

The Arginine Methyltransferase CARM1 Regulates the Coupling of Transcription and mRNA Processing

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SUMMARY

The coactivator-associated arginine methyltransferase CARM1 is recruited by many different transcription factors as a positive regulator. To understand the mechanism by which CARM1 functions, we sought to isolate its substrates. We developed a small-pool screening approach for this purpose and identified CA150, SAP49, SmB, and U1C as splicing factors that are specifically methylated by CARM1. We further showed that CA150, a molecule that links transcription to splicing, interacts with the Tudor domain of the spinal muscular atrophy protein SMN in a CARM1-dependent fashion. Experiments with an exogenous splicing reporter and the endogenous CD44 gene revealed that CARM1 promotes exon skipping in an enzyme-dependent manner. The identification of splicing factors that are methylated by CARM1, and protein-protein interactions that are regulated by CARM1, strongly implicates this enzyme in the regulation of alternative splicing and points toward its involvement in spinal muscular atrophy pathogenesis.

INTRODUCTION

Arginine methylation has been implicated in the regulation of transcription, translation and DNA repair (Bedford and Richard, 2005). This posttranslational modification is catalyzed by a family of protein arginine methyltransferases (PRMTs), of which there are at least nine members. CARM1 (also referred to as PRMT4) was identified in the yeast two-hybrid assay to associate with GRIP1, the p160 steroid receptor coactivator (Chen et al., 1999). The recruitment of CARM1 to transcriptional promoters results in the methylation of both histone H3 at arginine 17 (Chen et al., 1999) and the histone acetyltransferases (HATs) p300/CBP (Lee et al., 2005). This methylation has a positive effect on transcription, and therefore CARM1 is considered a coactivator. Gene targeting of CARM1 in

mice has been performed, and knockout mice, which are smaller than their wild-type littermates, die just after birth (Yadav et al., 2003). CARM1 knockout mouse embryonic fibroblasts (MEFs) were established and have been used to confirm CARM1's role as a coactivator for the estrogen receptor (Yadav et al., 2003) and NF- κ B (Covic et al., 2005).

In order to gain a mechanistic understanding of CARM1's role as a transcriptional coactivator, we need to know the repertoire of proteins it methylates and the functional significance that methylation has on these substrates. Apart from histone H3 and p300/CBP, additional CARM1 substrates have been identified by performing large-scale enzyme reactions on protein arrays (Lee and Bedford, 2002), which include the poly(A)-binding protein 1 (PABP1) and the T cell-specific factor TARPP. Candidate approaches have also been used to identify the RNA-binding proteins HuR and HuD as CARM1 substrates (Fujiwara et al., 2006). Thus, CARM1 methylates two classes of proteins—class 1 substrates include proteins that are involved in chromatin remodeling (histone H3 and p300/CBP), and class 2 substrates possess RNA-binding properties (PABP1, TARPP, HuR, HuD, and splicing factors identified in this study). The ability of CARM1 to methylate RNA-binding proteins points to its possible role in RNA processing, and indeed a recent study reported that CARM1 interacts with the U1C splicing factor and regulates alternative splicing (Ohkura et al., 2005). Importantly, we have identified several splicing and transcription elongation factors (SmB, SAP49, U1C, and CA150) as bona fide substrates of CARM1. The methylation sites on these factors map to a common protein interaction domain—the PGM motif (Bedford et al., 1998). This motif has been shown previously to be the dominant protein-protein interaction module in SmB that binds both SH3 and WW domains (Espejo et al., 2002), and here we show that this same region interacts with the Tudor domain of SMN. This interaction is subject to regulation by asymmetric, CARM1-mediated arginine methylation. Thus, Tudor domains are emerging as a major class of methyl-binding domain that interacts with symmetrically methylated arginine motifs (Friesen et al., 2001), methyllysine marks (Kim et al., 2006), and asymmetrically methylated arginine motifs (this study). We further show that the enzymatic activity of CARM1 alters the patterns of exon choice in splicing. These results

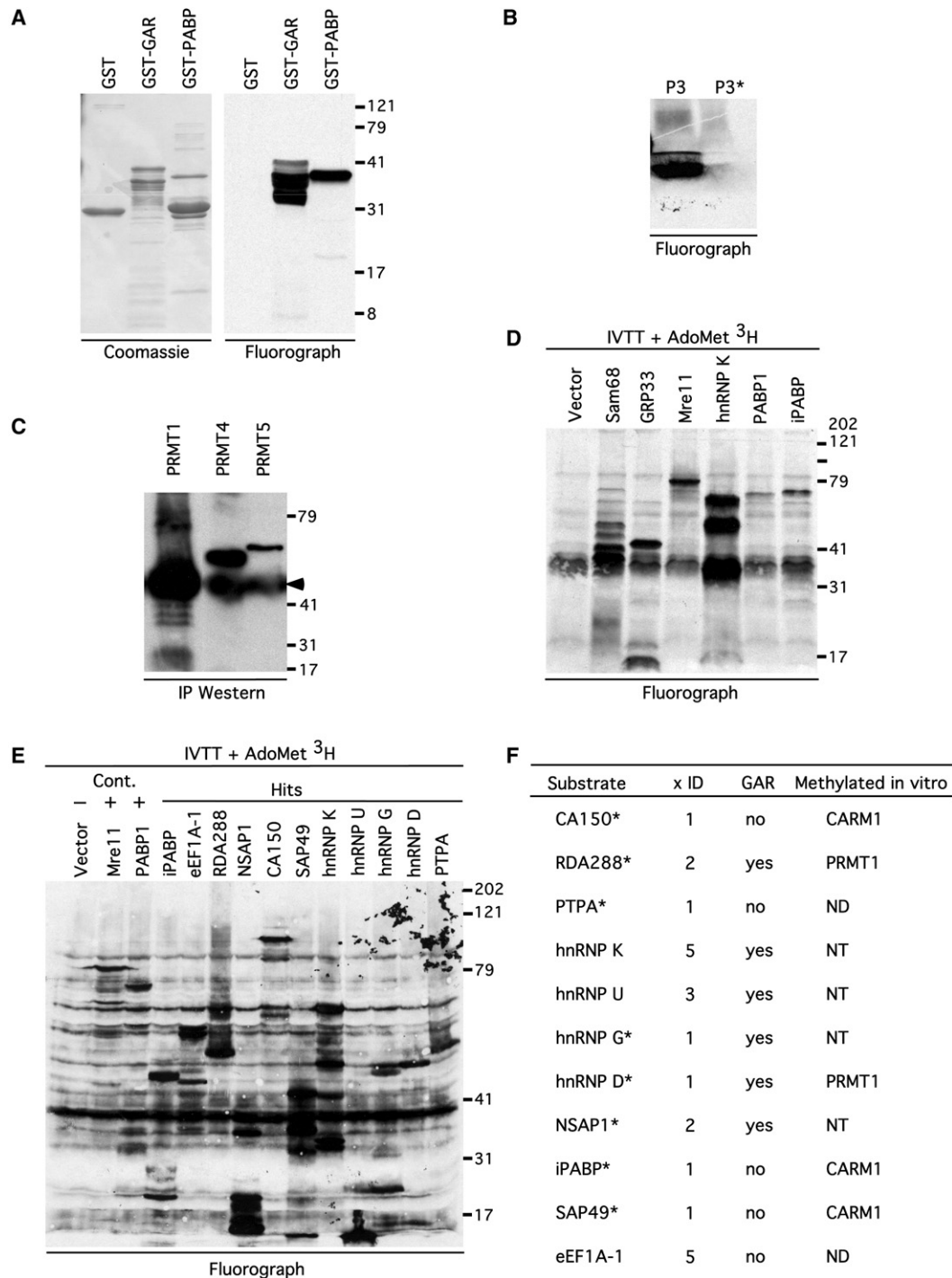


Figure 1. Developing a Small-Pool Screening Approach to Identify Methylated Protein

(A) GST fusions of known methylated proteins can be methylated when using reticulocyte lysate as an enzyme source.

(B) The unmethylated form of the P3 peptide is a methyl acceptor, whereas the methylated form (P3*) is not.

(C) Immunoprecipitation (IP) followed by western analysis demonstrates the presence of PRMT1, PRMT5, and CARM1 in reticulocyte lysate. IPs were performed with the indicated antibodies, and western analysis was performed with a mixture of the same three antibodies that were used to perform the IP. Three splice variants of PRMT1 are seen. Arrowhead, IgG heavy chain.

(D) An IVTT reaction of known arginine-methylated templates, in the presence of tritium-labeled AdoMet, results in labeled proteins. In certain lanes, more than one band is seen due to premature termination of the IVTT product.

(E) An IVTT reaction of all the methylated hits identified in the screen was carried out in the presence of tritium-labeled AdoMet. An empty vector (–) and vectors harboring cDNA from known arginine-methylated proteins (+) are included as controls.

suggest that the methylation by CARM1 of PGM motifs located on splicing and transcription elongation factors regulates protein-protein interactions that are involved in the ordered assembly of the spliceosome. Thus, CARM1-mediated methylation couples transcription to splicing and impacts alternative splicing.

RESULTS

Using Small-Pool Screening to Identify CARM1 Substrates

Small-pool screening is a systematic and broadly applicable approach to cloning genes based solely on the biological activities or biochemical properties of the gene product (Lustig et al., 1997). We modified this method to facilitate the identification of methylated proteins. A plasmid cDNA library of 14,000 cDNA clones was generated and divided into pools containing ten clones. Using a coupled transcription/translation system, we produce protein pools. The reticulocyte lysate is an enzyme “soup” that allows transcription/translation in vitro (IVTT). We predicted that this soup also contains copious amounts of protein methyltransferase activity—both lysine and arginine. Thus, if IVTTs are performed in the presence of tritium-labeled S-adenosyl-L-methionine (AdoMet), then the labeled proteins represent methyl acceptors. To identify the methylated proteins, single plasmids from a deconvoluted plasmid pool are sequenced.

To show that rabbit reticulocyte lysate contained at least active PRMT1 and CARM1, we carried out an in vitro methylation reaction, in the presence of labeled AdoMet, on GST, GST-GAR (a PRMT1, -3, -5, and -6 substrate), and GST-PABP (a CARM1 substrate), using rabbit reticulocyte lysate as the sole enzyme source. GST-GAR and GST-PABP were labeled in this reaction, while GST remained unlabeled, indicating the presence of protein methyltransferase activity (Figure 1A). To further establish the presence of PRMT activity in the lysate, we incubated a peptide (P3) from the methylated protein Sam68 with reticulocyte lysate and hot AdoMet. The P3 peptide is an acceptor of methyl groups, but the methylated P3* peptide remains unlabeled, as all the arginine residues are synthesized asymmetrically dimethylated (Figure 1B), thus further establishing that PRMT1-like activity is present in reticulocyte lysate. In addition, immunoprecipitation (IP) followed by western analysis demonstrated the presence of PRMT1, PRMT4 (CARM1), and PRMT5 in the reticulocyte lysate (Figure 1C).

We then performed an in vitro transcription/translation pilot experiment using expression vectors harboring known and predicted arginine-methylated proteins (Sam68, GRP33, Mre11, hnRNP K, PABP1, and the inducible poly(A)-binding protein, iPABP) (Bedford and Richard,

2005). IVTT reaction on these templates, in the presence of labeled AdoMet, resulted in labeled proteins (Figure 1D), thus demonstrating the feasibility of using a small-pool approach to screen for methylated proteins.

Using this approach, we then screened the 1400 plasmid pools and identified a number of methylated proteins (Figures 1E and 1F). Of the 11 identified substrates found, only hnRNP K and hnRNP U were previously described as being arginine-methylated proteins (Lee and Bedford, 2002; Ostareck-Lederer et al., 2006). In addition, eEF1A-1 has been reported to contain internal amino-methylated lysine residues (Dever et al., 1989) and to carry a C-terminal carboxymethyl ester (Zobel-Thropp et al., 2000).

The majority of the identified methylated proteins contained GAR motifs (glycine- and arginine-rich motifs) that are indicative of PRMT1, -3, -6, and -8 substrates. We were particularly interested in identifying substrates for CARM1 because it does not methylate a GAR motif and its substrates can only be identified empirically. We thus generated GST-fused recombinant proteins for all non-GAR motif-containing substrates (CA150, PTPA, iPABP, SAP49, and eEF1A-1) and a few of the GAR motif-containing substrates (RDA288 and hnRNP D). The GST fusion proteins were tested in in vitro methylation reactions with recombinant PRMTs (PRMT1, PRMT6, and CARM1) and recombinant lysine methyltransferases (SET 7/9, hDOT1L, and Suv39h1). In this fashion, we established which enzymes could methylate each substrate in vitro (Figure 1F). The type of methylation found on PTPA, and the putative lysine methyltransferase that modifies eEF1A-1, remain undetermined. Those substrates that harbor GAR motifs were indeed methylated by PRMT1 and PRMT6. CARM1 was able to efficiently methylate CA150, iPABP, and SAP49 (data not shown). CA150 is involved in transcriptional elongation and splicing and has been proposed to tether these two cellular processes (Goldstrohm et al., 2001; Lin et al., 2004; Sanchez-Alvarez et al., 2006; Smith et al., 2004). SAP49 is also a splicing factor (Das et al., 1999). iPABP is closely related to PABP1, which we have previously identified and characterized as a CARM1 substrate (Lee and Bedford, 2002).

CARM1 Methylates a Cohort of Splicing Factors

We next characterized CA150 as a CARM1 substrate. To establish the region of CA150 that is methylated by CARM1, in vitro methylation assays were performed on four GST-CA150 contigs that cover the coding region of CA150 (Figure 2A). Methylation of the N-terminal-most fusion, GST-CA150^a, was observed (Figure 2B). This N-terminal region is a proline-, glycine-, methionine-, and arginine-rich zone, has been termed a PGM motif, and has been previously noted in splicing factors (Bedford et al., 1998). We also saw this motif in SAP49 and at least two

(F) List of the identified substrates. Some of the substrates were identified multiple times. An asterisk (*) denotes those substrates that were identified as methylated proteins for the first time in this study. Substrates that contain a GAR motif are noted. The enzyme that can methylate the recombinant form of each substrate in vitro is reported. NT refers to substrates that were not tested in in vitro reactions. ND refers to recombinant substrates that were tested but for which a methylating enzyme was not determined.

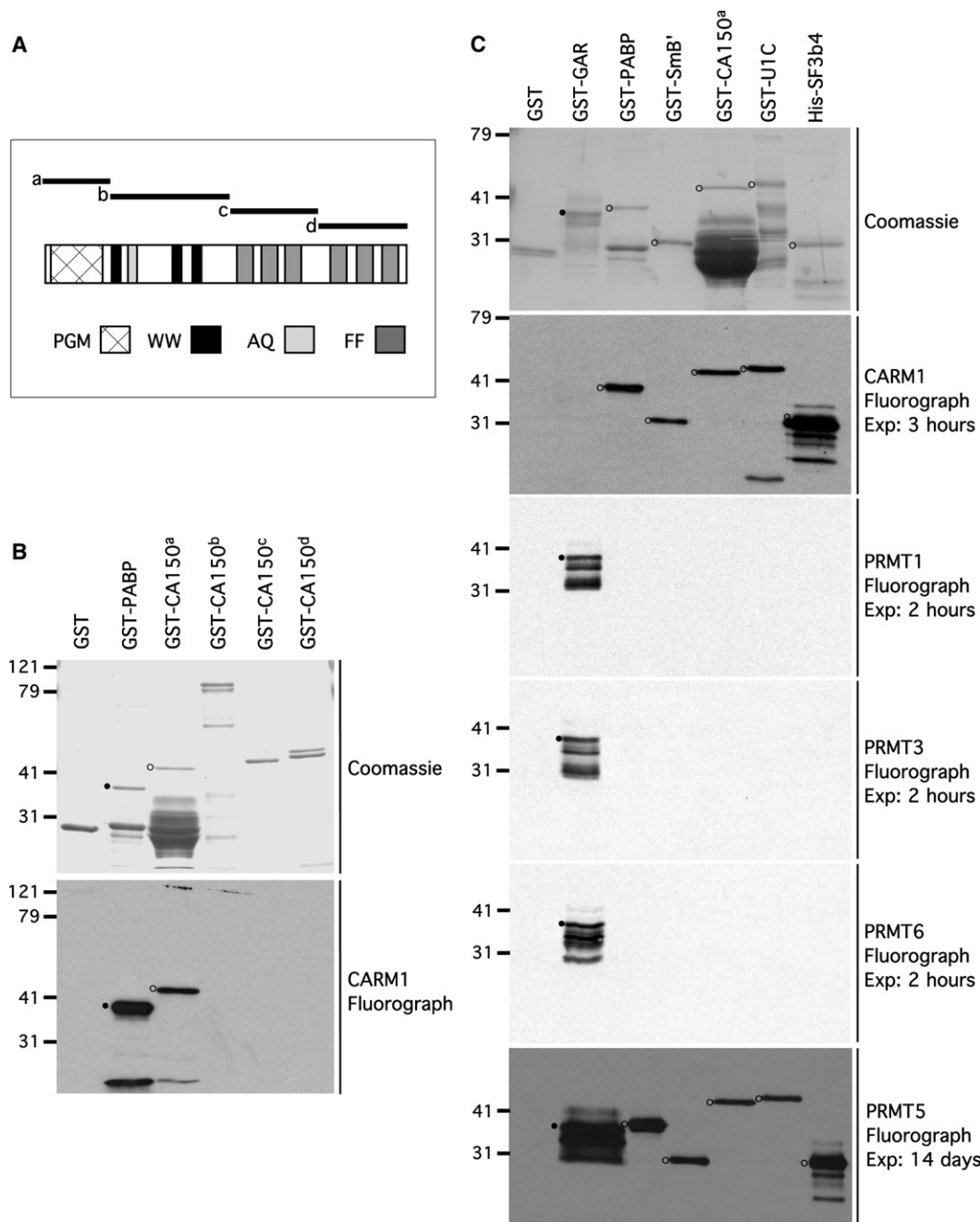


Figure 2. CA150 and Other PGM Motif-Containing Proteins Are Methylated by CARM1 In Vitro

(A) GST fusion proteins were generated from the indicated regions of CA150.

(B) CA150^a fragment is methylated in vitro by recombinant CARM1. GST-PABP serves as a positive control for recombinant CARM1 activity.

(C) A number of PGM motif-containing splicing factors are methylated by CARM1 in vitro, including CA150, SmB, U1C, and SAP49. PRMT5 (myc-tagged) that is expressed in CARM1 null cells also methylates PGM motif-containing splicing factors. The circles on the gels indicate the positions of the full-length GST fusion proteins that are methylated by the respective PRMTs.

other splicing factors, U1C and SmB (see [Figure S1](#) in the [Supplemental Data](#) available with this article online). We then tested the ability of CARM1 to specifically methylate the PGM motifs of CA150, SAP49, SmB, and U1C and

found that all of these splicing factors are in vitro substrates for CARM1 and not other type I PRMTs ([Figure 2C](#)). Importantly, the PGM motif-containing proteins are also in vitro substrates for PRMT5.

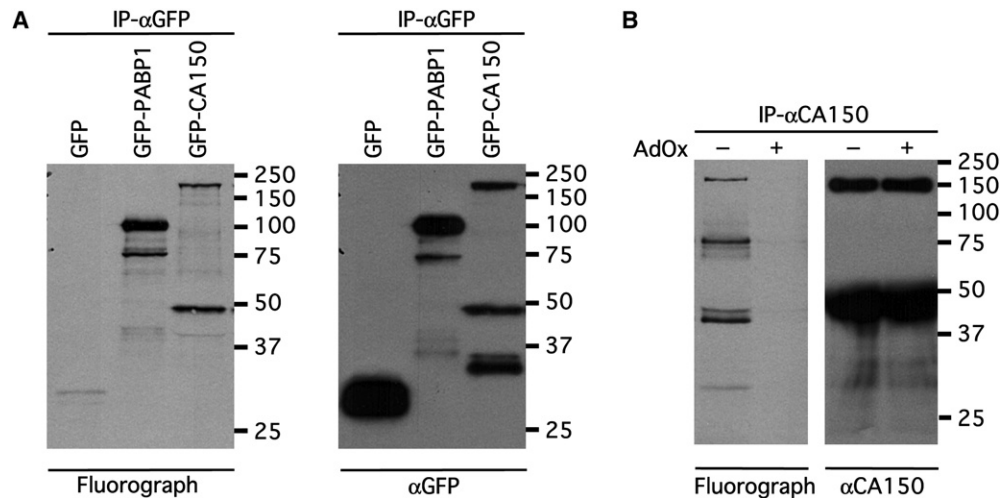


Figure 3. CA150 Is Methylated In Vivo

(A) HeLa cells transiently expressing GFP, GFP-PABP1, and GFP CA150 were incubated with cycloheximide and L-[methyl- 3 H]methionine for 3 hr. GFP fusion proteins were immunoprecipitated (IP) with anti-GFP antibodies (α GFP). In vivo methylation was visualized by fluorography (left). Expression of GFP fusion proteins was analyzed by western blot (right).

(B) Methylated proteins were labeled in vivo, and endogenous CA150 was immunoprecipitated. Methylated CA150 was visualized by fluorography (left), and the same membrane was immunoblotted with an α CA150 antibody (right). In the presence of 10 μ M AdOx, a global methylation inhibitor, methylation of endogenous CA150 is lost.

CA150 Is Methylated In Vivo

To determine whether endogenous CA150 is methylated in cells, we used an in vivo methylation assay (Lee and Bedford, 2002). HeLa cells were transiently transfected with GFP, GFP-PABP1, and GFP-CA150, and the total methylated protein pool was labeled by incubating cells with [methyl- 3 H]-L-methionine in the presence of the protein synthesis inhibitor, cycloheximide. After IP with α GFP antibodies, the proteins were separated by SDS-PAGE, transferred to a PVDF membrane, and subjected to fluorography (Figure 3A, left). GFP-PABP1 serves as a positive control for in vivo methylation. GFP is a negative control to confirm that translation was indeed inhibited. We found that the GFP fusions of PABP1 and CA150 were methylated in HeLa cells, indicating that CA150 (fused to GFP) is methylated in vivo. The same membrane was then subjected to western analysis using an α GFP antibody to confirm roughly equal loading (Figure 3A, right). To establish that endogenous CA150 is methylated, the same in vivo methylation assay was performed on HeLa cells. Endogenous CA150 was immunoprecipitated from these labeled cell extracts. CA150 was methylated in HeLa cells, and this methylation was inhibited in the presence of the global methylation inhibitor AdOx (Figure 3B).

CA150 Is Methylated by CARM1 and PRMT5

We have demonstrated that both ectopically expressed and endogenous CA150 are methylated in HeLa cells (Figure 3). To determine whether CA150 was methylated in the absence of CARM1, we performed an in vivo methylation reaction in *Carm1*^{+/+} and *Carm1*^{-/-} MEFs. CA150 was immunoprecipitated from these labeled cell extracts. Equiv-

alent amounts of CA150 were present in both cell lines, and CA150 from the wild-type and the knockout line was methylated (Figure 4A), suggesting that CA150 is methylated by at least one other enzyme apart from CARM1, likely PRMT5 (Figure 2C).

The first substrate to be identified for CARM1 was histone H3 (Chen et al., 1999), which is methylated at arginines 2, 17, and 26 on its N-terminal tail. Methyl-specific antibodies have been raised against the methyl-arginine 17 mark (α H3R17me2a, Upstate 07-214), and we have shown that this antibody crossreacts with a number of proteins in a CARM1-dependent manner (Yadav et al., 2003). Further studies using this antibody to perform western analysis on nuclear extracts from *Carm1*^{+/+} and *Carm1*^{-/-} MEFs reveal that there are at least 7 CARM1 substrates recognized by this antibody (Figure 4B, left). Two of these proteins migrate at the same position as SmB and CA150 (Figure 4B, middle and right), raising the possibility that the α H3R17me2a antibody recognizes these two CARM1 substrates. To investigate this likelihood, we immunoprecipitated SmB from *Carm1*^{+/+} and *Carm1*^{-/-} MEFs that had been subjected to an in vivo methylation reaction. Equivalent amounts of SmB were immunoprecipitated from both cell lines (Figure 4C, bottom), and SmB from the wild-type and the knockout line was methylated (Figure 4C, top), but the α H3R17me2a antibody only recognized SmB from wild-type cells. A similar result was seen for CA150 when the membrane depicted in Figure 4A was stripped and reprobbed with the α H3R17me2a antibody (Figure 4D).

Both CA150 and SmB remain methylated in CARM1 knockout cells but lose their immunoreactivity with the

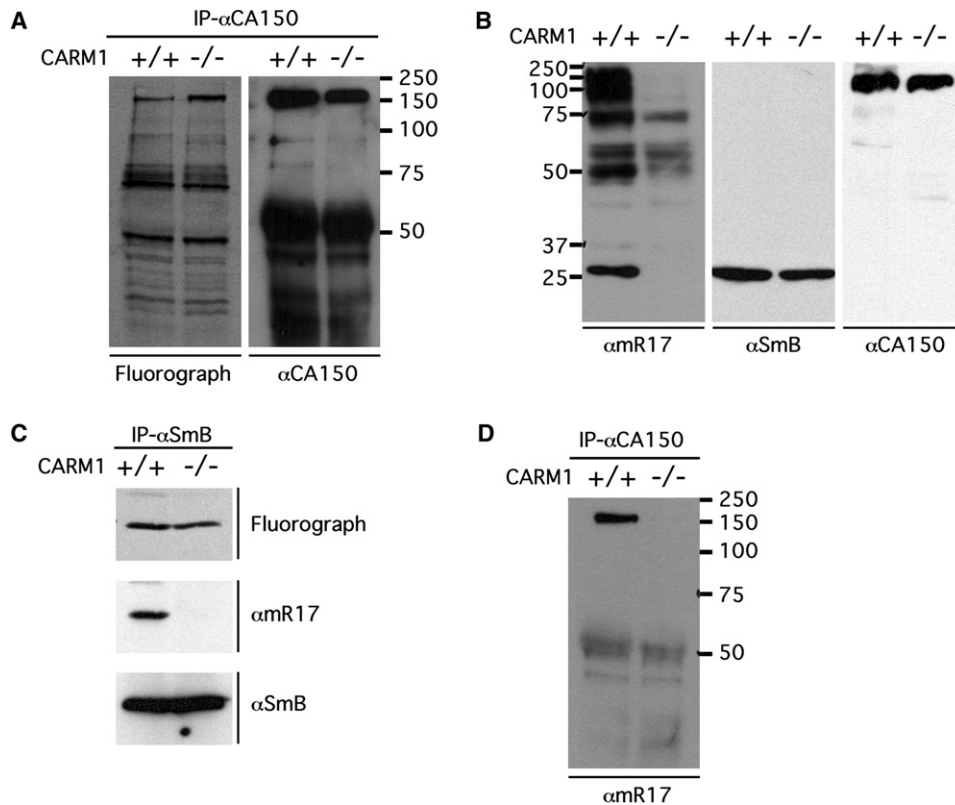


Figure 4. An Antibody Raised against Methylated Histone H3 Also Recognizes SmB and CA150 in a CARM1-Dependent Manner

(A) CA150 is methylated in CARM1 null cells. After an in vivo methylation reaction, CA150 was immunoprecipitated from CARM1 wild-type (+/+) and knockout (-/-) MEFs. Methylated CA150 was visualized by fluorography (left), and the same membrane was immunoblotted with an α CA150 antibody (right).

(B) Nuclear extracts from CARM1 wild-type (+/+) and knockout (-/-) MEFs were subjected to western analysis with α H3R17me2a (Upstate 07-214), α SmB (Y12), and α CA150.

(C) After an in vivo methylation reaction, SmB was immunoprecipitated from CARM1 wild-type (+/+) and null (-/-) MEFs. Methylated SmB was visualized by fluorography (top), and the same membrane was immunoblotted with the α H3R17me2a antibody (middle), then stripped and immunoblotted again with the α SmB antibody (bottom).

(D) The membrane from (A) was stripped and immunoblotted again with the α H3R17me2a antibody.

α H3R17me2a antibody in the absence of CARM1. This clearly demonstrates that these two substrates are being methylated by at least one other enzyme and that this methylation is likely not at the same site as the CARM1-mediated modification because it is not recognized by the α H3R17me2a antibody. It has been shown that SmB is symmetrically dimethylated by PRMT5 (Brahms et al., 2001). Importantly, amino acid analysis of SmB revealed that it is also asymmetrically dimethylated (Miranda et al., 2004). Here we show that this asymmetrical dimethylation is carried out by CARM1. Because CA150 also remains methylated in the absence of CARM1, we speculated that it is a substrate for PRMT5 as well. Indeed, the recombinant CA150-PGM domain can be methylated just as efficiently as GST-SmB, when we use myc-PRMT5 isolated from CARM1 knockout cells as the enzyme source (Figure 2C).

Thus, the α H3R17me2a antibody crossreacts with a CARM1-generated epitope on CA150 and SmB, and ex-

periments in knockout cells provide genetic proof that both CA150 and SmB are methylated by CARM1. Importantly, the α H3R17me2a antibody does not recognize all CARM1-methylated proteins, as it displays no immunoreactivity with PABP1 (data not shown).

CA150 Interacts with the Tudor Domain of SMN in a CARM1-Dependent Fashion

The methylation of SmB by PRMT5 facilitates an interaction with the Tudor domain of the spinal muscular atrophy gene product SMN (Brahms et al., 2001; Cote and Richard, 2005; Friesen et al., 2001). We speculated that CA150 may also interact with SMN in a similar methyl-dependent manner. To investigate this possibility, we transiently transfected GFP and GFP-CA150^a into *Carm1*^{+/+} and *Carm1*^{-/-} MEFs and performed an in vivo methylation reaction on these cells. After IP with α GFP antibodies, the proteins were separated by SDS-PAGE and transferred to a PVDF membrane. Copies of these blots were made and

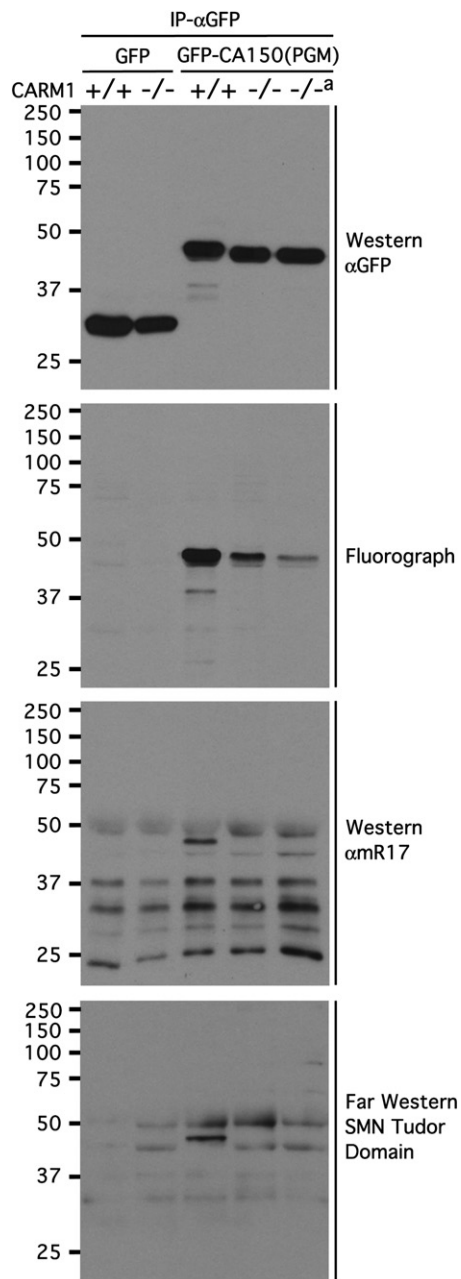


Figure 5. The PGM Motif of CA150 Is Recognized by the α H3R17me2a Antibody in a CARM1-Dependent Manner

CARM1 wild-type (+/+) and knockout (-/-) MEFs were transiently transfected with GFP or GFP-CA150 (PGM) and subjected to an *in vivo* methylation reaction. The expressed proteins were then immunoprecipitated. Methylation was visualized by fluorography, and the same membrane was immunoblotted with the α H3R17me2a antibody, then stripped and immunoblotted again with an α GFP antibody. A duplicate blot was subjected to far western analysis with a GST fusion of the SMN Tudor domain. In the presence of 10 μ M AdOx, almost all the methylation of GFP-CA150 (PGM) in the CARM1 knockout line is lost (-/-^a).

subjected to western analysis with an α GFP antibody as a loading control, to fluorography, to western analysis with the α H3R17me2a antibody, and to far western analysis using the GST-Tudor domain of SMN (Figure 5). These results demonstrate that the isolated PGM motif of CA150 is methylated in wild-type MEFs and methylation is reduced in CARM1 knockout MEFs. The residual methylation in the knockout MEFs is likely due to PRMT5 activity. The α H3R17me2a only reacts with GFP-CA150^a isolated from wild-type cells, as seen with the endogenous protein in Figure 4D. The Tudor domain of SMN can interact with GFP-CA150^a only when it is synthesized in wild-type cells. We have performed a deletion analysis of the CA150 PGM motif and found that both the α H3R17me2a antibody and the SMN Tudor domain interact with a region containing five arginine residues within the first 34 amino acids of CA150 (Figure S2). This region is necessary but not sufficient for these interactions to take place, and indicates a possible structural element to its recognition as a protein-interacting surface.

To further demonstrate that SMN interacts with CA150 in a CARM1-dependent manner, we performed a far western analysis on endogenous CA150 that was immunoprecipitated from either CARM1 wild-type or knockout MEFs. Equal amounts of CA150 are found in both cell lines, but the Tudor domain of SMN is only able to interact with CA150 derived from wild-type cells (Figure 6A). In pull-down experiments, the Tudor domain of SMN is able to interact with CA150 from CARM1 wild-type but not knockout MEFs (Figure 6B). Interestingly, the SmB-SMN interaction, which has been shown to be PRMT5 dependent, is not directly regulated by CARM1. Finally, we are able to coimmunoprecipitate CA150 and SMN (Figure 6C). In this experiment, CA150 interacts strongly with SMN in wild-type cells and much more weakly with CA150 from CARM1 knockout cells. This weak interaction can be eliminated with prior AdOx treatment of the knockout cells. These findings suggest that CA150 interacts directly with SMN in a CARM1-dependent manner (Figures 6A and 6B). The residual methylation of CA150 that is seen in CARM1 knockout cells may play a role in stabilizing this interaction, possibly through a linker protein or a protein complex (Figure 6C), as we only see this weak interaction in the context of the coimmunoprecipitation (CoIP) and not the pull-down.

CARM1 Regulates Splicing by Promoting Exon Skipping

The ability of transcriptional coactivators to impact splicing events has been previously reported by the O'Malley laboratory (Auboeuf et al., 2002, 2005), who showed that CoAA and CAPER coactivators can regulate alternative splicing. In addition, the nuclear receptor coactivator PGC-1 also has the ability to regulate mRNA processing (Monsalve et al., 2000). All three of these coactivators (PGC-1, CoAA, and CAPER) harbor RNA-recognition motifs (RRMs) and/or arginine-serine-rich domains characteristic of SR splicing factors, which pointed toward their

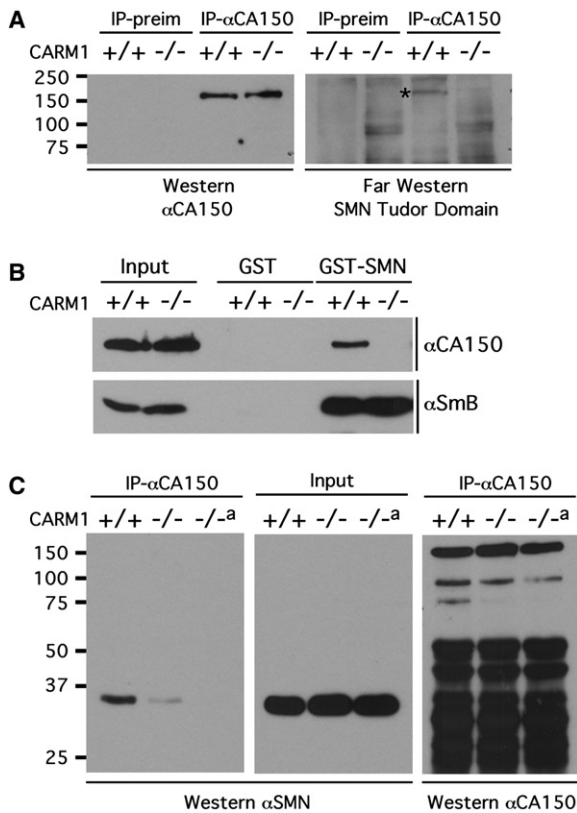


Figure 6. Endogenous CA150 Interacts with SMN in a CARM1-Dependent Manner

(A) Endogenous CA150 was immunoprecipitated from CARM1 wild-type (+/+) and null (-/-) cells and subjected to far western analysis with a GST fusion of the SMN Tudor domain. A weak band is seen in lysates from wild-type cells, but not knockout cells (*). Preimmune sera was used as a negative control.

(B) A pull-down experiment was performed using GST-SMN Tudor domain. Lysates from CARM1 wild-type (+/+) and null (-/-) MEF lines were subjected to this pull-down analysis and immunoblotted with α CA150 and α SmB.

(C) The CoIP of CA150 and SMN requires CARM1. Endogenous CA150 was immunoprecipitated from CARM1 (+/+ and -/-), and AdOx-treated MEFs (-/-^a). α SMN was used to demonstrate the CoIP of SMN with CA150 (left). There is equal SMN input (middle) and CA150 IP (right).

involvement in mRNA processing. CARM1 does not possess any structural elements that are indicative of coactivators that also impact splicing. However, CARM1 does specifically methylate a number of splicing factors (Figure 2C), a property that suggests it could also function as a regulator of alternative splicing.

To investigate the ability of CARM1 to influence alternative pre-mRNA splicing, we used the CD44v5 minigene as a reporter. This splicing reporter, which is driven by an estrogen response element (ERE-CD44) (Auboeuf et al., 2002), was cotransfected with ER α and either CARM1 or CARM1^{dead} expression vectors into CARM1 knockout MEFs. Because CARM1 homodimerizes, the use of knockout MEFs eliminates any confounding consequence that

may be due to the recruitment of the endogenous enzyme by either the transiently transfected wild-type or enzyme-dead CARM1 mutant (CARM1^{dead}). The transfected minigene gives rise to two major spliced RNA products, containing either both variable exons (inclusion) or neither variable exon (skipping), and a minor product harboring a single exon. We observed a 2- to 3-fold increase in the skipping/inclusion ratio of spliced mRNAs as compared with the ratio obtained in the total absence of CARM1 or in the presence of CARM1^{dead} (Figure 7A). This effect is not as pronounced in CARM1 wild-type cells, possibly due to the buffering ability of the endogenous CARM1. In addition, the base level of ERE-CD44 skipping is only slightly higher in the CARM1 wild-type as compared to the CARM1 knockout line (lanes 1 and 2), possibly because the levels of endogenous CARM1 are limiting, and thus the overexpression of CARM1 stimulates significantly more skipping.

Using the same reporter system in a different cell line, we can also observe the capacity of CARM1 to facilitate exon skipping. We generated a tetracycline (Tet)-inducible HEK293 cell line for this purpose. With the induction of CARM1, we see a 4-fold increase, at the 48 hr time point (lane 5 versus lane 10), in the skipping/inclusion ratio as compared with the ratio obtained in the presence of endogenous CARM1 levels (Figure 7B). A portion of the same samples that were used for the splicing analysis was subjected to western analysis to confirm that CARM1 levels are indeed induced at the time point where we see the strongest effects on splicing (Figure 7B). Importantly, as the CARM1 levels increase so does the methylation level of CARM1 substrates, as assayed using the α H3R17me2a antibody. Thus, CARM1 substrates are not fully methylated, and the recruitment of CARM1 to sites of transcriptional activation clearly has the capacity to increase the methylation of substrates and thereby impact alternative splicing.

Again, using the same reporter system we have demonstrated the importance of the PGM motif of CA150 in mediating the CARM1-driven exon skipping (Figure 7C). Tet-inducible HEK293 cells were transfected with the ERE-CD44 reporter construct, together with a CA150 expression construct that either contains (WT) or does not contain the PGM motif (Δ -PGM). Without the PGM motif, CA150 does not interact with SMN (Figure S3) and the skipping/inclusion ratio (Figure 7C, lanes 2–5) is similar to that seen with endogenous levels of CA150 (Figure 7B, lanes 7–10). The skipping/inclusion ratio is more than doubled when full-length CA150 is overexpressed (Figure 7C, lanes 7–10). Thus, the effects of CARM1 overexpression on splicing are partially mediated through the PGM motif of CA150.

We have gone on to show that not only the ERE-CD44 reporter but also the endogenous CD44 locus is subjected to alternative splice regulation by CARM1. We analyzed the expression of exon v5-containing endogenous CD44 mRNA in wild-type MEFs, two CARM1 knockout MEF lines, and a knockout line that has been engineered to

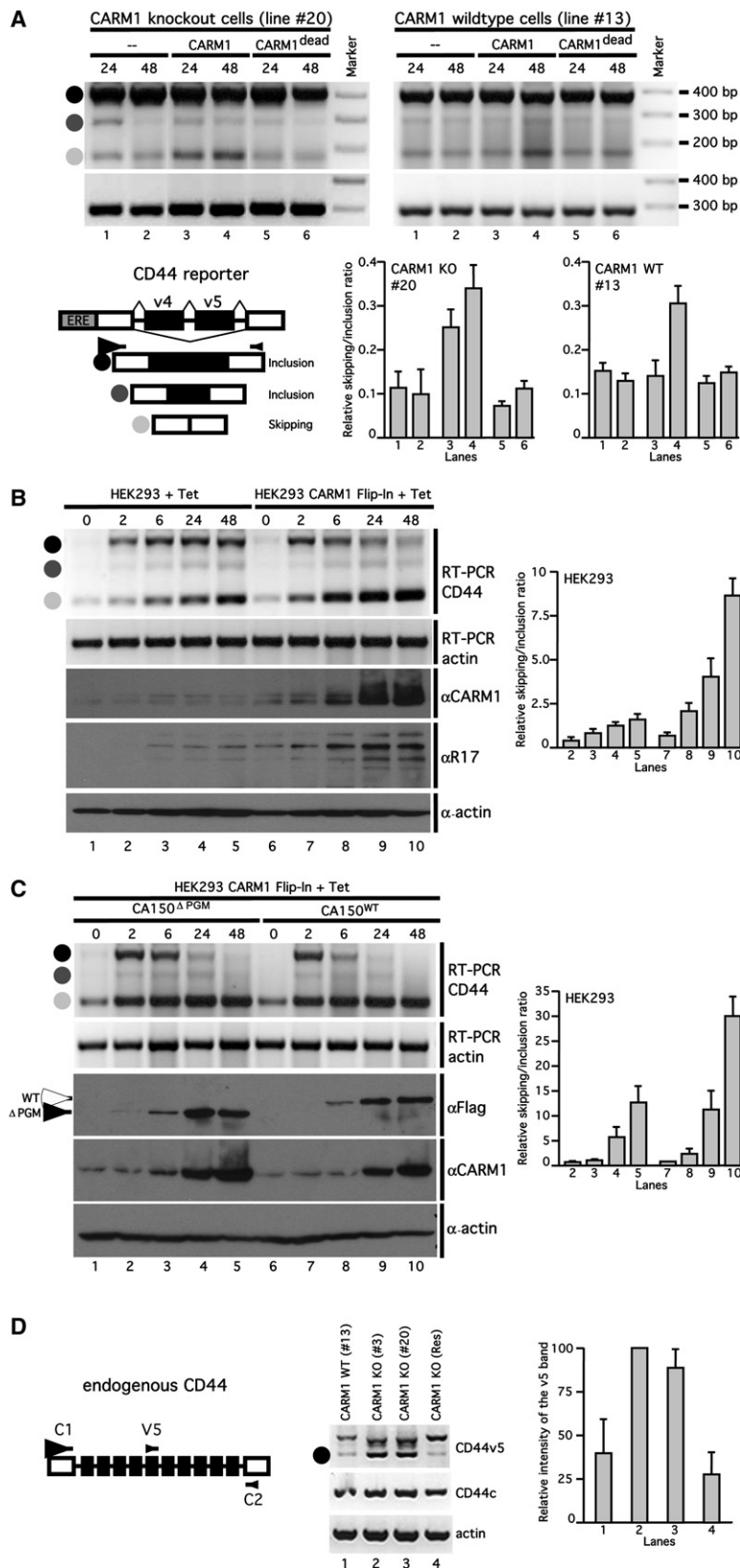


Figure 7. CARM1 Affects Splicing Decisions

(A) CARM1 wild-type and knockout MEFs were transfected with the CD44 minigene. Cotransfections were performed with a CARM1 expression vector (lanes 3 and 4) and an enzyme-dead form of CARM1 (lanes 5 and 6). Transfected cells were incubated for 24 and 48 hr in the presence of estradiol. A gel of the RT-PCR products is depicted. A β -actin PCR serves as a loading control. A mean (\pm SD, $n = 3$) quantification is graphically displayed; the values shown are a ratio of skipped product (lower band) and the two-exon-included product (upper band).

(B) Overexpression of CARM1 promotes exon skipping. HEK293 cells (lanes 1–5) and Tet-induced HEK293 cells (lanes 6–10) were transfected with the CD44 minigene. Western analysis confirmed that CARM1 expression was induced by Tet, and this correlated with an increase in arginine methylation levels as examined with α H3R17me2a. Splicing was monitored by RT-PCR after 2, 6, 24, and 48 hr. A mean (\pm SD, $n = 3$) quantification is graphically displayed.

(C) The PGM motif of CA150 is required to synergize with CARM1 to promote exon skipping. Expression vectors harboring the Δ -PGM (lanes 1–5) and wild-type forms (lanes 6–10) of CA150 were transfected with the CD44 minigene in Tet-inducible HEK293 cells. Western analysis confirmed the expression of Δ -PGM and wild-type CA150, and the induction of CARM1. A mean (\pm SD, $n = 4$) quantification is graphically displayed.

(D) CARM1 participates in regulating splicing of endogenous CD44. RT-PCR analysis was performed using exon-specific primers for CD44, on cDNA from wild-type MEFs (13), two CARM1 knockout MEF lines (3 and 20), and a rescued knockout line. The lower band represents v5 (black spot), and the additional bands represent v5/v6 and v5/v7 mRNA as determined by sequencing. A mean (\pm SD, $n = 3$) quantification of the effect of CARM1 loss is graphically displayed as a percentage of the band in lane 2.

re-express CARM1 (rescued) (Figure 7D). An increase in CD44v5 inclusion is seen in the two knockout lines, which indicates reduced exon skipping in the absence of CARM1—consistent with the results that we observe when using the splicing reporter (Figures 7A–7C).

DISCUSSION

CARM1 Is a Transcriptional Coactivator that Impacts Alternative Splicing

Transcription and downstream RNA processing events are highly coordinated, and specific transcription factors have been shown to play a role in pre-mRNA processing (Maniatis and Reed, 2002). A subset of transcriptional coactivators is involved in transcription-coupled splicing, and it contains either serine/arginine (SR) domains or RRM, or both, and physically links transcription to splicing. Examples of these SR/RRM proteins include the PPAR γ coactivator PGC-1 (Monsalve et al., 2000) and the steroid receptor coactivators p72, COAA, and CAPER α (Auboeuf et al., 2002; Dowhan et al., 2005). To date, more than twenty transcriptional coregulators have been structurally or functionally linked to splicing (Auboeuf et al., 2005), and recently CARM1 joined these ranks (Ohkura et al., 2005).

The link between transcription and splicing has focused on the C-terminal domain (CTD) of RNA polymerase II (Pol II). The CTD serves as a scaffold for many factors involved in nascent transcript maturation. CARM1 does not associate with the CTD directly and cannot be coimmunoprecipitated with Pol II (data not shown), but it does methylate CA150, which is a CTD-binding protein (Goldstrohm et al., 2001). The ability of CA150 to interact with the CTD is not affected by its methylation status (Figure S4). In addition, SMN is also associated with the CTD, and fractionation experiment using sucrose gradient centrifugation revealed that >30% of nuclear SMN cofractionates with Pol II (Pellizzoni et al., 2001). The tethering of SMN to the CTD of Pol II is mediated by RNA helicase A (Pellizzoni et al., 2001), NSAP1/hnRNP Q (Carty and Greenleaf, 2002; Mourelatos et al., 2001), and CA150 (this study). Importantly, all three of these SMN interaction nodes are arginine methylated. We identified RNA helicase A as a PRMT1 substrate (Lee and Bedford, 2002). NSAP1 also contains a GAR motif (Mourelatos et al., 2001) and is thus likely a PRMT1 substrate. Indeed, the small-pool screen identified NSAP1 and CA150 as methylated proteins (Figure 1).

The SMN Tudor domain binds CA150 in a CARM1-dependent manner (Figures 5 and 6). To the best of our knowledge, this is the first example of a protein-protein interaction that is stimulated by a CARM1 methylation event. Previously, CARM1 has been shown to play an inhibitory role in protein-protein interactions, specifically with regard to the GRIP1-p300 interaction (Lee et al., 2005). Importantly, CA150 needs to be methylated by CARM1 for it to interact with the Tudor domain of SMN. Thus, SMN protein interactions are regulated by both PRMT5 and CARM1, and this clearly adds another level of complexity to the

interpretation of SMA pathogenesis. Finally, the CARM1-dependent interaction of CA150 with SMN could provide a molecular mechanism for the proposed involvement of SMN in pre-mRNA splicing (Pellizzoni et al., 1998).

CARM1 Methylates the PGM Motifs of Splicing Factors

In vitro methylation assays confirm that the PGM motifs of CA150, SmB, U1C, and SAP49 are methylated by CARM1 (Figure 2C). CA150 also harbors WW domains and FF domains and is a key factor in linking transcription to the spliceosome (Bedford and Leder, 1999). Its WW domains bind the splicing factors SF1 and U2AF, the U2 snRNP, and the SF3 complex (Goldstrohm et al., 2001; Lin et al., 2004). The FF domains of CA150 tether it to the CTD of Pol II in addition to other proteins that are involved with the regulation of Pol II promoters (Carty et al., 2000; Smith et al., 2004). Importantly, CA150 copurifies with spliceosome fractions and colocalizes with splicing factor-rich nuclear speckles (Neubauer et al., 1998; Sanchez-Alvarez et al., 2006). In the freshwater midge, the ortholog of CA150 (hrp130) is concentrated in large chromosomal puffs called Balbiani rings, which are sites of high transcriptional activity and splicing (Sun et al., 2004). It has been shown that CA150 has the capacity to repress transcription elongation by Pol II in a promoter-selective fashion (Goldstrohm et al., 2001; Sune and Garcia-Blanco, 1999). This effect of CA150 on transcription elongation has been proposed to be through a mechanism that allows adjustments to be made to the transcription rate, thus ensuring the fidelity of splicing at highly transcribed loci.

Apart from CA150, the other CARM1 substrates SmB, U1C, and SAP49 also have discrete functions in pre-mRNA splicing. SmB is a common Sm protein that is a component of both the U1 snRNP and the U2 snRNP, which recognize the 5' and 3' splice site, respectively. U1C is part of the U1 snRNP, SAP49 is found in the SF3b complex that contributes to the U2 snRNP, and CA150 is associated with the U2 snRNP (Lin et al., 2004; Stanek and Neugebauer, 2006). Although these factors are components of the basic splicing machinery, modulating their activity could impact the use of specific splice sites. Recently, it was found that Brm, a component of the SWI/SNF chromatin-remodeling complex, regulates alternative splicing. Interestingly, Brm has the opposite effect on the CD44 splicing than CARM1 has—it induces the inclusion of variant exons (Batsche et al., 2006). Thus, factors that impact chromatin structure can clearly alter splicing patterns.

CARM1 Enzymatic Activity Contributes to Its Effects on Splicing

Four independent lines of evidence support the notion that CARM1's enzymatic activity is required for its effect on splicing. First, the ability of CARM1 to methylate at least four factors involved in splicing provides circumstantial evidence that its enzymatic activity is a key part of its

overall function in splicing (Figure 2C). Second, the SMN-CA150 interaction requires CARM1 enzymatic activity (Figure 6). Third, CARM1 promotes exon skipping in *Carm1*^{-/-} MEFs only when the enzyme-active form of the molecule is expressed (Figure 7A). Finally, a synergistic effect on exon skipping is observed between CA150 and CARM1 only when the PGM motif of CA150 is a component of the transiently expressed protein (Figures 7B and 7C).

These findings are at odds with data recently published by the Tsukada laboratory (Ohkura et al., 2005). Their data support an enzyme-independent role of CARM1 in splicing, and they propose that, in the context of splicing, CARM1 functions as a scaffolding molecule and not an enzyme. The CARM1-v3 splice form focused on in their study has a unique 34 amino acid stretch at its C-terminal end. This is due to the retention of intronic sequence and not an active splicing event. Thus, the v3 form of CARM1 may be representative of a very rare form of unspliced pre-mRNA. Indeed, when we perform a blast analysis using the unique 34 amino acid stretch of CARM1-v3 to search the translated EST database, we obtain only one hit. Thus, we feel that the CARM1-v3 isoform represents unspliced pre-mRNA and that it will play an insignificant role in the overall biological function of CARM1.

In summary, CARM1 methylates multiple nodes, all of which impact gene expression. At the level of transcription initiation, CARM1 modifies histone H3 and p300/CBP (Chen et al., 1999; Lee et al., 2005). With the identification of CA150, SmB, U1C, and SAP49 in this study, we show that pre-mRNA splicing is also a target for regulation by this enzyme, and certain data from the Tsukada group (Ohkura et al., 2005) support this link. Postsplicing, the methylation of the RNA-binding proteins HuR and HuD plays a role in regulating the stability of labile mRNAs carrying AU-rich elements (Fujiwara et al., 2006), and PABP1 is also a major CARM1 substrate (Lee and Bedford, 2002). Thus, CARM1 lays an Ariadnean thread through the gene expression pathway.

EXPERIMENTAL PROCEDURES

Antibodies, Plasmids, and Peptides

CA150 antibodies were a gift from Woan-Yuh Tarn (Institute of Biomedical Sciences, Taiwan) and were purchased from Bethyl Laboratory. The PRMT1, PRMT5, and CARM1 antibodies were a gift from Stéphane Richard (McGill University). Y12 was a gift from Robin Reed (Harvard Medical School). The following antibodies were obtained commercially: α GFP (Molecular Probes), α H3R17me2a (Upstate Biotechnology), α SMN (BD Transduction Laboratories), H5 (Covance), and β -actin (Sigma). For small-pool screening, a cDNA mouse B cell hybridoma (LK35.2) library was constructed in the pZeoSV2 vector (Invitrogen) and was a gift from Yasumasa Ishida (Nara Institute of Science and Technology). GST-PABP1, GST-GAR (Lee and Bedford, 2002), GST-U1C, and GST-SmB (Bedford et al., 1998) have been described previously, as have GST-PRMT1, GST-PRMT3, GST-PRMT4, GST-PRMT6 (Frankel et al., 2002), and GST-SMN Tudor (Kim et al., 2006). SAP49 encoding amino acid residues 190–424 was amplified and subcloned in frame into pET-28a(+) (Novagen) to generate a histag PGM motif. pEFBOST7-CA150 missing the first 60 nucleotides of

the full-length CA150 was a gift from Mariano Garcia-Blanco (Duke University). The missing 60 base pairs were added by PCR. PCR was used to generate the following GST constructs: a, 1–136; b, 137–665; c, 660–845; and d, 894–1084 in Figure 2. For splicing assays, human full-length CARM1 was cloned into pCEP4 vector (Invitrogen). A QuikChange Site-Directed Mutagenesis Kit (Stratagene) was used to generate CARM1(E266Q)-pCEP4. ERE-CD44 minigene was a gift from Bert O'Malley (Baylor College of Medicine). Full-length CA150 was subcloned into p3 \times Flag-CMV-7.1 vector (Sigma), and PCR was used to generate the Δ -PGM version. P3 and P3* peptides have been described previously (Bedford et al., 2000).

Small-Pool Screen

The pZeoSV2-B cell library was transformed into competent bacteria. Pools of five colonies were cultured overnight, and the plasmids were purified. Two pools (ten plasmids) were then combined and transcribed and translated in vitro (IVTT) with the TNT system (Promega). In addition, 1.0 μ Ci S-adenosyl-L-[methyl-³H]methionine (85 Ci/mmol from a 0.5 mCi/ml stock solution; PerkinElmer) was added into this system during the IVTT.

In Vitro and In Vivo Methylation Assay

In vitro methylation reactions (Frankel et al., 2002) and the in vivo methylation assay (Lee and Bedford, 2002) were performed as previously described.

Cell Lines

The generation of wild-type and *Carm1*^{-/-} MEFs has been described (Yadav et al., 2003). A stable/inducible T-Rex-CARM1 cell line was established by transfection with pcDNA/FRT/TO-CARM1 and pOG44 plasmids into Flip-in T-Rex HEK293 cells (Invitrogen). Transfected cells were maintained in culture media supplemented with 15 μ g/ml blasticidin and 100 μ g/ml hygromycin. The resistant colonies were pooled.

GST Pull-Down and CoIP Assays

MEFs were lysed in 0.5 ml of mild lysis buffer (150 mM NaCl, 5 mM EDTA, 1% Triton X-100, and 10 mM Tris/HCl [pH 7.5]). Fifteen micrograms of fusion protein bound to beads was incubated with MEF cell extracts (10 cm plate) for 2.5 hr at 4°C. After five washes with lysis buffer, the beads were boiled in loading buffer, separated by SDS-PAGE, and transferred onto PVDF membranes. For the CoIP assay, cells (10 cm plate) were lysed in 0.5 ml of mild lysis buffer.

Far Western Blotting

CA150 was immunoprecipitated from MEFs, separated by SDS-PAGE, and transferred onto PVDF membranes. Blots were blocked in PBS-Tween 20 containing 5% nonfat dry milk and then incubated with 5 μ g/ml of GST-SMN Tudor protein in the blocking buffer overnight at 4°C. The blots were then washed and probed with an anti-GST antibody, incubated with an HRP-labeled secondary antibody, and detected using enhanced chemiluminescence (Amersham).

Splicing Assays

Splicing experiments were done in triplicate. *Carm1*^{-/-} MEFs were grown on 10 cm dishes and transfected with either 15 μ g of pCEP4-CARM1 or pCEP4-CARM1(E266Q) and BlueScript as a control, 10 μ g of ERE-CD44 minigene plasmid, and 5 μ g of ER plasmid using Fugene 6 (Roche) as the transfection agent. After 5 hr of incubation, the transfection media were replaced with media (without phenol red) containing 10% of stripped fetal bovine serum and E2 (2×10^{-9} M).

The transfection of HEK293 and Flip-in T-Rex CARM1/HEK293 cells was performed in 6-well plates using 2 μ g of ERE-CD44 minigene plasmid and 1 μ g ER plasmid. After 5 hr of incubation, E2 (2×10^{-9} M) and 1 μ g/ml Tet were added in the replacement medium. At the indicated time of incubation, cells were collected. Three-fifths of the cells were used for RNA analysis, and two-fifths were lysed in 100 μ l of RIPA buffer for western blotting.

Total RNA was extracted using the RNeasy kit (QIAGEN). RT-PCR was performed using the SuperScript One-Step RT-PCR kit (Invitrogen). As loading controls, primers for β -actin were used (Ambion). The spliced CD44 isoforms from ERE-CD44 minigene were identified by PCR as described previously (Auboeuf et al., 2002). The primers for constant region (c) and specific for variant exons of endogenous CD44 have been described for the human gene (Konig et al., 1996). Mouse primers were as follows: (C1) 5'-ACCCAGAAGGCTACATT TGCAC-3' and (V5) 5'-TAGACAGAATCAGCACCAGTGCTC-3', and the reverse (C2) is 5'-TTCCGGGTCGTCAGCTGTCATA-3'. PCR products were separated on a 2% agarose gel and visualized with ethidium bromide. The gels were scanned using an image analyzer (Typhoon 9410, Amersham).

Supplemental Data

Supplemental Data include four figures and can be found with this article online at <http://www.molecule.org/cgi/content/full/25/1/71/DC1/>.

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REFERENCES

- Auboeuf, D., Honig, A., Berget, S.M., and O'Malley, B.W. (2002). Coordinate regulation of transcription and splicing by steroid receptor coregulators. *Science* 298, 416–419.
- Auboeuf, D., Dowhan, D.H., Dutertre, M., Martin, N., Berget, S.M., and O'Malley, B.W. (2005). A subset of nuclear receptor coregulators act as coupling proteins during synthesis and maturation of RNA transcripts. *Mol. Cell. Biol.* 25, 5307–5316.
- Batsche, E., Yaniv, M., and Muchardt, C. (2006). The human SWI/SNF subunit Brm is a regulator of alternative splicing. *Nat. Struct. Mol. Biol.* 13, 22–29.
- Bedford, M.T., and Leder, P. (1999). The FF domain: a novel motif that often accompanies WW domains. *Trends Biochem. Sci.* 24, 264–265.
- Bedford, M.T., and Richard, S. (2005). Arginine methylation: an emerging regulator of protein function. *Mol. Cell* 18, 263–272.
- Bedford, M.T., Reed, R., and Leder, P. (1998). WW domain-mediated interactions reveal a spliceosome-associated protein that binds a third class of proline-rich motif: the proline glycine and methionine-rich motif. *Proc. Natl. Acad. Sci. USA* 95, 10602–10607.
- Bedford, M.T., Frankel, A., Yaffe, M.B., Clarke, S., Leder, P., and Richard, S. (2000). Arginine methylation inhibits the binding of proline-rich ligands to Src homology 3, but not WW, domains. *J. Biol. Chem.* 275, 16030–16036.
- Brahms, H., Meheus, L., de Brabandere, V., Fischer, U., and Luhrmann, R. (2001). Symmetrical dimethylation of arginine residues in spliceosomal Sm protein B/B' and the Sm-like protein LSm4, and their interaction with the SMN protein. *RNA* 7, 1531–1542.
- Carty, S.M., and Greenleaf, A.L. (2002). Hyperphosphorylated C-terminal repeat domain-associating proteins in the nuclear proteome link transcription to DNA/chromatin modification and RNA processing. *Mol. Cell. Proteomics* 1, 598–610.
- Carty, S.M., Goldstrohm, A.C., Sune, C., Garcia-Blanco, M.A., and Greenleaf, A.L. (2000). Protein-interaction modules that organize nuclear function: FF domains of CA150 bind the phosphoCTD of RNA polymerase II. *Proc. Natl. Acad. Sci. USA* 97, 9015–9020.
- Chen, D., Ma, H., Hong, H., Koh, S.S., Huang, S.M., Schurter, B.T., Aswad, D.W., and Stallcup, M.R. (1999). Regulation of transcription by a protein methyltransferase. *Science* 284, 2174–2177.
- Cote, J., and Richard, S. (2005). Tudor domains bind symmetrical dimethylated arginines. *J. Biol. Chem.* 280, 28476–28483.
- Covic, M., Hassa, P.O., Sacconi, S., Buerki, C., Meier, N.I., Lombardi, C., Imhof, R., Bedford, M.T., Natoli, G., and Hottiger, M.O. (2005). Arginine methyltransferase CARM1 is a promoter-specific regulator of NF-kappaB-dependent gene expression. *EMBO J.* 24, 85–96.
- Das, B.K., Xia, L., Palandjian, L., Gozani, O., Chung, Y., and Reed, R. (1999). Characterization of a protein complex containing spliceosomal proteins SAPs 49, 130, 145, and 155. *Mol. Cell. Biol.* 19, 6796–6802.
- Dever, T.E., Costello, C.E., Owens, C.L., Rosenberry, T.L., and Merrick, W.C. (1989). Location of seven post-translational modifications in rabbit elongation factor 1 alpha including dimethyllysine, trimethyllysine, and glycerylphosphorylethanolamine. *J. Biol. Chem.* 264, 20518–20525.
- Dowhan, D.H., Hong, E.P., Auboeuf, D., Dennis, A.P., Wilson, M.M., Berget, S.M., and O'Malley, B.W. (2005). Steroid hormone receptor coactivation and alternative RNA splicing by U2AF65-related proteins CAPEAlpha and CAPEBeta. *Mol. Cell* 17, 429–439.
- Espejo, A., Cote, J., Bednarek, A., Richard, S., and Bedford, M.T. (2002). A protein-domain microarray identifies novel protein-protein interactions. *Biochem. J.* 367, 697–702.
- Frankel, A., Yadav, N., Lee, J., Branscombe, T.L., Clarke, S., and Bedford, M.T. (2002). The novel human protein arginine N-methyltransferase PRMT6 is a nuclear enzyme displaying unique substrate specificity. *J. Biol. Chem.* 277, 3537–3543.
- Friesen, W.J., Massenet, S., Paushkin, S., Wyce, A., and Dreyfuss, G. (2001). SMN, the product of the spinal muscular atrophy gene, binds preferentially to dimethylarginine-containing protein targets. *Mol. Cell* 7, 1111–1117.
- Fujiwara, T., Mori, Y., Chu, D.L., Koyama, Y., Miyata, S., Tanaka, H., Yachi, K., Kubo, T., Yoshikawa, H., and Tohyama, M. (2006). CARM1 regulates proliferation of PC12 cells by methylating HuD. *Mol. Cell. Biol.* 26, 2273–2285.
- Goldstrohm, A.C., Albrecht, T.R., Sune, C., Bedford, M.T., and Garcia-Blanco, M.A. (2001). The transcription elongation factor CA150 interacts with RNA polymerase II and the pre-mRNA splicing factor SF1. *Mol. Cell. Biol.* 21, 7617–7628.
- Kim, J., Daniel, J., Espejo, A., Lake, A., Krishna, M., Xia, L., Zhang, Y., and Bedford, M.T. (2006). Tudor, MBT and chromo domains gauge the degree of lysine methylation. *EMBO Rep.* 7, 397–403.
- Konig, H., Moll, J., Ponta, H., and Herrlich, P. (1996). Trans-acting factors regulate the expression of CD44 splice variants. *EMBO J.* 15, 4030–4039.
- Lee, J., and Bedford, M.T. (2002). PABP1 identified as an arginine methyltransferase substrate using high-density protein arrays. *EMBO Rep.* 3, 268–273.
- Lee, Y.H., Coonrod, S.A., Kraus, W.L., Jelinek, M.A., and Stallcup, M.R. (2005). Regulation of coactivator complex assembly and function by protein arginine methylation and demethylation. *Proc. Natl. Acad. Sci. USA* 102, 3611–3616.
- Lin, K.T., Lu, R.M., and Tarn, W.Y. (2004). The WW domain-containing proteins interact with the early spliceosome and participate in pre-mRNA splicing in vivo. *Mol. Cell. Biol.* 24, 9176–9185.
- Lustig, K.D., Stukenberg, P.T., McGarry, T.J., King, R.W., Cryns, V.L., Mead, P.E., Zon, L.I., Yuan, J., and Kirschner, M.W. (1997). Small pool expression screening: identification of genes involved in cell cycle control, apoptosis, and early development. *Methods Enzymol.* 283, 83–99.

- Maniatis, T., and Reed, R. (2002). An extensive network of coupling among gene expression machines. *Nature* *416*, 499–506.
- Miranda, T.B., Khusial, P., Cook, J.R., Lee, J.H., Gunderson, S.I., Pestka, S., Zieve, G.W., and Clarke, S. (2004). Spliceosome Sm proteins D1, D3, and B/B' are asymmetrically dimethylated at arginine residues in the nucleus. *Biochem. Biophys. Res. Commun.* *323*, 382–387.
- Monsalve, M., Wu, Z., Adelmant, G., Puigserver, P., Fan, M., and Spiegelman, B.M. (2000). Direct coupling of transcription and mRNA processing through the thermogenic coactivator PGC-1. *Mol. Cell* *6*, 307–316.
- Mourelatos, Z., Abel, L., Yong, J., Kataoka, N., and Dreyfuss, G. (2001). SMN interacts with a novel family of hnRNP and spliceosomal proteins. *EMBO J.* *20*, 5443–5452.
- Neubauer, G., King, A., Rappsilber, J., Calvio, C., Watson, M., Ajuh, P., Sleeman, J., Lamond, A., and Mann, M. (1998). Mass spectrometry and EST-database searching allows characterization of the multi-protein spliceosome complex. *Nat. Genet.* *20*, 46–50.
- Ohkura, N., Takahashi, M., Yaguchi, H., Nagamura, Y., and Tsukada, T. (2005). Coactivator-associated arginine methyltransferase 1, CARM1, affects pre-mRNA splicing in an isoform-specific manner. *J. Biol. Chem.* *280*, 28927–28935.
- Ostareck-Lederer, A., Ostareck, D.H., Rucknagel, K.P., Schierhorn, A., Moritz, B., Huttelmaier, S., Flach, N., Handoko, L., and Wahle, E. (2006). Asymmetric arginine dimethylation of heterogeneous nuclear ribonucleoprotein K by protein-arginine methyltransferase 1 inhibits its interaction with c-Src. *J. Biol. Chem.* *281*, 11115–11125.
- Pellizzoni, L., Kataoka, N., Charroux, B., and Dreyfuss, G. (1998). A novel function for SMN, the spinal muscular atrophy disease gene product, in pre-mRNA splicing. *Cell* *95*, 615–624.
- Pellizzoni, L., Charroux, B., Rappsilber, J., Mann, M., and Dreyfuss, G. (2001). A functional interaction between the survival motor neuron complex and RNA polymerase II. *J. Cell Biol.* *152*, 75–85.
- Sanchez-Alvarez, M., Goldstrohm, A.C., Garcia-Blanco, M.A., and Sune, C. (2006). Human transcription elongation factor CA150 localizes to splicing factor-rich nuclear speckles and assembles transcription and splicing components into complexes through its amino and carboxyl regions. *Mol. Cell. Biol.* *26*, 4998–5014.
- Smith, M.J., Kulkarni, S., and Pawson, T. (2004). FF domains of CA150 bind transcription and splicing factors through multiple weak interactions. *Mol. Cell. Biol.* *24*, 9274–9285.
- Stanek, D., and Neugebauer, K.M. (2006). The Cajal body: a meeting place for spliceosomal snRNPs in the nuclear maze. *Chromosoma* *115*, 343–354.
- Sun, X., Zhao, J., Kylberg, K., Soop, T., Palka, K., Sonnhammer, E., Visa, N., Alzhanova-Ericsson, A.T., and Daneholt, B. (2004). Conspicuous accumulation of transcription elongation repressor hrp130/CA150 on the intron-rich Balbiani ring 3 gene. *Chromosoma* *113*, 244–257.
- Sune, C., and Garcia-Blanco, M.A. (1999). Transcriptional cofactor CA150 regulates RNA polymerase II elongation in a TATA-box-dependent manner. *Mol. Cell. Biol.* *19*, 4719–4728.
- Yadav, N., Lee, J., Kim, J., Shen, J., Hu, M.C., Aldaz, C.M., and Bedford, M.T. (2003). Specific protein methylation defects and gene expression perturbations in coactivator-associated arginine methyltransferase 1-deficient mice. *Proc. Natl. Acad. Sci. USA* *100*, 6464–6468.
- Zobel-Thropp, P., Yang, M.C., Machado, L., and Clarke, S. (2000). A novel post-translational modification of yeast elongation factor 1A. Methylesterification at the C terminus. *J. Biol. Chem.* *275*, 37150–37158.