin occupancy affects cell cycle specific transcriptomes by influencing splicing and/or gene expression in proximity to the site of gene occupancy, we determined overlap between genes enriched in three different datasets: G1/S-specific chromatin binding (ChIP-Seq), gene expression (Cufflinks) and alternative splicing (rMATS analysis). To determine if SF3B1 occupancy of chromatin positively influences transcription of genes in those regions or change alternative splicing, we determined overlap of genes co-localized with G1/S-specific peaks with transcripts or alternative splicing events increased in G1/S compared to G2/M. In contrast to our expectations, as shown in Figure 5E, there appeared to be little overlap among these gene-sets, not reaching statistical significance by Fisher’s exact test. To examine the possibility that SF3B1 binding of nucleosomes decreased transcription or alternative splicing, a similar analysis was performed for transcripts and splicing events that decreased in G1/S compared to G2/M (Supplementary Figure 5). Little overlap was evident in this analysis as well. Importantly, no G2/M peaks were found to be specific when compared to G1/S when analyzed with similar HOMER parameters. Taken together,
while RNA found in the sensitive effect on polymerase mediated CDKs regulate SF3B1-chromatin interactions.

8 genome-wide integrative analyses suggest that change in... change in... two studies: Kfir et al’s conclusions were not based on correlation of genome occupancy and changes in splicing, threonine 187, a CDK substrate motif, is substrate motifs. Phosphorylation at cycle and contains a number of CDK phosphorylation of linker histone H1, and have a positive effect on transcription (45). Similar to SF3B1, H1.4 also undergoes phosphorylation during cell cycle and contains a number of CDK substrate motifs. Phosphorylation at threonine 187, a CDK substrate motif, is associated with RNA polymerase mediated transcription (38) and phosphorylation of H1.4 at serine 27 inhibits HP1 binding and potentially heterochromatin formation (43). Given that SF3B1 and histone H1 exist in a complex together, are both phosphorylated during cell cycle progression by CDKs and the recognized role of H1 in regulating transcription and chromatin organization, the potential for SF3B1 and histone H1 acting collaboratively on chromatin in regulating chromatin structure and transcription should be further explored. Additionally, phosphorylated H1.4 has been found in the nucleolus, suggesting a sequestration function to regulate the activity of binding partners (46). Studies utilizing high-resolution imaging to examine whether H1 isoforms interact with SF3B1 differentially during cell cycle progression and alter SF3B1 subcellular localization may provide additional mechanistic detail on the regulation of SF3B1-chromatin interactions.

We have also learned that SF3B1-nucleosome interactions in vitro are dependent on the presence and phosphorylation of linker histone H1, especially the H1.4 isoform. Our in vivo data and previously reported MS-based studies (36) demonstrate that H1.4, which plays an important role in the regulation of chromatin organization and transcription, interacts with SF3B1. H1.4 methylation at lysine 26 promotes its binding to HP1 and L3MBTL1 leading to the recruitment of these factors, with roles in heterochromatin formation and transcriptional repression, to chromatin (43,44). H1.4 acetylation at lysine 34, on the other hand, is known to co-localize on active promoters with a transcriptional activator, TAF1, and have a positive effect on transcription (45). Similar to SF3B1, H1.4 also undergoes phosphorylation during cell cycle and contains a number of CDK substrate motifs. Phosphorylation at threonine 187, a CDK substrate motif, is associated with RNA polymerase mediated transcription (38) and phosphorylation of H1.4 at serine 27 inhibits HP1 binding and potentially heterochromatin formation (43). Given that SF3B1 and histone H1 exist in a complex together, are both phosphorylated during cell cycle progression by CDKs and the recognized role of H1 in regulating transcription and chromatin organization, the potential for SF3B1 and histone H1 acting collaboratively on chromatin in regulating chromatin structure and transcription should be further explored. Additionally, phosphorylated H1.4 has been found in the nucleolus, suggesting a sequestration function to regulate the activity of binding partners (46). Studies utilizing high-resolution imaging to examine whether H1 isoforms interact with SF3B1 differentially during cell cycle progression and alter SF3B1 subcellular localization may provide additional mechanistic detail on the regulation of SF3B1-chromatin interactions.

Discussion:

SF3B1 is phosphorylated by CDKs (19,20) and SF3B1 phosphorylation is known to be both associated with active spliceosomes and important for mediating protein-protein interactions (23,24). Our data now provide a new function for SF3B1 phosphorylation by CDKs in regulating SF3B1-nucleosome interactions. SF3B1 phosphorylation at proline-directed serine/threonine sites peaks at G2/M, which causes it to dissociate from nucleosomes. Consistent with the varied functions of cyclin B/CDK1 in mitosis initiation, we speculate that CDK1-dependent SF3B1 disassociation from chromatin may be necessary to ensure efficient chromatin condensation, given the size of the spliceosome, which could serve as an impediment to this process if remaining associated.

We have also learned that SF3B1-nucleosome interactions in vitro are dependent on the presence and phosphorylation of linker histone H1, especially the H1.4 isoform. Our in vivo data and previously reported MS-based studies (36) demonstrate that H1.4, which plays an important role in the regulation of chromatin organization and transcription, interacts with SF3B1. H1.4 methylation at lysine 26 promotes its binding to HP1 and L3MBTL1 leading to the recruitment of these factors, with roles in heterochromatin formation and transcriptional repression, to chromatin (43,44). H1.4 acetylation at lysine 34, on the other hand, is known to co-localize on active promoters with a transcriptional activator, TAF1, and have a positive effect on transcription (45). Similar to SF3B1, H1.4 also undergoes phosphorylation during cell cycle and contains a number of CDK substrate motifs. Phosphorylation at threonine 187, a CDK substrate motif, is associated with RNA polymerase mediated transcription (38) and phosphorylation of H1.4 at serine 27 inhibits HP1 binding and potentially heterochromatin formation (43). Given that SF3B1 and histone H1 exist in a complex together, are both phosphorylated during cell cycle progression by CDKs and the recognized role of H1 in regulating transcription and chromatin organization, the potential for SF3B1 and histone H1 acting collaboratively on chromatin in regulating chromatin structure and transcription should be further explored. Additionally, phosphorylated H1.4 has been found in the nucleolus, suggesting a sequestration function to regulate the activity of binding partners (46). Studies utilizing high-resolution imaging to examine whether H1 isoforms interact with SF3B1 differentially during cell cycle progression and alter SF3B1 subcellular localization may provide additional mechanistic detail on the regulation of SF3B1-chromatin interactions.

To understand the functional outcomes of cell cycle dependent SF3B1-nucleosome interactions, we performed ChIP-seq and RNA-seq in synchronized cells. ChIP-seq revealed a cell cycle stagespecific pattern of SF3B1 chromatin occupancy with increased binding within transcribed regions (exons, introns as well as transcription start and termination sites) in G1/S compared G2/M with genome-wide analysis (Figure 5A). While RNA-Seq of matched samples revealed cell cycle stagespecific splicing and expression changes, in agreement with a previous report (25), we found no evidence that SF3B1 occupancy on chromatin within these intragenic regions influences their expression level or alternative splicing in corresponding transcripts in a cell cycle stage-specific manner. Our findings were surprising since they differ from a previous report by Kfir et al. (18) that suggested a direct, positive effect on the splicing of exons located near SF3B1-bound regions of nucleosomes. It is important to note some differences between the design of these two studies: Kfir et al’s conclusions were not based on correlation of genome occupancy and changes in splicing,
but rather by comparing exon utilization after SF3B1 knock-down or trichostatin A treatment. Our results are based on direct correlation of SF3B1 occupancy to changes to the transcriptome in two cell cycle states with the maximal identified difference in SF3B1-nucleosome interaction. We did not observe significant alterations in cell cycle kinetics or apoptosis with overexpressing the compound phosphosite-mutant (6A) SF3B1 versus wild-type (Supplemental Figure 6), though a relatively limited impact of these amino acid substitutions on overall phosphorylation (Supplemental Figure 1) and histone binding (Figure 3E) may account for the absence of an obvious phenotype with overexpression of this mutant protein. Further investigations into the functional importance of SF3B1-nucleosome interactions and their possible role in regulation of transcription (independent of splicing) and downstream biology are warranted.

SF3B1 associates with a number of proteins involved in control of chromatin modifications and transcription. For example, SF3B1 interacts with NIPP1 in a phosphorylation-dependent manner, and NIPP1 is implicated in the regulation of EZH2 occupancy at promoter regions (47, 48). SF3B1 and PHF5a are found together in the SF3B complex and PHF5a has been shown to regulate RNA polymerase-dependent transcription of pluripotency genes (35). Furthermore, it has been shown that splicing promotes the recruitment of methyltransferase HYPB/Setd2 to chromatin resulting in histone H3 lysine 36 methylation at actively transcribed intron-containing genes (15). A potential non-splicing related role for cell cycle-dependent SF3B1-nucleosome interactions might be to enable key chromatin modifications by facilitating the recruitment of chromatin modifying proteins and complexes to transcriptionally active chromatin. Thus, our data may point to a previously unappreciated role for CDKs: coordinators of chromatin modifications via the modulation of SF3B1-nucleosome interactions during cell cycle progression.

**Cell Culture:**

HeLa cells were cultured in DMEM (Gibco Life Technologies) supplemented with 10% fetal bovine serum (FBS) (Gemini Bioproducts) and 1X penicillin/streptomycin (Pen/Strep) (Gibco Life Technologies). K562 cells were cultured in RPMI-1640 (Gibco Life Technologies) supplemented with 10% FBS and 1X Pen/Strep. HeLa and K562 cells were synchronized in G2/M by culturing in their respective growth media with 40 ng/mL Nocodazole (Sigma-Aldrich, M1404) for 20 hours. For G1 and G1/S synchronizations, HeLa cells were cultured in growth media with 40 ng/mL Nocodazole for 24 hours. For G2/M synchronizations, HeLa cells were cultured in growth media with 40 ng/mL Nocodazole for 16 hours. For all synchronization experiments, cycle status was verified using flow cytometry as described below. Cells were then washed with growth media once and cultured in growth media for 20 hours (G1) or 24 hours (G1/S). K562 cells were cultured in growth media with 40 ng/mL Nocodazole for 20 hours, washed once with growth media and cultured in growth media for 4 hours (G1) or 7.5 hours (G1/S). Pharmacologic inhibitors were added to G1/S synchronized cells for the indicated times following release from Nocodazole treatment at the following concentrations: Roscovitine (Sigma-Aldrich, R7772) 20 µM; Purvalanol-A (Sigma-Aldrich, P4484) 15 µM; Harmine (Sigma-Aldrich, 286044) 5 µM; Okadaic Acid (OA) (Sigma-Aldrich, O7760) 20 nM. Retroviral supernatants were prepared by transfecting Phoenix cells (G. Nolan, Stanford) by calcium phosphate precipitation method. K562 cells were spinoculated with retroviral supernatants for 90 minutes at room temperature (RT).

**Flow cytometry:**

For cell cycle analysis, cells were fixed with 75% ethanol and incubated at 4°C overnight. Cells were then washed once with 1X phosphate-buffered saline (PBS) (Gibco, Life Technologies) and stained with 10 µg propidium iodide (PI) (Sigma-Aldrich 81845), 0.1% bovine serum albumin (BSA) (Sigma-Aldrich A2153) and 10 µg RNase-A (Sigma-
Aldrich, R6513) in 1X PBS. Flow cytometry was performed on LSR II flow cytometer (BD Biosciences) and analysis used FlowJo software. Apoptosis detection was performed using Annexin V Apoptosis Detection Kit I (BD Biosciences).

**Plasmids:**
Codon optimized FLAG-tagged SF3B1 (11) was cloned into BamHI and EcoRI sites of pBABE-Puro and pUC57 plasmids. Threonine-to-alanine point mutations in SF3B1 were introduced by site-directed mutagenesis using the following primers:

- T142A: 5' GCC GAC GGA GGC AAA GCA CCT GAT CCA AAG ATG 3'
- T211A: 5' CAG ACA CCT GGA GCA GCT CCA AAG AAA CTG AGT 3'
- T257 261A: 5' AGC AAG ATT TGG GAC CCC GCA CCT TCT CAT G 3'
- T426A: 5' TAC GTC CCA ATC AGA GCT CCC GCC AGG AAA CTG 3'
- T434A 5' AGG AAA CTG ACC GCT GCA CCC ACT CCT CTG 3'.

N-term SF3B1 (amino acids 1-500) were PCR amplified from pBABE-Puro-SF3B1 and sub-cloned into pGEX-6P2 (GE Healthcare Lifescience) using BamHI and EcoRI sites.

**Immunoprecipitation (IP) and Immunoblots:**
Whole cell lysates were prepared by lysis cells in RIPA buffer (150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% Sodium Deoxycholate, 50mM Tris pH7.5 and 2mM EDTA) supplemented with protease and phosphatase inhibitors (PPI) (10 mg/ml each of aprotinin, leupeptin and pepstatin, 50 mM sodium fluoride and 1 mM sodium orthovanadate). Immunoprecipitations were performed by rotating lysates at 4 °C overnight with the indicated antibodies (2 µg/sample) conjugated to Protein Sepharose A (Life Technologies) or Protein Sepharose G (GE Healthcare) beads for antibodies produced in rabbit or mouse respectively. Beads were washed 3x with RIPA buffer and equal amounts of samples were electrophoresed and transferred to nitrocellulose membranes. Immunoblots were performed using the indicated antibodies. Nucleosome-enriched lysates were prepared as described in (18) with some modifications. Cells were lysed using nucleosome lysis buffer (60 mM KCl, 15 mM NaCl, 5 mM MgCl₂, 0.1 mM EGTA, 15 mM Tris-HCl pH 7.5, 0.2% NP-40) supplemented with 0.1 mM DTT and PPI. Lysates were then passed through a sucrose cushion (50 mM Tris-HCl pH 7.5, 5 mM MgCl₂, 25 mM KCl, 1.2M Sucrose) by centrifugation at 3.5g for 15 minutes at 4 °C. The pellet (containing the nuclei) was resuspended in the digestion buffer (50 mM Tris-HCl pH 7.5, 4mM MgCl₂, 1mM CaCl₂, 0.32 M Sucrose) with 200U micrococcal nuclease (Mnase) (Worthington Biochemical) and incubated for 10 minutes at 37 °C. Digestion was stopped by the addition of 1 mM each of EDTA and EGTA. Nuclei were pelleted by centrifugation at 3.5g for 10 minutes at 4 °C. Pellet was resuspended in 500 µl solubilization buffer (50mM HEPES pH 7.6, 500mM LiCl, 1mM EDTA, 0.7% sodium deoxycholate, 0.1% SDS and 1% NP-40) and rotated at 4 °C for 1 hour followed by centrifugation at 12,000g for 10 minutes at 4 °C. The pellet was discarded and the supernatant, the nucleosome enriched fraction, was used for immunoprecipitations and immunoblot assays. DNA was extracted from a small aliquot of the nucleosome-enriched fraction using phenol: chloroform: isoamyl alcohol (Thermo Fisher Scientific 15593031) and run on a 2% agarose gel to confirm the expected size of the digested DNA (147 bp and multiples thereof). For immunoprecipitations, nucleosome enriched fractions were diluted to 1 ml in dilution buffer (0.005% SDS, 0.1% Triton X-100, 1.2 mM EDTA, 1.67 mM Tris HCl pH8.0, 167 mM NaCl) and incubated with antibodies (2 µg/sample) conjugated to Sepharose A or G beads with rotation at 4 °C overnight. Beads were washed with dilution buffer 3x and immunoblots were performed as described above. RNase-A treatment was performed as described in (18).

The following antibodies were used: SF3B1 (Abcam ab172634 (immunoblot), MBL D221-3 (IP and ChIP)), phospho-serine/threonine-Proline (Abcam ab9344), phospho-threonine 313 SF3B1 (Cell
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Signaling Technologies 25009s), histone H3 (Abcam ab1791 (immunoblot), Active Motif 61475 (IP)), histone H1 (Active Motif 39707), histone H1.4 (Abcam ab105522), phospho-histone H1 (Abcam ab4270), U1-70k (Abcam ab51266, Santa Cruz Biotechnology sc-390998) and an antibody produced and provided as a gift by Dr. Douglas Black’s Lab), beta-Actin (Sigma-Aldrich A5441), SF3B2 (Abcam ab56800 and Novus 79848), Anti-Flag tag (Sigma-Aldrich F1804).

**GST-tagged protein expression and immunoaffinity purification:**
pGEX-6P2-N-term-SF3B1^{1-500} was transformed into BL21 competent E.coli cells (Life Technologies C-6000-03) by heat-shock method. Cells were grown in LB-media at 37°C until they reached an O.D of 0.4-0.6. SF3B1^{1-500} expression was induced by treatment with 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Fisher Scientific) for 4 hours at 37°C. Cells were pelleted by centrifugation and frozen at -80°C. Pellets were thawed and lysed in 1% Triton X-100 in 1X PBS, sonicated using a Diagenode Bioruptor (12 cycles of 10s pulse, 30s rest). Glutathione agarose beads (25 µL/ml lysate) (Thermo 16100) were washed 2X with 1X PBS (10X bead volume). Lysates were incubated with glutathione beads at 4°C overnight with rotation. Beads were washed 3X with 1X PBS (10X bead volume).

**In vitro Binding Assay:**
N-Term, GST-tagged SF3B1 (SF3B1^{1-500}) was expressed in bacteria and bound to glutathione beads as described above. Beads were then washed 3X with lambda-phosphatase wash buffer (25 mM Tris-HCl pH 7.5, 50 mM NaCl, 2.5 mM MnCl\(_2\), 2 mM DTT) and incubated with lambda-phosphatase (NEB P0753) for 20 minutes according to the manufacturer’s protocol. Phosphorylation by cyclin E-CDK2 in vitro was performed as described below. Next, beads were washed 1X with binding buffer (0.1% Triton X-100, 1.2 mM EDTA, 1.67 mM Tris HCl pH8.0, 100 mM NaCl), resuspended in 1ml binding buffer followed by incubation with 1 µg purified histone H1 (Sigma-Aldrich, Calf Thymus), recombinant human histone H1.0 (NEB M2501) or recombinant human histone H1.4 (MyBioSource MBS963223) and 1 µg purified HeLa mono-nucleosomes (Epicypher 16-0002) at 4°C with rotation for 12 hours. Histone H1 was treated with lambda-phosphatase for 20 minutes according to the manufacturer’s protocol or phosphorylated for 30 minutes in kinase assay buffer as described below. Beads were washed 3X with binding buffer followed by immunoblot analysis as described before.

**In vitro kinase reactions:**
Substrates for cyclin E-CDK2 included FLAG-tagged full-length SF3B1 expressed in mammalian cells as above or GST-SF3B1^{1-500}. Immunoprecipitation used either protein sepharose G or glutathione beads, and these were then washed 3X with kinase wash buffer (25 mM Tris-HCl pH 7.5, 70 mM NaCl, 10 mM MgCl\(_2\), 1mM DTT) and resuspended in kinase reaction buffer (kinase wash buffer, 10 µM ATP (NEB P0756), [γ-32P]ATP for phosphorylation of full-length SF3B1 only (5Ci per reaction; 1Ci=37GBq), 0.5 µg active cyclin E-CDK2 (Millipore 14-475) followed by incubation at 37°C.

**RNA sequencing:**
Synchronized HeLa cells were resuspended in TRIzol (Thermo Fisher Scientific 15596026). Total RNA was extracted from TRIzol using the manufacturer’s protocol and quantified using a Qubit fluorometer (Thermo Fisher Scientific). Poly-A mRNA was enriched using NEBNext Poly-A mRNA isolation kit (NEB E7490). Libraries for paired-end sequencing were prepared using NEBNext Ultra RNA Library Preparation Kit (NEB E7530) and sequenced on Illumina NextSeq 500. Approximately 511 million reads passing filter were obtained with an average of approximately 122 million reads per sample. Reads were aligned to human genome hg19.

**ChIP sequencing:**
Nucleosome-enriched lysates were prepared from synchronized HeLa cells as described...
CDKs regulate SF3B1-chromatin interactions above. Lysates were diluted to 1 ml with dilution buffer and incubated with 7.5 µg/sample SF3B1 antibody (MBL D221-3) at 4 °C with rotation overnight. Sheep anti-mouse M-280 Dynabeads (Life Technologies 11201D) were washed and pre-cleared by incubation with 0.5% BSA in PBS at 4 °C with rotation overnight. Beads were then added to the immunoprecipitated lysates and incubated at 4 °C with rotation for 4 hours. Beads were washed four times with dilution buffer, magnetically isolated and resuspended in 150 µl SDS elution-buffer (1% SDS, 10mM EDTA, 50 mM Tris pH 8.0), then eluted by incubation at 65 °C overnight. Samples were treated with RNase-A and proteinase-K followed by DNA isolation using phenol: chloroform: isoamyl alcohol. Libraries for sequencing were prepared using NEBNext Ultra DNA Library Preparation Kit (NEB E7370) and sequenced on Illumina NextSeq 500. Approximately 393 million total reads passing filter were generated with an average of 75 million reads passing filter per sample. Reads were aligned to human genome hg19.

**Bioinformatic Analysis:**
All Illumina reads were preprocessed with Trimmomatic (49) and aligned with Bowtie2 (50) to hg19 genome (ChIP-Seq) or STAR (51) to hg19 transcriptome (RNA-Seq). Peak calling for individual ChIP-Seq files was performed with HOMER (40) using the respective input files as controls. For differential peak calls and overlap of peaks, biological replicates were merged and analyzed using HOMER (getDifferentialPeaks or mergePeaks functions of HOMER). Differential enrichment of reads in the exonic region and correlation between ChIP-Seq files were performed using deepTools2 (52). Differential gene expression was determined from aligned RNA-Seq files using Cufflinks (41). Analysis of differential splicing was performed using rMATS (42). Custom perl scripts were used to integrate results of HOMER, Cufflinks and rMATS analyses.

Detailed parameters for analysis and scripts will be made available on request.

**Accession Number:**
ChIP sequencing and RNA sequencing raw data are accessible at NCBI Gene Expression Omnibus under accession number GSE108347.

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Table 1

| #  | SITE | MOTIF | #  | SITE | MOTIF |
|----|------|-------|----|------|-------|
| 1  | S129 | IIsPE | 18 | T326 | GEtPt |
| 2  | T142 | GKtPD | 19 | T328 | tPtPg |
| 3  | T207 | DQtPG | 20 | T341 | DETPA |
| 4  | T211 | GAtPK | 21 | T350 | GstPV |
| 5  | T223 | AEtPG | 22 | T354 | VltPG |
| 6  | T227 | GHtPs | 23 | T362 | IGitPA |
| 7  | T235 | DETPG | 24 | T369 | MATPt |
| 8  | T244*| sEtPG | 25 | T371 | tPtPg |
| 9  | T248*| GAitPG | 26 | T379 | sMtPE |
| 10 | T257 | DPtPs | 27 | T426 | IRTPA |
| 11 | T261 | sHtPA | 28 | T434*| tAtPt |
| 12 | T267 | AAitPG | 29 | T436 | tPtPL |
| 13 | T273 | GDtPG | 30 | S488 | TLPsPE |
| 14 | T278 | HAtPG | 31 | T508 | NGtPP |
| 15 | T296 | DETPK | 32 | S541 | LMsPT |
| 16 | T303 | RDtPG | 33 | T1021 | RLtPI |
| 17 | T313*| AEtPR | 34 | T1170 | AVtPL |

Table 1. Proline-directed Serine/Threonine CDK substrate motifs in SF3B1.

Proline-directed serine/threonine phosphorylation sites in SF3B1 listed by PhosphositePlus tool (53) and previously identified by mass spectrometry (MS). All serine and threonine residues are shown in lowercase letters. Predicted CDK phosphosites have been underlined. (*) indicates that MS data was confirmed in studies utilizing site-specific methods (20,21,34). Six threonine residues were mutated to alanine in order to understand their impact on SF3B1-nucleosome interactions and are denoted by bolded and italicized text.
CDKs regulate SF3B1-chromatin interactions

Figure 1

A. HeLa - Ab IP: SF3B1
   G1 G1/S G2/M
   p-Ser/Thr-Pro
   pSF3B1(T313)
   SF3B1
   INPUT:
   SF3B1
   G2/M
   G1
   G1/S
   DNA Content

B. K562 - Ab IP: SF3B1
   G1 G1/S G2/M
   p-Ser/Thr-Pro
   pSF3B1(T313)
   SF3B1
   INPUT:
   SF3B1
   G2/M
   G1
   G1/S
   DNA Content

C. HeLa (G2/M) - Ab IP: SF3B1
   Purv-A (hrs) 0 0.5 4
   p-Ser/Thr-Pro
   SF3B1
   INPUT:
   SF3B1
   β-Actin

D. K562 (G2/M) - Ab IP: SF3B1
   Purv-A (hrs) 0 0.5 4
   p-Ser/Thr-Pro
   SF3B1
   INPUT:
   SF3B1
   B-Actin

E. HeLa (G1/S) - Ab IP: SF3B1
   OA (hrs) 0 12
   p-Ser/Thr-Pro
   SF3B1
   INPUT:
   SF3B1
   β-Actin
Figure 1. SF3B1 phosphorylation is dynamic during cell cycle progression.

SF3B1 was immunoprecipitated from whole-cell lysates of synchronized (A) HeLa and (B) K562 cells. Immunoblot analysis to assay SF3B1 phosphorylation was performed using an antibody that detects phosphorylation at all serine/threonine residues with a proline at the +1 position (pSer/Thr-Pro) and a site-specific antibody that detects phosphorylation at threonine 313 of SF3B1 (Thr313). Lower panels in (A) and (B) show flow cytometric analysis of cells synchronized and harvested for analysis in G2/M, G1 and G1/S phases. Cells were labeled with propidium iodide (PI) to measure DNA content and model cell cycle. Histogram peaks representing diploid and tetraploid DNA content are labeled as 2N and 4N respectively. G2/M arrested (C) HeLa and (D) K562 cells were treated with Purvalanol-A (Purv-A) for indicated times and SF3B1 was immunoprecipitated from whole-cell lysates followed by immunoblot analysis to assay SF3B1 phosphorylation. (E) G1/S HeLa cells were treated with 20 nM okadaic acid (OA) for 12 hours and SF3B1 was immunoprecipitated from whole-cell lysates. SF3B1 phosphorylation was determined as shown.
CDKs regulate SF3B1-chromatin interactions
Figure 2. SF3B1-nucleosome interactions are dynamic during cell cycle progression.

SF3B1 protein abundance was assayed in nucleosome-enriched and whole-cell lysates from (A) HeLa and (B) K562 cells synchronized in G1, G1/S and G2/M. Histone H3 (H3) and beta-Actin were used as nuclear and whole-cell lysate loading controls respectively. Inset: DNA was isolated from micrococcal nuclease (Mnase)-digested nuclear lysates using phenol: chloroform extraction and run on an agarose gel to assess the size of the DNA fragments. (C) Total protein abundance of SF3B2 and U1 subunit-70K was assayed in nucleosome-enriched and whole-cell lysates of HeLa cells synchronized in G1/S and G2/M. U1 subunit-70K Santa Cruz Biotechnology sc-390988 and SF3B2 Abcam ab56800 antibodies were used in this experiment. H3 and beta-Actin were used as nuclear and whole-cell lysate loading controls respectively. (D) H3 was immunoprecipitated from nucleosome-enriched lysates of HeLa cells synchronized in G1/S and G2/M. SF3B1 co-immunoprecipitation was assayed by immunoblot assay. (E) H3 was immunoprecipitated from nucleosome-enriched lysates from HeLa cells synchronized in G1/S and G2/M. SF3B2 and U1-70K-subunit co-immunoprecipitation was assayed by immunoblot using the U1 subunit-70K antibody from Dr. Doug Black’s Lab and SF3B2 (Novus 79848) antibody. (F) G1/S HeLa cells were treated with 20 nM okadaic acid (OA) for 12 hours and H3 was immunoprecipitated from nucleosome-enriched lysates. SF3B1 co-immunoprecipitation (co-IP) was assayed by immunoblot assay. SF3B1 in co-IP was quantified and normalized to immunoprecipitated histone H3. The experiment was performed in triplicate and relative SF3B1 abundance in co-IP quantified using NIH ImageJ and standard deviation (SD) calculated. (G) H3 was immunoprecipitated from nucleosome-enriched lysates of HeLa cells synchronized in G1/S and G2/M. SF3B1, SF3B2 and U1-70K subunit co-immunoprecipitation was assayed after RNase-A treatment of the immunoprecipitation reaction as shown. The immunoblot displayed is representative of three independent assays.
CDKs regulate SF3B1-chromatin interactions

Figure 3

A. -Ab IP: Histone H3

| Purv-A (hrs) | 0 | 0.5 | 4 |
|-------------|---|-----|---|
| SF3B1       |   |     |   |
| H3          |   |     |   |
| INPUT:      |   |     |   |

G2/M

B. -Ab IP: Histone H3

| RCV (20µM) | - | + |
|------------|---|---|
| SF3B1      |   |   |
| H3         |   |   |
| INPUT:     |   |   |

G1/S

C. -Ab IP: Histone H3

| HRM (5µM) | - | + |
|-----------|---|---|
| SF3B1     |   |   |
| H3        |   |   |
| INPUT:    |   |   |

G1/S

D. SF3B1-histone binding

| SF3B1 in co-IP relative to vehicle-treated samples |
|--------------------------------------------------|
| RCV                                             |
| HRM                                             |

E. IP: Histone H3

| V | WT | 6A |
|---|----|----|
|   |    |    |
| FLAG (SF3B1) |   |   |
| H3           |   |   |

Normalized SF3B1 in co-IP (SD)

| 1.0 | 0.58 | (0.19) |

INPUT:
Figure 3. CDK activity regulates SF3B1-chromatin interactions.

(A) Histone H3 was immunoprecipitated from nucleosome-enriched lysates of HeLa cells synchronized in G2/M and treated with CDK1 inhibitor Purvalanol-A (Purv-A) for the indicated times. SF3B1 co-immunoprecipitation was assessed by immunoblot analysis. No significant changes in cell cycle status of Purv-A treated cells were observed by flow cytometric analysis (not shown). (B) Upper panel- H3 was immunoprecipitated from nucleosome-enriched lysates of HeLa cells synchronized in G1/S and treated with CDK2 inhibitor Roscovitine (RCV) for 12 hours. SF3B1 co-IP was assessed by immunoblot analysis. No significant changes in cell cycle status of RCV treated cells were observed by flow cytometric analysis. Lower panel- RCV-mediated inhibition of CDK2 activity was confirmed by analysis of cyclin E auto-phosphorylation (54) using similar conditions as in panel (B). (C) Upper panel- H3 was immunoprecipitated from G1/S HeLa cells treated with DYRK1a inhibitor Harmine (HRM) for 12 hours. SF3B1 co-immunoprecipitation was assessed by immunoblot assay. Lower panel - DYRK1a inhibition was confirmed by analysis of cyclin D2 protein levels in whole cell lysates of HRM treated G1/S HeLa cells by immunoblot assay. (D) SF3B1 abundance in H3 co-IPs from drug-treated relative to vehicle-treated cells was quantified from 3-4 experiments and is displayed with means (thick bars) and SDs (thin bars). (E) FLAG-tagged wild-type (WT) and compound phosphosite mutant SF3B1 (6A) were overexpressed in K562 cells by retroviral transduction. Cells were synchronized in G1/S, and H3 was immunoprecipitated from nucleosome-enriched lysates. SF3B1 co-immunoprecipitation was assayed by immunoblot analysis using an antibody against the FLAG tag in triplicate experiments.
CDKs regulate SF3B1-chromatin interactions

Figure 4

A.

- Ab IP: SF3B1

SF3B1  H1

H3

INPUT:

SF3B1  H1

H3

B.

HEAT-containing

NTD

SF3B1

500 aa  1304 a.a

75 kDa

SF3B1^1-500

C.

GST pull-down

CDK2 (min)  0  15  30

p-Ser/Thr-Pro

pSF3B1(T313)

SF3B1^1-500

D.

GST pull-down

IPTG  +  +  +  +  +

Phospho-Histone H1  +  +  +

Mono-nucleosomes  +  +  +  +  +

CDK2 (min)  0  15  30  15  30

E.

GST pull-down

P'tase +Histone H1

CDK2+Histone H1

Mono-nucleosomes  +  +  +  +  +

SF3B1^1-500

H3

F.

- Ab IP: Histone H3

G1/S G2/M

Phospho-H1

H1

H3

INPUT:

H3

25
Figure 4. SF3B1-chromatin interactions are dependent on the phosphorylation status of both SF3B1 and linker histone H1.

(A) SF3B1 was immunoprecipitated from nucleosome-enriched HeLa lysates. Co-IP of histone H3 and histone H1 were assessed by western blot analysis. Histone H1 (Active Motif 39707) antibody was used in this experiment. Data shown are representative of three independent experiments. (B) Upper panel - Schematic highlighting N-terminal domain (NTD) of SF3B1 protein (SF3B11-500) that is encoded by an inducible expression construct used in panels B-F is shown in relationship to the HEAT motif-containing region spanning most of the protein (amino acids 463-1304) (55). Lower panel - N-term GST-tagged SF3B11-500 expression in E.coli after IPTG induction was assessed by immunoblot analysis using an antibody that recognizes an N-terminus region of SF3B1 (Abcam ab172634). (C) SF3B11-500 expressed in E.coli was isolated from whole cell lysates after IPTG induction and phosphorylated in vitro using purified cyclin E-CDK2 for the indicated incubation times. SF3B1 phosphorylation was assessed by immunoblot analysis. (D) Upper panel - N-term GST-SF3B1 expressed in E.coli was phosphorylated in vitro with purified cyclin E-CDK2 for the indicated times and incubated in vitro with purified mono-nucleosomes in the presence or absence of phosphorylated native calf thymus histone H1. * - slower migrating species of SF3B11-500 with 30 min cyclin E-CDK2 incubation, compared to o - faster migrating species without phosphorylation in vitro. N-term GST-SF3B1-mono-nucleosome interactions were determined by immunoblot analysis. Lower panel - equal levels of total H3 and H1 and phosphorylated histone H1 added to the in vitro binding reaction were confirmed by immunoblot analysis. Histone H1 (Active Motif 39707) antibody was used, and H1 phosphorylation was assessed using the phospho-serine/threonine-Proline (Abcam ab9344) antibody. Representative result of three independent replicates is shown. (E) Upper panel - N-term GST-SF3B1 expressed in E.coli was phosphorylated in vitro with purified cyclin E-CDK2 for 15 minutes and incubated in vitro with purified mono-nucleosomes in the presence of lambda
phosphatase treated or cyclin E-CDK2-phosphorylated native calf-thymus H1 containing a mix of isoforms (H1m), purified human histone H1.0 or purified human histone H1.4. **Lower panel** – immunoblot showing levels of H3, total H1 and phosphorylated H1. Mononucleosomes and linker histone H1 (H1m, H1.0, or H1.4) were added to the binding assays as shown in equal quantities by mass. Data displayed are representative of two independent experiments. (F) H3 was immunoprecipitated from nucleosome-enriched lysates of G1/S- and G2/M- synchronized HeLa cells. Abundance and phosphorylation of co-immunoprecipitated histone H1.4 was assessed by immunoblot analysis. Antibodies recognizing H1.4 specifically (histone H1.4 (Abcam ab105522)) and phosphorylated H1 (phospho-histone H1 (Abcam ab4270)) were used. Representative result of three independent replicates is shown.
Figure 5

A. Distribution of ChIP-Seq Peaks (G1/S vs G2/M)

B. Peak enrichment normalized to total length of region

C. Differential enrichment of Peaks (G1/S vs. G2/M)

D. Altered Splicing Detected (G1/S vs G2/M)

E. Overlap of Events

CDKs regulate SF3B1-chromatin interactions
CDKs regulate SF3B1-chromatin interactions

Figure 5. Integrative analysis of SF3B1 genome occupancy and cell cycle-dependent splicing.

(A) Distribution of ChIP-Seq peaks as a proportion of genomic regions in G1/S and G2/M. Peaks determined by HOMER algorithm located within promoter regions (promoter-TSS), transcriptional termination site-containing regions (TTS), exons, introns, ncRNA (non-coding RNA) and intergenic regions are shown as a percentage. (B) Enrichment of peaks normalized to relative length of genomic regions. Length of Peaks determined to be in exon, intron or intergenic regions were normalized to the total length of these regions in the hg19 genome build. Values for G1/S and G2/M ae plotted side by side. (C) Differential enrichment of peaks (G1/S vs G2/M). A total of 456 peaks were found to be differentially enriched in G1/S compared to G2/M peaks using the HOMER algorithm. Their relative distribution across genomic regions is plotted. (D) Analysis of altered splicing in G1/S vs. G2/M. Using the rMATS algorithm, aligned RNA-Seq files were analyzed for altered splicing. Distribution of 2340 events that met statistical cut-off (FDR <0.05 and delta PSI > 5%) across 5 different types of events (A3SS: alternative 3’ splice site; A5SS: alternative 5’ splice site; RI: retained intron; MXE: mutually exclusive exon; SE: skipped exon) are shown on the left. Distribution of these events (over represented in G1/S or G2/M) is shown in the right panel. (E) Overlap of genes between rMATS, Cuffdiff and HOMER analysis of G1/S and G2/M datasets. Genes with significant changes in splicing (FDR <0.05) in any of the 5 splice event types (A3SS, A5SS, SE, MXE or RI) with a higher isoform ratio in G1/S compared to G2/M were included in the rMATS set. Cuffdiff set includes genes significantly over-expressed in G1/S compared to G2/M (p <0.05). The HOMER set contained genes co-localized with intragenic peaks (290 of the total 456) enriched in G1/S compared to G2/M. Overlaps were not found to be significant by Fisher's exact test.
Cyclin-dependent kinase 1 (CDK1) and CDK2 have opposing roles in regulating interactions of splicing factor 3B1 with chromatin

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