Published in final edited form as:

Cancer Res. 2010 May 15; 70(10): 3975–3984. doi:10.1158/0008-5472.CAN-09-3468.

Identification of ASF/SF2 as a critical, allele-specific effector of the cyclin D1b oncogene

Nicholas A. Olshavsky^{a,c}, Clay E.S. Comstock^{b,c}, Matthew J. Schiewer^{b,c}, Michael A. Augello^{b,c}, Terry Hyslop^{b,e}, Claudio Sette^f, Jinsong Zhang^a, Linda M. Parysek^a, and Karen E. Knudsen^{b,c,d,*}

^aDepartment of Cancer and Cell Biology, University of Cincinnati, Cincinnati, OH 45267

^bKimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA 19107

^cDepartment of Cancer Biology, Thomas Jefferson University, Philadelphia, PA 19107

^dDepartment of Urology, Thomas Jefferson University, Philadelphia, PA 19107

^eDepartment of Pharmacology and Experimental Therapeutics, Thomas Jefferson University, Philadelphia, PA 19107

^fDepartment of Public Health and Cell Biology, Section of Anatomy, University of Rome Tor Vergata, 00133 Rome, Italy

Abstract

The cyclin D1b oncogene arises from alternative splicing of the *CCND1* transcript, and harbors markedly enhanced oncogenic functions not shared by full-length cyclin D1 (cyclin D1a). Recent studies showed that cyclin D1b is selectively induced in a subset of tissues as a function of tumorigenesis; however, the underlying mechanism(s) that control tumor-specific cyclin D1b induction remain unsolved. Here, we identify the RNA binding protein SF2/ASF as a critical, allele-specific, disease-relevant effector of cyclin D1b production. Initially, it was observed that SF2 associates with cyclin D1b mRNA (*transcript-b*) in minigene analyses and with endogenous transcript in prostate cancer (PCa) cells. SF2-association was altered by the *CCND1* G/A870 polymorphism, which resides in the splice donor site controlling *transcript-b* production. This finding was significant, as the A870 allele promotes cyclin D1b in benign prostate tissue, but in primary PCa, cyclin D1b production is independent of A870 status. Data herein provide a basis for this disparity, as tumor-associated induction of SF2 predominantly results in binding to and accumulation of G870-derived *transcript-b*. Finally, relevance of SF2 function was established, as SF2 strongly correlated with cyclin D1b (but not cyclin D1a) in human PCa. Together, these studies identify a novel mechanism by which cyclin D1b is induced in cancer, and reveal significant evidence of a factor that cooperates with a risk-associated polymorphism to alter cyclin D1 isoform production. Identification of SF2 as a disease-relevant effector of cyclin D1b provides a basis for future studies designed to suppress the oncogenic alternative splicing event.

Introduction

The cyclin D1b variant, produced via alternative splicing of the *CCND1* transcript (1,2), is a potent oncogene that harbors distinct functions from full-length cyclin D1 (cyclin D1a) (3–5). Unlike cyclin D1a, cyclin D1b independently confers cellular transformation (4,5). In addition, only the cyclin D1b isoform has the capacity to promote anchorage-independent

*To whom correspondence should be addressed karen.knudsen@kimmelcancercenter.org, Phone: 215-503-8574.

growth and cell invasiveness (6). Further confirmation of novel oncogenic capabilities was identified in mouse models, wherein animals expressing human cyclin D1b under the bovine K5 promoter demonstrated increased papilloma multiplicity (3). Given the enhanced oncogenic function of cyclin D1b, it is imperative to define the mechanisms that regulate cyclin D1b production in systems of clinical relevance.

The alternative splicing event that produces the cyclin D1b transcript (referred to as *transcript-b*) arises from failure to splice at the *CCND1* exon 4-intron 4 boundary. Due to intronic transcriptional termination, cyclin D1b lacks exon 5 encoded sequences and contains a novel C-terminal domain of unknown function (1,2). Previous studies showed that *transcript-b/a* ratios are enhanced in selected tumor types, thus providing evidence that the alternative splicing event may be altered as a function of tumorigenesis or tumor progression (6–10). Lending support to this posit, recent analyses of a large cohort of prostate cancer (PCa) specimens revealed that cyclin D1b (but not cyclin D1a) is induced in PCa as compared to non-neoplastic tissue (11). These findings were of interest, as cyclin D1b has specialized functions in this tumor type that are hypothesized to promote tumor progression (7).

Despite the compelling evidence identifying cyclin D1b as a potent, novel oncogene, the tumor-associated factor(s) that promote the alternative splicing event remain poorly defined. It has been long suggested that a polymorphism within the exon 4 splice donor site (G/A870) may contribute to *transcript-b* production, wherein the A-allele was suggested to favor the alternative splicing event (1,12,13). Recent analysis using minigenes supported this contention, and analysis of non-neoplastic prostate tissue demonstrated that the presence of the A-allele predicted for higher *transcript-b* production; however, the impact of the A-allele was lost in tumor tissue, thus indicating that tumor-associated factor(s) likely bypass or modify the impact of the G/A870 polymorphism with regard to *transcript-b* production (11). Here, the present study identifies the SF2 (also known as ASF or SRp30a) RNA binding protein, as a critical, allele-selective factor that associates directly with *transcript-b* and modulates cyclin D1b production in model systems of cancer relevance. Importantly, analyses of tumor tissue further support a model wherein tumor-associated elevation of SF2 specifically enhances cyclin D1b expression in human disease. Together, these findings provide a mechanism by which *CCND1* alternative splicing is controlled in tumorigenesis, and identify SF2 as a critical regulator of cyclin D1b oncogene production.

Materials and Methods

Cell Culture, Transfections, Generation of Stables

LNCaP, C33A, and LAPC4 cell lines were obtained, cultured and transfected as previously described (7,14–17). The DT40-ASF cell line was a generous gift from James Manley (Columbia University) and maintained as previously described (18). DT40-ASF cells were transfected using the Amaxa Nucleofector protocol. C33A stable cell lines were generated by transfecting the indicated expression constructs encoding empty vector, or individual cyclin D1 minigenes and selected with 400 µg/ml G418 (MP Biomedicals). Clonal isolates were screened for expression by immunoblot. Isolates utilized herein are denoted as C33A-Vec, C33A-G1 (containing the G870 allele minigene) and C33A-A1 (containing the A870 allele minigene).

Plasmids

The pCGT7, pCGT7-ASF/SF2 and pCGT7-SRp40 expression constructs were generous gifts from Adrian Krainer (Cold Spring Harbor) and previously described (19). pCEP4-ASF/SF2 was a generous gift from Woan Yuh-Tarn (Institute of Biomedical Sciences, Taiwan) and was previously described (20). The cyclin D1 minigenes were previously described (11).

Immunoblotting

Cells were harvested, lysed in NETN, and subjected to SDS-PAGE and immunoblotting as previously described (14,15). Immunoblots were performed using antisera against ASF/SF2 (Invitrogen), β -tubulin (Sigma), cyclin D1a (Neomarker, Ab3), cyclin D1b (7), GAPDH (Invitrogen), or T7 (Novagen), as indicated.

RNA Isolation, PCR, PCR-RFLP and Real Time PCR

RNA was isolated using TRIzol per manufacturer's protocol. PCR analyses and primer sequences for *transcript-a*, *transcript-b* and GAPDH were previously described (7,15,21). The PCR-RFLP was performed as previously described (7,22); briefly, RIP recovered products were subjected to a second amplification using primers designed for RFLP analyses. The product was then digested and visualized after electrophoresis by ethidium bromide. Real-time PCR for *transcript-b* was performed using the Syber Green protocol from Applied Biosystems and a 7900F Real Time PCR machine.

Immunohistochemistry and AQUA system analysis

IHC and AQUA analyses were performed as previously described (11) using a human prostate cancer array (SuperBio Chips Labs) and antisera indicated above. Comparison of relative SF2 levels from microarrays were analyzed using one way analysis of variance. Correlations of SF2 with cyclin D1a and D1b AQUA expression levels was completed using Spearman correlation coefficients, with Bonferroni correction of p-values for multiple comparisons.

RNA Immunoprecipitation (RIP)

RIPs were performed as previously described (23). Briefly, cells (LNCaP, LAPC4, C33A and the C33A-derived stables cell lines) were harvested and nuclear extracts prepared by resuspending in isotonic buffer. After a 7 min incubation on ice, samples were centrifuged at 700g for 7 min and nuclei were isolated, resuspended in buffer supplemented with 90 mM NaCl and 0.5% Triton X-100, and sonicated. After centrifugation at 5,000g for 15 min, nuclear extracts were precleared for 1h and immunoprecipitated with ASF/SF2 antibody or mouse IgG antibody. The antibody-antigen complex was precipitated by the addition of protein G-sepharose beads (Invitrogen) for 3h at 4° with rotation. Beads were washed thrice with lysis buffer and an aliquot was eluted in SDS sample buffer for immunoblot analysis. The remaining beads were incubated with lysis buffer in the presence of (RNase-free) DNase (Ambion) for 15 min at 37°C and washed thrice with lysis buffer before incubation with 50 μ g proteinase K (Roche) for 15 min at 37°C. Co-precipitated RNA was then extracted by the TRIzol procedure and used for RT-PCR analyses.

Results

G/A870 Polymorphism alters cyclin D1b production and SF2 binding

Given the enhanced oncogenic function of the cyclin D1b variant (3–5), and the established observation that cyclin D1b levels are enhanced as a function of prostate tumorigenesis (7, 11), it is imperative to discern the mechanism(s) underpinning the alternative splicing event. As was recently reported, the *CCND1* G/A870 polymorphism plays a context-specific role in the alternative splicing event, wherein the A-allele favors cyclin D1b production in minigene expression studies in which the alleles were individually examined (11). These studies were validated here, wherein minigenes harboring either the G- or A870 allele (Figure 1A) were independently introduced by stable transfection into C33A cells, which have been previously shown to harbor low to undetectable levels of endogenous cyclin D1 isoforms (24). Introduction of the G-allele minigene (stable line C33A-G1), resulted in detectable expression of both *CCND1* transcripts (*a* and *b*), but a preference towards *transcript-a* and cyclin D1a

protein was observed (lanes 2). *Transcript-b* levels were comparatively increased in the presence of the A-allele minigene (lanes 3), consistent with previous studies wherein the minigenes were individually analyzed in parallel (11). Subsequent analyses of individual minigene studies in spontaneously immortalized CV1 cells or viral oncoprotein immortalized RWPE-1 prostate epithelial cells (25,26) were also carried out. Similar findings were observed in both non-tumorigenic model systems, wherein the A-allele predisposed to *transcript-b* and cyclin D1b production (Supplemental Figure S1). These studies further support the premise that in isolated comparison, the G/A870 polymorphism influences *CCND1* alternative splicing.

As G/A870 lies within the splice donor site, it was reasoned that the polymorphism may influence RNA binding protein recognition or activity. The splicing factor binding resource ESEfinder3.1 (27,28) was therefore utilized to predict distinctions in the profile of associated splicing factors between the G- and A-870 allele splice donor sites. As shown, binding of SF2 was suggested to be altered in both position and strength by changes in the polymorphic site (Figure 1C). To initially assess whether SF2 could play a role in *CCND1* alternative splicing, an RNA immunoprecipitation (RIP) was performed using the same stable cell lines described in Figure 1B. Using standard techniques, RNA associated with either SF2-specific or control antisera was isolated, reverse transcribed, and detected by PCR amplification. As illustrated in Figure 1D (top panel), *transcript-b* was only weakly detected in control cells and immunoprecipitates thereof (lanes 1–3). In cells containing the G-allele minigene (C33A-G1), SF2 readily associated with *transcript-b*, providing the first evidence that SF2 associates with this mRNA species (lanes 4–6). SF2 association with *transcript-b* was reduced in cells containing the A-allele minigene (lanes 7–9), as was confirmed by quantitative-RT-PCR (Fig. 1D, bottom panel). For these analyses, signal observed in C33A-Vec cells was set to “1” for ease of comparison. The finding that SF2 binding is influenced by the polymorphism was subsequently confirmed in additional stable isolates of minigene integration (Supplemental Figure S2, panel A) as well through transient analyses (panel B). Together, these data strongly indicate for the first time that the G/A870 polymorphism influences recognition by the SF2 splicing factor.

SF2 specifically correlates with cyclin D1b in prostate cancer

The observation that the G/A870 polymorphism may alter SF2 association with *transcript-b* was of interest, given reports that cyclin D1b, not cyclin D1a, is induced as a function of prostate tumorigenesis (7,11), and that cyclin D1b has oncogenic activity (3–5). Intriguingly, analyses of gene microarray data from human PCa demonstrated that SF2 expression increases with tumor progression (Figure 2A) (29–31). This observation was further validated upon screening a panel of PCa cell lines, wherein it was revealed that SF2 expression is lowest in non-transformed RWPE-1, and as compared to cells derived from primary and distant metastases of prostate cancer (Supplemental Figure S3). Among the cancer cell lines representative of hormone-therapy sensitive disease, SF2 was relatively low in cells known to express lower levels of cyclin D1b (LNCaP) and higher in cells of this subtype known to express high levels of cyclin D1 (LAPC4) (7,11). For the androgen-receptor negative cells, unexpected high levels of expression were observed in PC3 cells, which exhibit the most aggressive phenotype *in vivo* with regard to metastases (32). To probe the impact of SF2 on cyclin D1b production in human tumors, comparative analyses of nuclear SF2 and cyclin D1 (a and b isoform) expression was objectively analyzed using serial sections of PCa specimens and quantitative AQUA analyses. Representative images of tumors scoring with low SF2 (Figure 2B, top panels) or high SF2 (lower panels) are shown. Signal was quantified across each specimen within the nuclear compartment (DAPI positive, blue) of epithelial-derived carcinoma cells (cytokeratin-positive; green), and resultant data are plotted in Figure 2C. As shown, tumors expressing low SF2 exhibited low cyclin D1b expression; conversely, tumors with high SF2 scored high for cyclin D1b. Quantification of all tumors examined revealed a significant correlation between

SF2 and cyclin D1b ($p=0.023$), but not cyclin D1a ($p=0.19$). Co-expression of cyclin D1b and SF2, in serial sections, was observed in glandular epithelial cells (data not shown). Together, these data demonstrate that SF2 expression is strongly correlated with only the cyclin D1b isoform in human disease.

SF2 modulates cyclin D1b production in multiple model systems

Since SF2 predicted for high cyclin D1b in PCa, the effect of SF2 on cyclin D1b production was examined in model systems of disease relevance. Initially, LNCaP cells were utilized, which express low endogenous levels of cyclin D1b (7). Epitope tagged SR proteins (SRp40 or SF2) were individually introduced (Supplemental Figure S4) by transient assays with high transfection efficiency (Supplemental Figure S5), and the impact on *CCND1* mRNA isoform expression determined (Figure 3A). Consistent with previous results, these cells express low levels of endogenous *transcript-b* (lane 1), and SRp40 failed to elicit changes in either *CCND1* transcript (lane 2). Significantly, SF2 promoted a dramatic increase in *transcript-b* expression without altering *transcript-a* (lane 3). Quantification revealed an approximate 2.7-fold increase in *transcript-b* following SF2 introduction (lower panel), thus providing the first functional evidence for SF2-mediated regulation of cyclin D1b. Increased expression of SF2 in LNCaP cells also resulted in an increase in cyclin D1b protein expression (Supplemental Figure S4). Unfortunately, depletion of endogenous SF2 in PCa models using siRNA were unsuccessful (data not shown), consistent with previous observations that knockdown of SF2 induces cell death (33). As such, a previously developed DT40-ASF/SF2 cell line was utilized (DT40-ASF), in which endogenous SF2 was replaced with a tet-repressible SF2 construct (Figure 3B) (18). In this system, SF2 expression is undetectable approximately 24 h following addition of Dox. Previous reports indicated that after 48 h post-knockdown, the DT40-ASF cell line begins to undergo apoptosis (33). Thus, experiments analyzed endpoints after 24 hrs, whereupon no loss of cell viability was noted (data not shown). As expected, no detectable cyclin D1 isoforms were recognized (Figure 3B, left panel lanes 1, 2). Stable introduction of the G870 minigene revealed that although both isoforms were produced (left panel, lanes 4, 5), suppression of endogenous SF2 (left panel, lane 5) diminished cyclin D1b (but not cyclin D1a) production. Similar results were observed at the level of the transcripts following depletion of SF2 (Figure 3B, right panel). Collectively, these results identify SF2 as an effector of cyclin D1b production in multiple model systems.

Endogenous SF2 associates with both alleles in PCa cells

As it was observed that elevated SF2 induces cyclin D1b in PCa (Figure 2), and that SF2 differentially binds the transcript dependent on G/A870 (Figure 1), the impact of the polymorphism on SF2 association was investigated in PCa cells. First, LAPC4 cells were utilized, which are homozygous for the A870 allele (7). Consistent with the individual minigene analyses in Fig. 1, endogenous SF2 associates with the endogenous *transcript-b* in these cells (Figure 4A). RFLP (Restriction Fragment Length Polymorphism) analyses of the SF2-associated transcript was also performed (Figure 4B). These observations show that in PCa, SF2 can associate with *transcript-b* generated by the A870 allele; however, as individual minigene analyses in Fig. 1 indicated a preference for SF2 to associate with *transcript-b* from the G870 allele (when compared in isolation), the most critical analyses were generated in LNCaP cells, which are heterozygous for the polymorphism (shown as genotype control in Figure 4B). This model system afforded the first opportunity to dissect the impact of SF2 on G870- versus A870-derived transcripts in the same model system. As expected, SF2 was associated with total *transcript-b* in this model system (Figure 4C). Using the SF2 bound and recovered transcript, two key determinations were made. First, RFLP analyses of the total transcript (Figure 4D, lane 1) revealed that approximately 61% of the endogenous *transcript-b* was derived from the G-allele, and 39% from the A-allele, thus indicating that in the endogenous setting, both alleles significantly contribute to *transcript-b* production.

Intriguingly, RFLP analyses and quantification of the SF2 bound fraction (Figure 4D, lane 2) resulted in a similar ratio of transcript generated from the G- and A-alleles, based on at least five independent analyses and quantification (Figure 4D, lower panel). Thus, these data show that endogenous SF2 can associate with both transcripts when present, but demonstrates a slight preference for the G870-derived transcript.

SF2 predominantly associates with and produces *transcript-b* from the G-allele

Given the observation that elevated SF2 correlates with high cyclin D1b in human PCa and is sufficient in PCa model systems to induce cyclin D1b production, the impact of the G/A870 polymorphism for this event was determined. As shown, SF2 levels were elevated though ectopic expression of an HA-tagged allele in cells heterozygous for the polymorphism and that express low endogenous cyclin D1b (Supplemental Figure S6), so as to mimic tumor-associated SF2 induction. Subsequent analyses (Figure 5A) confirmed SF2-mediated enhancement of overall *transcript-b* (compare lanes 1, 4). In addition, SF2 association with *transcript-b* was markedly enhanced in the SF2-transfected cells (compare lanes 2, 5), demonstrating that elevated SF2 results not only in enhanced *transcript-b* production, but also enhanced SF2-associated transcript.

Furthermore, these findings also allowed determination of: 1) the allele composition of *transcript-b* levels elevated by SF2; and 2) whether elevated SF2 altered the likelihood of either allele to directly associate with SF2. To answer both questions, RFLP analyses were performed with the SF2-associated transcripts after SF2 induction or in control cells. First, it was observed that SF2 predominantly resulted in an induction of *transcript-b* from the G870 allele (panel 5B, lanes 1 vs. 3; quantified in panel C). These data implicate SF2 as an allele-selective modifier of the alternative splicing event. Second, to determine whether this action of SF2 was direct, RFLP analyses of the SF2-bound fraction was compared in cells with steady-state SF2 (control) vs. those with elevated SF2 expression (Figure 5B, compare lanes 2, 4). For ease of comparison, the percent of SF2 bound to the A870-derived transcript (relative to input) in control cells was set to “1”, and relative association determined for each (Figure 5D). As shown, SF2 association with the G-allele was statistically unchanged under conditions mimicking tumor-associated SF2 elevation. By contrast, SF2 association with the A-allele was enhanced by approximately 2-fold under these conditions.

Collectively, these data suggest a model (summarized in Figure 6) wherein SF2 elicits allele-selective effects on cyclin D1b production in prostate cancer that are dose dependent. Under steady state conditions, SF2 binds with slight preference to transcript generated from the G870 allele; concordantly, the G-allele accounts for 61% of *transcript-b* production with the A-allele accounting for the remaining 39%. These data are consistent with the supposition that SF2 acts (potentially directly) to suppress splicing at the intron-4/exon-4 boundary, and that this event is facilitated by the G870 allele. Elevated SF2, such as observed in human disease, can modestly enhance SF2 association with the A-allele transcript, but induced SF2 remains predominantly associated with and produces cyclin D1b from the G-allele transcript.

Discussion

Here, we identify SF2 as a dose-dependent effector of cyclin D1b production, and provide evidence of a tumor-associated mechanism that alters the influence of the G/A870 polymorphism. While minigene analyses of individual alleles (Figure 1) and evaluation of non-neoplastic human tissue support a role for the A870 allele in promoting *transcript-b* and resultant cyclin D1b production, the influence of the A-allele is thought to be relieved in PCa (11). The present data demonstrate that the RNA binding protein SF2, which is induced as a function of PCa progression, predicts for cyclin D1b (not cyclin D1a) elevation in human disease (Figure 2). By contrast, cyclin D1b production was attenuated in model systems of SF2

depletion (Figure 3). Functional studies in PCa cells heterozygous for the polymorphism unexpectedly demonstrated that SF2 predominantly associates with and induces *transcript-b* derived from the G870-allele, however, association with and production of *transcript-b* from the A-allele can still occur (Figures 4 and 5). Together, these studies provide a novel mechanism by which cyclin D1b oncogene production induced in human disease, and identify tumor-associated SF2 as an allele selective effector of cyclin D1b.

Despite the potent oncogenic activity of cyclin D1b, knowledge of the factor(s) that regulate the *CCND1* alternative splicing event are poorly defined. The present identification of SF2 as an effector of *transcript-b* and cyclin D1b production using both *in vitro* models and analyses of human tumors provides strong evidence linking splicing factor deregulation to oncogene activation. It is tempting to speculate as to whether SF2 can cooperate with few effectors of cyclin D1b production that were identified in other tissue types. For example, in colorectal cells it was observed that knockdown of the SWI/SNF chromatin remodeling complex subunit Brahma (BRM) increased *transcript-b* production without altering *transcript-a* (34). These findings are of note, as BRM was recently shown to be significantly downregulated in human PCa (35), and it has yet to be determined whether BRM loss might also impact SF2 levels. In a separate study, a chromosomal translocation-derived transcription factor known to be upregulated in Ewing's sarcoma (EWS-FLI1) was found to enhance cyclin D1b production by diminishing the rate of transcriptional elongation (10). More recently, it was demonstrated that Sam68 promotes cyclin D1b production through a splicing repressive mechanism by blocking U1-70k association, a constitutive spliceosome accessory factor of the U1 snRNP that is necessary for 5' splice site recognition (36,37). It is well established that chromatin remodeling complexes can alter RNA Pol II accessibility by alteration of the native chromatin structure (38); moreover, pre-mRNA splicing occurs co-transcriptionally, and is aided by the function of SR proteins (including SF2) which can bind to Pol II and selected SWI/SNF subunits (BAF155 and BAF53A) (39). Therefore, an attractive hypothesis is that SF2 could act in concert with either BRM or EWS-FLI1 to modulate the *CCND1* splicing event. Given the marked pro-tumorigenic activity of cyclin D1b, these collective observations further underscore the importance of delineating the mechanisms that regulate or influence the SF2-mediated alternative splicing event in models of disease relevance.

With regard to clinical relevance, it is notable that in PCa cells, SF2 exhibited an allele selective effect on the alternative splicing event. Previous studies showed that in non-neoplastic tissue, the A870 allele is associated with higher *transcript-b* production but that the influence of the A-allele was lost in PCa specimens (11); these findings suggested tumor-associated factors may either bypass the impact of the polymorphism or bolster production of *transcript-b* from the G-allele. The present data support the hypothesis that SF2 may serve as such a factor, since SF2 induction (such as occurs in human disease) predominantly binds to *transcript-b* derived from the G-allele and promotes accumulation of this transcript. Production of *transcript-b* still occurs from the A-allele (about 40%), however, appears to remain unaltered following modulation of SF2 expression, thus it will be of interest to examine the functional relationship between SF2 and the A-allele. Examination of how SF2 influences allele-specific cyclin D1b production in other tumor types in which cyclin D1b levels are elevated as a function of tumorigenesis (e.g. colon, bladder or breast carcinoma) will be critical (6,9,40,41). Given the propensity of SF2 to preferentially bind to and induce cyclin D1b from the G-allele, the present data indicate that SF2 may promote intron inclusion at the *CCND1* exon4-intron4 boundary, and precedence for a splicing repressor function of SF2 was previously established for the MNK2 kinase (42). It cannot be presently ruled out that the impact of SF2 could be manifest through other means, given the ability of SF2 to affect mRNA metabolism, mRNA transport and/or stability, and mTOR-mediated translation (43,44). Future analyses will be directed at defining the action of SF2 at the exon4-intron4 boundary.

Finally, the present findings provide new insight into a potential means through which SF2 promotes cellular transformation. It is noteworthy that SF2 can independently induce transformation and induce tumor growth *in vivo* (42), thus demonstrating phenotypes similar to those observed with cyclin D1b (3,5). As SF2 levels correlated with cyclin D1b in human PCa, these data implicate cyclin D1b as a possible downstream effector of SF2-mediated cellular transformation in the prostate. Additional mechanisms are predicted to contribute to this event, as perturbations in spliceosome function and RNA processing proteins have been recently identified as major contributors to genomic instability (45). Importantly, dysregulation of splicing factors has also been shown to accelerate PCa progression and metastases, such as observed by deregulated expression of SRp40, or in the presence of specific, cancer-risk associated polymorphisms in the intronic region of the KLF6 tumor suppressor that create novel SRp40 binding sites (46). Given the disease relevance of this event, it will be imperative to determine not only the consequence of SR protein dysregulation, but whether these events act in concert to promote tumor progression. Ongoing studies suggest that tumor-associated SF2 induction may induce and enhanced migration and invasion phenotype in prostate cancer cells (data not shown), providing the impetus to discern the impact of SF2 on development of tumor metastases.

In summary, our study identifies SF2 as a novel, clinically relevant effector of *CCND1* alternative splicing, capable of promoting allele-specific induction of cyclin D1b in prostate cancer. The findings presented are among the first to determine how the cyclin D1b oncogene is enhanced in human disease, and provide the foundation for future studies directed at developing mechanisms to target oncogene induction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank the K.Knudsen lab for ongoing discussions, Dr. E. Knudsen for critical commentary, Drs. J. Bruzik, C. Chalfant, and R. Carstens for valuable discussions, and J. Tulenko for assistance with figure presentation. This study was supported by NIH grants R01 CA09996 and CA116777 (to K.E.K.), Associazione Italiana Ricerca sul Cancro and Association for International Cancer Research (to C.S.), a DOD New Investigator Award (to C.E.S.C.). M.J.S. and M.A.A. are supported by pre-doctoral fellowships from the Department of Defense, and N.A.O. is supported by NIEHS training grant 2T32ES007250-21A1.

References

1. Betticher DC, Thatcher N, Altermatt HJ, Hoban P, Ryder WD, Heighway J. Alternate splicing produces a novel cyclin D1 transcript. *Oncogene* 1995;11:1005–1011. [PubMed: 7675441]
2. Hosokawa Y, Tu T, Tahara H, Smith AP, Arnold A. Absence of cyclin D1/PRAD1 point mutations in human breast cancers and parathyroid adenomas and identification of a new cyclin D1 gene polymorphism. *Cancer letters* 1995;93:165–170. [PubMed: 7621424]
3. Rojas P, Benavides F, Blando J, et al. Enhanced skin carcinogenesis and lack of thymus hyperplasia in transgenic mice expressing human cyclin D1b (CCND1b). *Molecular carcinogenesis* 2009;48:508–516. [PubMed: 18942117]
4. Solomon DA, Wang Y, Fox SR, et al. Cyclin D1 splice variants. Differential effects on localization, RB phosphorylation, and cellular transformation. *The Journal of biological chemistry* 2003;278:30339–30347. [PubMed: 12746453]
5. Lu F, Gladden AB, Diehl JA. An alternatively spliced cyclin D1 isoform, cyclin D1b, is a nuclear oncogene. *Cancer research* 2003;63:7056–7061. [PubMed: 14612495]
6. Kim CJ, Nishi K, Isono T, et al. Cyclin D1b variant promotes cell invasiveness independent of binding to CDK4 in human bladder cancer cells. *Molecular carcinogenesis* 2009;10:953–964. [PubMed: 19415719]

7. Burd CJ, Petre CE, Morey LM, et al. Cyclin D1b variant influences prostate cancer growth through aberrant androgen receptor regulation. *Proceedings of the National Academy of Sciences of the United States of America* 2006;103:2190–2195. [PubMed: 16461912]
8. Li R, An SJ, Chen ZH, et al. Expression of cyclin D1 splice variants is differentially associated with outcome in non-small cell lung cancer patients. *Human pathology* 2008;39:1792–1801. [PubMed: 18715616]
9. Millar EK, Dean JL, McNeil CM, et al. Cyclin D1b protein expression in breast cancer is independent of cyclin D1a and associated with poor disease outcome. *Oncogene* 2009;28:1812–1820. [PubMed: 19287456]
10. Sanchez G, Bittencourt D, Laud K, et al. Alteration of cyclin D1 transcript elongation by a mutated transcription factor up-regulates the oncogenic D1b splice isoform in cancer. *Proceedings of the National Academy of Sciences of the United States of America* 2008;105:6004–6009. [PubMed: 18413612]
11. Comstock CE, Augello MA, Benito RP, et al. Cyclin D1 Splice Variants: Polymorphism, Risk, and Isoform-Specific Regulation in Prostate Cancer. *Clin Cancer Res* 2009;17:5338–5349. [PubMed: 19706803]
12. Holley SL, Heighway J, Hoban PR. Induced expression of human CCND1 alternative transcripts in mouse Cyl-1 knockout fibroblasts highlights functional differences. *International journal of cancer* 2005;114:364–370.
13. Howe D, Lynas C. The cyclin D1 alternative transcripts [a] and [b] are expressed in normal and malignant lymphocytes and their relative levels are influenced by the polymorphism at codon 241. *Haematologica* 2001;86:563–569. [PubMed: 11418364]
14. Petre CE, Wetherill YB, Danielsen M, Knudsen KE. Cyclin D1: mechanism and consequence of androgen receptor co-repressor activity. *The Journal of biological chemistry* 2002;277:2207–2215. [PubMed: 11714699]
15. Petre-Draviam CE, Cook SL, Burd CJ, Marshall TW, Wetherill YB, Knudsen KE. Specificity of cyclin D1 for androgen receptor regulation. *Cancer research* 2003;63:4903–4913. [PubMed: 12941814]
16. Petre-Draviam CE, Williams EB, Burd CJ, et al. A central domain of cyclin D1 mediates nuclear receptor corepressor activity. *Oncogene* 2005;24:431–444. [PubMed: 15558026]
17. Chen C, Okayama H. High-efficiency transformation of mammalian cells by plasmid DNA. *Molecular and cellular biology* 1987;7:2745–2752. [PubMed: 3670292]
18. Wang J, Takagaki Y, Manley JL. Targeted disruption of an essential vertebrate gene: ASF/SF2 is required for cell viability. *Genes & development* 1996;10:2588–2599. [PubMed: 8895660]
19. Caceres JF, Misteli T, Screaton GR, Spector DL, Krainer AR. Role of the modular domains of SR proteins in subnuclear localization and alternative splicing specificity. *The Journal of cell biology* 1997;138:225–238. [PubMed: 9230067]
20. Lai MC, Tarn WY. Hypophosphorylated ASF/SF2 binds TAP and is present in messenger ribonucleoproteins. *The Journal of biological chemistry* 2004;279:31745–31749. [PubMed: 15184380]
21. Burd, CJ.; Petre, CE.; Moghadam, H.; Wilson, EM.; Knudsen, KE. *Molecular endocrinology*. Vol. 19. Baltimore, Md: 2005. Cyclin D1 binding to the androgen receptor (AR) NH2-terminal domain inhibits activation function 2 association and reveals dual roles for AR corepression; p. 607-620.
22. Sawa H, Ohshima TA, Ukita H, et al. Alternatively spliced forms of cyclin D1 modulate entry into the cell cycle in an inverse manner. *Oncogene* 1998;16:1701–1712. [PubMed: 9582018]
23. Paronetto MP, Achsel T, Massiello A, Chalfant CE, Sette C. The RNA-binding protein Sam68 modulates the alternative splicing of Bcl-x. *The Journal of cell biology* 2007;176:929–939. [PubMed: 17371836]
24. Wang Y, Dean JL, Millar EK, et al. Cyclin D1b is aberrantly regulated in response to therapeutic challenge and promotes resistance to estrogen antagonists. *Cancer research* 2008;68:5628–5638. [PubMed: 18632615]
25. Jensen FC, Girardi AJ, Gilden RV, Koprowski H. Infection of Human and Simian Tissue Cultures with Rous Sarcoma Virus. *Proceedings of the National Academy of Sciences of the United States of America* 1964;52:53–59. [PubMed: 14192657]

26. Webber MM, Bello D, Kleinman HK, Hoffman MP. Acinar differentiation by non-malignant immortalized human prostatic epithelial cells and its loss by malignant cells. *Carcinogenesis* 1997;18:1225–1231. [PubMed: 9214606]
27. Cartegni L, Wang J, Zhu Z, Zhang MQ, Krainer AR. ESEfinder: A web resource to identify exonic splicing enhancers. *Nucleic acids research* 2003;31:3568–3571. [PubMed: 12824367]
28. Smith PJ, Zhang C, Wang J, Chew SL, Zhang MQ, Krainer AR. An increased specificity score matrix for the prediction of SF2/ASF-specific exonic splicing enhancers. *Human molecular genetics* 2006;15:2490–2508. [PubMed: 16825284]
29. Lapointe J, Li C, Higgins JP, et al. Gene expression profiling identifies clinically relevant subtypes of prostate cancer. *Proceedings of the National Academy of Sciences of the United States of America* 2004;101:811–816. [PubMed: 14711987]
30. Vanaja DK, Chevillet JC, Iturria SJ, Young CY. Transcriptional silencing of zinc finger protein 185 identified by expression profiling is associated with prostate cancer progression. *Cancer research* 2003;63:3877–3882. [PubMed: 12873976]
31. Welsh JB, Sapinoso LM, Su AI, et al. Analysis of gene expression identifies candidate markers and pharmacological targets in prostate cancer. *Cancer research* 2001;61:5974–5978. [PubMed: 11507037]
32. Crowley CW, Cohen RL, Lucas BK, Liu G, Shuman MA, Levinson AD. Prevention of metastasis by inhibition of the urokinase receptor. *Proceedings of the National Academy of Sciences of the United States of America* 1993;90:5021–5025. [PubMed: 8389464]
33. Li X, Wang J, Manley JL. Loss of splicing factor ASF/SF2 induces G2 cell cycle arrest and apoptosis, but inhibits internucleosomal DNA fragmentation. *Genes & development* 2005;19:2705–2714. [PubMed: 16260492]
34. Batsche E, Yaniv M, Muchardt C. The human SWI/SNF subunit Brm is a regulator of alternative splicing. *Nature structural & molecular biology* 2006;13:22–29.
35. Shen H, Powers N, Saini N, et al. The SWI/SNF ATPase Brm is a gatekeeper of proliferative control in prostate cancer. *Cancer research* 2008;68:10154–10162. [PubMed: 19074882]
36. Black DL. Mechanisms of alternative pre-messenger RNA splicing. *Annual review of biochemistry* 2003;72:291–336.
37. Paronetto MP, Cappellari M, Busa R, et al. Alternative splicing of the cyclin D1 proto-oncogene is regulated by the RNA-binding protein Sam68. *Cancer research* 70:229–239. [PubMed: 20028857]
38. Clapier CR, Cairns BR. The biology of chromatin remodeling complexes. *Annual review of biochemistry* 2009;78:273–304.
39. Das R, Yu J, Zhang Z, et al. SR proteins function in coupling RNAP II transcription to pre-mRNA splicing. *Molecular cell* 2007;26:867–881. [PubMed: 17588520]
40. Bala S, Peltomaki P. CYCLIN D1 as a genetic modifier in hereditary nonpolyposis colorectal cancer. *Cancer research* 2001;61:6042–6045. [PubMed: 11507050]
41. Hong Y, Eu KW, Seow-Choen F, Fook-Chong S, Cheah PY. GG genotype of cyclin D1 G870A polymorphism is associated with increased risk and advanced colorectal cancer in patients in Singapore. *Eur J Cancer* 2005;41:1037–1044. [PubMed: 15862753]
42. Karni R, de Stanchina E, Lowe SW, Sinha R, Mu D, Krainer AR. The gene encoding the splicing factor SF2/ASF is a proto-oncogene. *Nature structural & molecular biology* 2007;14:185–193.
43. Michlewski G, Sanford JR, Caceres JF. The splicing factor SF2/ASF regulates translation initiation by enhancing phosphorylation of 4E-BP1. *Molecular cell* 2008;30:179–189. [PubMed: 18439897]
44. Zhong XY, Wang P, Han J, Rosenfeld MG, Fu XD. SR proteins in vertical integration of gene expression from transcription to RNA processing to translation. *Molecular cell* 2009;35:1–10. [PubMed: 19595711]
45. Paulsen RD, Soni DV, Wollman R, et al. A genome-wide siRNA screen reveals diverse cellular processes and pathways that mediate genome stability. *Molecular cell* 2009;35:228–239. [PubMed: 19647519]
46. Narla G, Difeo A, Reeves HL, et al. A germline DNA polymorphism enhances alternative splicing of the KLF6 tumor suppressor gene and is associated with increased prostate cancer risk. *Cancer research* 2005;65:1213–1222. [PubMed: 15735005]

Fig. 1. SF2 associates with *transcript-b* and shows allele preference in minigene analyses (A) Schematic of the pcDNA3.1-cyclin D1-Intron 4 minigenes. Primers used to amplify *transcript-a* (bars; exon 1 and exon 5) and *transcript-b* (arrows; exon 4 and intron 4) are indicated. Clonal isolates C33A-Vec, C33A-G1 and C33A-A1 are as described in the Materials and Methods section. (B) Representative mRNA (left panel) and protein (right panel) analyses of cell lines engineered to individually express the G870 or A870-containing minigenes. Non-template (NT) is the negative control. (C) Analyses of predicted SF2 binding to the CCND1 splice donor site as determined using the ESEfinder3.1 resource. Exonic (upper case) and intronic (lower case) sequence is shown, and the G/A870 polymorphic site underlined. (D) C33A-derived stable cell lines shown in panel B were subjected to a RIP. Representative RT-PCR reaction of input and SF2 or IgG-associated transcript is shown (top panel), and quantification by qPCR of at least 3 independent experiments provided (bottom panel). Statistical significance ($p < 0.05$) was determined by ANOVA.

Fig. 2. SF2 is enhanced in PCa and positively correlates with cyclin D1b

(A) Analyses of independent microarray analyses were determined using the Oncomine resource. Relative SF2 expression in non-neoplastic (N), primary PCa (PCa) and metastatic PCa (Met) are shown and p-values given. (B) Representative immunostaining of tissue microarrays using the AQUA platform. As previously described, cells of epithelia origin were identified using a pan-cytokeratin antisera (green), nuclei were stained with DAPI (blue), and cyclin D1 isoforms detected in serial sections using well characterized, isoform specific antisera (red). (C) Quantification of all samples analyzed ($n=49$) across each core was used to determine the relevance of SF2 for cyclin D1a (left) and cyclin D1b (right) levels. P-values were determined via Spearman Rank Correlation.

Fig. 3. Cyclin D1b production is induced in multiple models systems by SF2

(A) LNCaP cells were transfected with constructs encoding empty vector, T7-SRp40 or T7-SF2, harvested after 48 h, and examined for expression of indicated transcripts. Representative PCR (upper panel) and quantification by qPCR of at least 3 independent experiments (bottom panel) are shown. (B) DT40-ASF/SF2 cells were transfected with plasmids encoding either empty vector or the G870 minigene. Transfected cells were split after 24 h into populations treated with vehicle or doxycycline for 24 h. Cells were harvested, lysed and subjected to immunoblot analyses with the indicated antisera (left panel) or RNA was isolated and subjected to RT-PCR analyses for the indicated transcripts (right panel).

Fig. 4. Endogenous SF2 can associate with transcript generated from both G/A870 alleles in PCa (A) Asynchronous LAPC4 cells were harvested and subjected to a RIP. An aliquot of immunoprecipitated complexes was subjected to SDS-PAGE and immunoblot to validate SF2 recovery (left panel). From the remaining sample, RNA was isolated, converted to cDNA, and analyzed for the presence of *transcript-b* by PCR (representative sample, right panel). qPCR quantification of at least 4 independent experiments is shown (lower left). P-value determined by ANOVA. (B) Input and SF2-recovered transcripts from Fig. 4A were amplified with primers required for RFLP analyses of the *CCND1* polymorphism, which have been previously described (38). Following amplification, the PCR product was ScrFI digested and visualized on an agarose gel. Genotype control is from amplification of genomic DNA from LNCaP cells. (C) Asynchronous LNCaP cells were treated as per panel A. Relative SF2 pulldown and *transcript-b* recovery are shown. Quantification was from at least 5 independent RIP experiments. (D) Analyses of input and SF2-associated transcripts by RFLP from panel C is shown (top panel). Relative band intensities of endogenous, overall *transcript-b* levels as a

function of the G/A870 allele (generated from the input band) or SF2-associated transcript (generated from the SF2-RIP studies) were quantified from at least five independent experiments (lower panel). Averages and SEM are shown. The ratio of *transcript-b* as A to G (A/G) is shown for the representative image.

Fig. 5. Elevated expression of SF2 predominately associates with and promotes *transcript-b* production from the G-allele

(A) LNCaP cells were transfected with expression constructs encoding empty vector or HA-SF2. 48 h later, cells were harvested and subjected to a RIP. *Transcript-b* expression was analyzed as previously described (Fig. 1). (B) Input and SF2-associated RNA from panel A were then subjected to RFLP analyses as in Fig. 4D. Representative analyses are shown with their respective ratios of *transcript-b* as A to G (A/G). (C) To assess the impact of SF2 elevation on allele-specific *transcript-b* production, relative change in transcript abundance generated from the G or A-allele after SF2 introduction was determined by quantification of the “input” RFLP signal (represented by Fig. 5B, percent change in lane 3 vs. lane 1) from four independent experiments. Average change in relative G or A-allele derived transcript per experiment was averaged and plotted as percent D1b change over vector control. (D) To assess the impact of SF2 elevation on allele-specific SF2-association with *transcript-b*, relative band intensities from the SF2 pulldowns (lanes 2 vs. 4) from four independent experiments were plotted setting SF2 association in the control (vector) with the A-allele (lane 2, top band relative to input) as “1”.

Fig. 6. Model of SF2 activity

Under steady state conditions, approximately 60% of detected *transcript-b* is generated from the G870 allele, and SF2 predominantly binds to the G870-derived transcript. SF2 elevation, as associated with PCa progression, enhances association with the A870 allele, but the observed increase in *transcript-b* production is exquisitely associated with the G870 allele. “No Δ” = no change.