Crosstalk between C/EBPβ phosphorylation, arginine methylation, and SWI/SNF/Mediator implies an indexing transcription factor code

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Cellular signalling cascades regulate the activity of transcription factors that convert extracellular information into gene regulation. C/EBPβ is a ras/MAPK kinase signal-sensitive transcription factor that regulates genes involved in metabolism, proliferation, differentiation, immunity, senescence, and tumorigenesis. The protein arginine methyltransferase 4 PRMT4/CARM1 interacts with C/EBPβ and dimethylates a conserved arginine residue (R3) in the C/EBPβ N-terminal transactivation domain, as identified by mass spectrometry of cell-derived C/EBPβ. Phosphorylation of the C/EBPβ regulatory domain by ras/MAPK kinase signalling abrogates the interaction between C/EBPβ and PRMT4/CARM1. Differential proteomic screening, protein interaction studies, and mutational analysis revealed that methylation of R3 constrains interaction with SWI/SNF and Mediator complexes. Mutation of the R3 methylation site alters endogenous myeloid gene expression and adipogenic differentiation. Thus, phosphorylation of the transcription factor C/EBPβ couples ras signalling to arginine methylation and adipogenic differentiation. This, phosphorylation of the transcription factor C/EBPβ and epigenetic gene regulatory protein complexes during cell differentiation.

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Introduction

The transcription factor C/EBPβ is a member of the CCAAT/enhancer-binding protein family that is composed of C/EBPα, β, δ, γ, ε, and ζ. C/EBPβ is expressed in a variety of cell types and participates in tissue-specific gene expression, proliferation, and differentiation in a hormone, cytokine, and nutrient-dependent manner. C/EBPβ controls important functions in liver homeostasis, regeneration, acute phase response, female reproduction, innate and adopted immunity, senescence, and receptor tyrosine kinase/ras oncoprotein-mediated tumorigenesis (Roesler, 2001; Farmer, 2006; Sebastian and Johnson, 2006; Nerlov, 2007; Zahnow, 2009). C/EBPβ carries at its N-terminus a modular, composite transactivation domain (TAD) that consists of four conserved regions (CR1–4), a composite regulatory domain (RD) consisting of CR5–7 at its centre that governs TAD functions, and a basic DNA binding and leucine dimerization domain (bZip) at the C-terminus (Kowenz-Leutz et al., 1994; Williams et al., 1995) (scheme in Figure 1A). Three C/EBPβ protein isoforms are expressed from a single, intronless transcript by signal-dependent alternative translation initiation from in-frame positioned start sites (Descombes and Schibler, 1991; Calkhoven et al., 2000). These N-terminally variant C/EBPβ isoforms of 38, 35, and 20 kDa are termed LAP*/C/EBPβ1, LAP/C/EBPβ2, and LIP/C/EBPβ3, respectively (Descombes and Schibler, 1991; Bundy and Sealy, 2003).

The C/EBPβ isoforms harbour distinct parts of the TAD and RD and display diverse or even opposite gene regulatory functions (Timchenko et al., 1999; Baer and Johnson, 2000; Calkhoven et al., 2000; Eaton et al., 2001). The two long isoforms (LAP*/C/EBPβ1 and LAP/C/EBPβ2) differ by a conserved sequence of 21–23 N-terminal amino acids in different species that represents CR1. CR1 functions as a gene regulatory module involved in the recruitment of the chromatin-remodelling SWI/SNF complex and the transcription regulatory Mediator complex (Kowenz-Leutz and Leutz, 1999; Pedersen et al., 2001; Mo et al., 2004). The short isoform (LIP/C/EBPβ3) lacks the TAD and part of the RD and represents a dominantly interfering protein that may neutralize both, transcriptional and transrepression by all C/EBPs (Descombes and Schibler, 1991; Zahnow et al., 1997; Luedde et al., 2004).

The transcriptional activity of C/EBPβ is diversified by a multitude of interactions with other C/EBP family members, unrelated transcription factors, and transcription co-factors (Sebastian and Johnson, 2006; Nerlov, 2008; Zahnow, 2009). A major post-translational modification has been attributed to signalling through receptor tyrosine kinase–ras/mitogen-activated protein kinase (MAPK) pathway that phosphorylates an evolutionary conserved MAPK consensus site in the C/EBPβ RD (Thr-235 in human (Nakajima et al., 1993), Thr-188 in rat (Hanlon et al., 2001), Thr-220 in chicken (Kowenz-Leutz et al., 1994)). In its non-phosphorylated form, the RD of C/EBPβ is involved in masking the TAD...
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through intra-molecular interaction (Kowenz-Leutz et al., 1994; Williams et al., 1995) and in binding a repressive or attenuated form of the Mediator complex. Phosphorylation of the MAPK site is accompanied by a conformational change in the protein, exchange of interacting Mediator components, and transcriptional activation (Mo et al., 2004).

Alignment and sequence comparison of vertebrate C/EBP\(\beta\) revealed a number of highly conserved arginine residues in their N-termini that we considered to be involved in regulating C/EBP\(\beta\) activity. Here, we show that the TAD of C/EBP\(\beta\) interacts with the protein arginine methyltransferase PRMT4/CARM1 that methylates C/EBP\(\beta\) (Supplementary Figure 1). A small amount of the truncated LIP/C/EBP\(\beta\) isoform was detected with the ASYM24 antibody, but not with SYM10 (Supplementary Figure 1B). Metabolic labelling with \(\text{L-}[\text{methyl-}^{3}\text{H}]\)methionine in the presence of cycloheximide and chloramphenicol revealed tracer incorporation only in the long C/EBP\(\beta\) isoforms (data not shown) (Pless et al., 2008). These results supported the idea that C/EBP\(\beta\) is post-translationally modified by both, asymmetrical and symmetrical dimethylation on arginines. This notion was scrutinized by mass spectrometric analysis. A histidine-tagged LAP\(^*/\text{C/EBP}\beta\) construct was expressed in fibroblasts and enriched under denaturing conditions by nickel chelating affinity chromatography (Figure 1C). LAP\(^*/\text{C/EBP}\beta\) fractions were digested to peptides, separated on a nano-HPLC system and analysed by MRM mass spectrometry from cell lysates approved the occurrence of arginine dimethylation on R3 of endogenous LAP\(^*/\text{C/EBP}\beta\) in cells, as shown in Supplementary Figure 2. As the N-terminally LAP\(^*\)-specific peptide has a clearly defined modular function in chromatin remodelling and gene regulation, we focused on the molecular biology and functions of C/EBP\(\beta\) R3 dimethylation;

Results

C/EBP\(\beta\) is methylated at arginine residues

Alignment and sequence comparison of vertebrate C/EBP\(\beta\) family members revealed several conserved arginine residues in low complexity regions of the N-terminus. We considered the possibility that arginine side chains of C/EBP\(\beta\) are post-translationally modified by methylation. As shown in Figure 1B, the LAP\(^*/\text{C/EBP}\beta\) isoform was immunoprecipitated with an antibody specific to methylated arginine (Rme). No Rme-specific immunoprecipitation was observed in the presence of adenosine dialdehyde (AdOx), a homocysteine hydrolase inhibitor that blocks the regeneration of the cellular methyl donor S-adenosyl methionine (Bartel and Borchardt, 1984; Chen et al., 2004). The two long C/EBP\(\beta\) isoforms reacted with antibodies that specifically recognize asymetrically dimethylated arginine (ASYM24) or symmetrically dimethylated arginine (SYM10) side chains (Supplementary Figure 1). A small amount of the truncated LIP/C/EBP\(\beta\) isoform was detected with the ASYM24 antibody, but not with SYM10 (Supplementary Figure 1B). Metabolic labelling with \(\text{L-}[\text{methyl-}^{3}\text{H}]\)methionine in the presence of cycloheximide and chloramphenicol revealed tracer incorporation only in the long C/EBP\(\beta\) isoforms (data not shown) (Pless et al., 2008). These results supported the idea that C/EBP\(\beta\) is post-translationally modified by both, asymmetrical and symmetrical dimethylation on arginines. This notion was scrutinized by mass spectrometric analysis. A histidine-tagged LAP\(^*/\text{C/EBP}\beta\) construct was expressed in fibroblasts and enriched under denaturing conditions by nickel chelating affinity chromatography (Figure 1C). LAP\(^*/\text{C/EBP}\beta\) fractions were digested to peptides, separated on a nano-HPLC system and analysed by MS/MS mass spectrometry, as shown in Figure 1D. The mass shift (28 m/z) of the modified R3 residue in the b-series of fragment ions identified a dimethylated arginine at position 3. Multiple-reaction monitoring (MRM) mass spectrometry from cell lysates approved the occurrence of arginine dimethylation on R3 of endogenous LAP\(^*/\text{C/EBP}\beta\) in cells, as shown in Supplementary Figure 2. As the N-terminally LAP\(^*\)-specific peptide has a clearly defined modular function in chromatin remodelling and gene regulation, we focused on the molecular biology and functions of C/EBP\(\beta\) R3 dimethylation;

Figure 1 C/EBP\(\beta\) is post-translationally methylated on arginine residues. (A) Scheme of C/EBP\(\beta\) isoforms that arise by alternative translation initiation from in-frame start codons termed as LAP\(^*/\text{C/EBP}\beta\)1, LAP\(^*/\text{C/EBP}\beta\)2 and LIP/C/EBP\(\beta\)3. (B) LAP\(^*/\text{C/EBP}\beta\)1 is precipitated by the ASYM24 anti-Rme2a antibody. His-tagged C/EBP\(\beta\) was purified under denaturing conditions by affinity chromatography on a nickel chelating resin. Blot shows purified fractions of C/EBP\(\beta\)1-transfected fibroblasts were treated with adenosine dialdehyde (AdOx) for 12 h (as indicated) and immunoprecipitated with the ASYM24 antibody. Precipitated proteins were analysed by immunoblotting using an ASYM24 anti-Rme2a antibody in the absence of AdOx. (C) Purification scheme of C/EBP\(\beta\) Briefly, His-tagged C/EBP\(\beta\) was purified under denaturing conditions by affinity chromatography on a nickel chelating resin. Blot shows purified fractions of C/EBP\(\beta\)1. (D) Tandem mass spectrum of an R3me2 and oxidized N-terminal C/EBP\(\beta\) peptide. b- and y-series of the LysC-generated peptides are indicated. Mass shifts indicate a 28 Da modification, corresponding to dimethylation at position R3. Underneath: alignment of conserved region 1 (CR1) of LAP\(^*/\text{C/EBP}\beta\)1 from various species shows conserved R3 (magenta box) and start sites (arrows) of the two long isoforms.

Future studies will address the functional implications of this post-translational modification.
however, several other Rme/Rme2 residues were discovered and will be described elsewhere (data not shown). Taken together, the data showed that cellular C/EBPβ is modified by dimethylation of the conserved R3 (Figure 1D, lower part).

Proteomic screening reveals C/EBPβ R3 methylation-sensitive protein interactions

Protein interactions with non-modified and R3 asymmetrically dimethylated peptides (aa 1–41) were analysed by proteomic screening of a UNIPEX human cDNA expression library that covers approximately one third of the human proteome. Bacterially expressed His-tagged recombinant proteins, immobilized in duplicates on PVDF membrane were screened in replicate libraries with C-terminally biotinylated peptides that encompassed C/EBPβ CR1–2 with R3 either in its unmethylated or asymmetrically dimethylated form (R3me2a), followed by detection with streptavidine coupled to horseradish peroxidase, as shown in Figure 2A. Numerous interactions detected with methylated and unmethylated peptides fall into three distinct categories: protein interactions with no preference for either peptide, interactions that favoured R3 methylation, and interactions that favoured unmodified R3 (data not shown). Thus, the methylation status of C/EBPβ R3 directs the interaction between CR1 and other proteins.

Of particular interest was the interaction with two core components of the SWI/SNF chromatin-remodelling complex, Brg1 (Figure 2A) and BAF47/Ini1 (data not shown). Both recombinant Brg1 and BAF47/Ini1 proteins interacted preferentially with non-modified peptide and weakly with the methylated peptide. Differential interaction between the SWI/SNF complex and unmethylated versus dimethylated peptide was confirmed in pull-down assays with cell lysates, as shown by retention of SWI/SNF subunits hBrm, BAF155, and BAF47/Ini1 (Figure 2B). In addition, Mediator components MED23/Sur2 and MED26 (not contained in the proteomic library), earlier shown to interact with CR1, likewise bound to unmodified LAP*/C/EBPβ1 N-terminal peptide (Figure 2B, bottom). These results are in accordance with the earlier genetic and biochemical results (Kowenz-Leutz and Leutz, 1999; Mo et al, 2004) and we conclude that methylation of R3 affects the interaction with SWI/SNF and Mediator complexes.

The effect of R3 modifications on protein complex binding was examined by substituting R3 with alanine (R3A) or leucine (R3L) to eliminate the methylation target or to mimic increased hydrophobicity after methylation, respectively. Immunoprecipitation and GST-pull-down assays with LAP*/C/EBPβ1 WT, R3A, and R3L mutants and SWI/SNF or Mediator components (Supplementary Figure 3A and B) showed preferential interaction of the R3A LAP*/C/EBPβ1 mutant with hBrm and with the Mediator component MED23, whereas R3L displayed decreased interactions with both complexes. Thus, the R3A and the R3L C/EBPβ1 mutant proteins reflect the binding specificity of the screening peptides and may serve as suitable tools for functional investigations.

Alterations in R3 specify gene regulation by LAP*/C/EBPβ1

The interaction between CR1 and SWI/SNF is critical for the activation of a subset of C/EBPβ target genes, whereas other C/EBPβ target genes remain unaffected by CR1 (Kowenz-Leutz and Leutz, 1999). Therefore, the effects of R3 mutations were examined on the CR1-dependent endogenous myeloid target gene mim-1 and the CR1-independent goose-type lysosome gene #325. As shown in Figure 3, the R3A LAP*/C/EBPβ1 mutant strongly activated endogenous mim-1 expression in comparison with WT LAP*/C/EBPβ1 that can be methylated at R3. In contrast, the R3L mutant was barely active. Importantly, WT and both mutants activated the #325 gene to similar extents. Thus, the gene regulatory activity of R3A and R3L mutants reflected CR1-specific co-factor interactions and CR1 R3-specific functions on chromatin-embedded target gene regulation.

PRMT4/CARM1 binds to and methylates C/EBPβ

Lymphoid and myeloid cells that express C/EBPβ also highly express the protein arginine methyltransferases PRMT3, PRMT4/CARM1 (both: asymmetric arginine methylation),...
and PRMT5 (symmetric arginine methylation) (BioGPS). We, therefore, examined whether R3 of the LAP\(^*\)/C/EBP\(\beta\) isoform represents a target for one or more of these PRMTs. PRMT3, 4, and 5 were transiently expressed in HEK-293 cells, immunoadfinity purified from cell lysates, and incubated in the presence of S-adenosyl-L-[methyl-\(^3\)H]methionine with peptides P1, P2, P3, and P4, tiling the TAD of the rat C/EBP\(\beta\) N-terminus (Figure 4A; P1: aa 1–21; P2: aa 22–56; P3: aa 50–82; P4: aa 81–113). As shown in Figure 4B, radioactivity was specifically incorporated with PRMT4/CARM1 in P1, representing CR1 of LAP\(^*\)/C/EBP\(\beta\). Label incorporation was approximately 20-fold higher in P1 than in P2, P3, or P4 that also contained arginine and/or lysine residues. All three PRMTs were equally expressed (Supplementary Figure 4A) and functionally active, as determined by incorporation of \(^3\)H-methyl into recombinant GST-Histone H3 and H4 N-termini, but not into the GST moiety (Supplementary Figure 4B). These data show that PRMT4/CARM1 specifically methylates the CR1 N-terminus of LAP\(^*\)/C/EBP\(\beta\).

Methylation assays with the entire GST-C/EBP\(\beta\) TAD (CR1–4; aa 1–113) showed that the C/EBP\(\beta\)-WT, but not the R3A mutant TAD, was methylated by PRMT4/CARM1 (Figure 4C). The PRMT4/CARM1 enzyme activity and the C/EBP\(\beta\) GST-protein expression were approved by auto-methylation and protein staining, respectively (Supplementary Figure 4C). In summary, these data confirm R3 of C/EBP\(\beta\) as a PRMT4/CARM1 methylation target.

Immunoprecipitation of C/EBP\(\beta\) from K562 cells revealed that endogenous PRMT4/CARM1 and C/EBP\(\beta\) interacted in myeloid cells (Figure 5A). Co-immunoprecipitations of WT PRMT4/CARM1 or a catalytically inactive PRMT4/CARM1 mutant (amino-acids 189–191 VLD to AAA, dubbed PRMT4/CARM1\(^{\text{mut}}\)) (Chen et al, 1999) with LAP\(^*\)/C/EBP\(\beta\) showed that interaction did not depend on the catalytic activity of the methyltransferase (Supplementary Figure 5A). Deletion mapping of C/EBP\(\beta\) revealed PRMT4/CARM1 association with the C/EBP\(\beta\) TAD (Supplementary Figure 5B) and GST-pull downs showed that PRMT4/CARM1 interacts preferentially with C/EBP\(\beta\) TAD CR3 and CR4 (Supplementary Figure 5C).

Examination of the functional consequences of PRMT4/CARM1 interaction with C/EBP\(\beta\) showed that the catalytically defective PRMT4/CARM1\(^{\text{mut}}\) enhanced activation of the myeloid LAP\(^*\)/C/EBP\(\beta\) target gene mim-1, whereas WT PRMT4/CARM1 decreased activation of mim-1 (Figure 5B). Expression of the CR1-independent #325 myeloid gene remained indifferent to WT PRMT4/CARM1 or its mutant, excluding a general repressive effect of PRMT4/CARM1 on the transcriptional machinery or on C/EBP\(\beta\) target genes. Importantly, co-expression of WT PRMT4/CARM1 did not suppress mim-1 activation by the non-methylatable C/EBP\(\beta\) R3A mutant (Figure 5B, right), providing further evidence that methylation of R3 specifically interferes with the activation of LAP\(^*\)/C/EBP\(\beta\) CR1-dependent target genes. The results suggest that the methylation status of the R3 side chain has a decisive function in the regulation of SWI/SNF complex-dependent target genes.

C/EBP\(\beta\) phosphorylation abrogates PRMT4/CARM1 interaction

C/EBP\(\beta\) is a repressed transcription factor that can be activated by EGF receptor tyrosine kinase and ras signalling through the MAP kinase Erk1/2 pathway (Nakajima et al, 1993; Kowenz-Leutz et al, 1994; Fan et al, 2009). Phosphorylation of the conserved MAP kinase site in the
The diagram in Figure 5 illustrates the physical and functional interaction between C/EBPβ and PRMT4/CARM1 in eukaryotic cells. (A) K562 cell lysates were incubated with antibodies (IgG, negative control) or anti-C/EBPβ and antigen–antibody complexes were immunoprecipitated. Proteins were analysed by immunoblotting with anti-PRMT4/CARM1 or anti-C/EBPβ as indicated. IB: immunoblot. (B) LAP*/C/EBPβ WT or R3A mutants, as indicated, were transfected in QT6 fibroblasts together with WT or PRMT4/CARM1mut constructs. RNA blots were subjected to serial hybridization to the GAPDH gene probes.

Central RD of C/EBPβ is accompanied by a conformational change in C/EBPβ that alters interactions with protein complexes (Kowenz-Leutz et al., 1994; Williams et al., 1995; Mo et al., 2004). We, therefore, examined whether ras signalling also alters interaction between PRMT4/CARM1 and C/EBPβ. As shown in Figure 6A, co-expression of activated rasV12 with C/EBPβ increased phosphorylation at the MAPkinase site. Concomitantly, binding of PRMT4/CARM1 to C/EBPβ was diminished. Immunoprecipitation of endogenous C/EBPβ from K562 cells approved that PRMT4/CARM1 interacts with C/EBPβ in untreated, but not in phorbol ester (phorbol-12-myristate-13-acetate, PMA)-treated (Figure 6B). MRM of endogenous C/EBPβ from K562 cells showed inverse correlation between phosphorylation at the MAPK-site and dimethylation of R3 of LAP*/C/EBPβ and disappearance of R3 methylation after PMA treatment (Figure 6C). Taken together, these results suggested that phosphorylation of the C/EBPβ RD abolishes the interaction between the TAD of C/EBPβ and PRMT4/CARM1 and as a consequence leads to abrogation of methylation of R3 LAP*/C/EBPβ.

**Alteration of the LAP*/C/EBPβ R3 residue affects myeloid and adipogenic differentiation**

C/EBPβ associates with the human neutrophil elastase gene (hELA2) and activates hELA2 expression in an MAPKine signal-dependent manner (Nuchprayoon et al., 1997; Lausen et al., 2006; Pless et al., 2008). Myeloid gene activation in heterologous cells and reprogramming by C/EBPβ are well established (Laiosa et al., 2006; Zahnow, 2009). Therefore, we examined whether the myeloid hELA2 gene can be activated by LAP*/C/EBPβ1 in fibroblasts. Expression of hELA2 in NIH 3T3 fibroblasts was strongly enhanced after PMA treatment with LAP*/C/EBPβ1, but not with LAP*/C/EBPβ2 or LIP/C/EBPβ3. The LAP*/C/EBPβ1 R3A mutant was significantly more active than WT LAP*/C/EBPβ1, yet barely induced by PMA (Figure 7A, expression controls in 7B). These data are consistent with the notion that R3 in its unmethylated and methylated form is involved in activation and repression processes and that replacement of R3 (R3A) partially compromises both functions. The LAP*/C/EBPβ1 R3L mutant did not display significant activity with or without PMA, consistent with the notion that replacement of R3 with leucine stabilizes the inhibitory state of LAP*/C/EBPβ1. These data show that CR1 of LAP*/C/EBPβ1 has an important function in the activation of the endogenous hELA2 gene in fibroblasts.

Endogenous C/EBPβ in human U937 myeloid cells binds to a 117 bp fragment upstream of the hELA2 TATA-box (Nuchprayoon et al., 1997; Lausen et al., 2006) and PMA-induced phosphorylation of the C/EBPβ MAPKine site upregulates hELA2 expression (Pless et al., 2008). As shown in Figure 7C, both C/EBPβ and PRMT4/CARM1 were found associated with the hELA2 promoter in U937 cells. Reciprocal re-immunoprecipitation of either C/EBPβ or PRMT4/CARM1 confirmed simultaneous occupancy of the hELA2 promoter (Supplementary Figure 6). PMA treatment abrogated association of PRMT4/CARM1 with the hELA2 promoter, whereas C/EBPβ occupancy persisted (Figure 7C). In addition, the Mediator complex component MED23/Sur2 was associated with the hELA2 promoter before PMA stimulation and increased after PMA treatment. In contrast, CDK5, a component of attenuated Mediator, was five-fold decreased after PMA treatment, probably reflecting the earlier described exchange between Mediator complex modules (Mo et al., 2004). Taken together, these data support the notion that C/EBPβ phosphorylation is accompanied by dissociation from PRMT4/CARM1 and loss of the Mediator CDK8 module during hELA2 gene activation.

C/EBPβ also regulates adipogenesis and controls early activation of a cascade of transcription factors that finally orchestrate adipogenic differentiation (Cao et al., 1991; Wu et al., 1995; Yeh et al., 1995; Mandrup and Lane, 1997; Birsoy et al., 2007). Earlier, it was reported that EGF receptor tyrosine kinase signalling inhibits PRMT4/CARM1 methyltransferase activity and subsequent histone H3 methylation (Higashimoto et al., 2007). We, therefore, examined whether activated C/EBPβ, as represented by the LAP*/C/EBPβ phospho-mimetic MAPKine site mutant T220D, interacts with PRMT4/CARM1. As shown in Figure 6D, interaction between PRMT4/CARM1 and C/EBPβ T220D was strongly diminished, as compared with WT C/EBPβ. In accordance, the C/EBPβ T220D mutant activated adipogenic target gene expression and fat cell differentiation, as compared with WT, whereas the C/EBPβ T220A displayed strongly diminished activity (Supplementary Figure 7). Taken together, these results suggested that phosphorylation of the C/EBPβ RD abolishes the interaction between the TAD of C/EBPβ and PRMT4/CARM1 and as a consequence leads to abrogation of methylation of R3 LAP*/C/EBPβ1.
et al., 2008). Recruitment of the SWI/SNF complex is an essential prerequisite for induced onset of the adipogenic program (Pedersen et al., 2001; Caramel et al., 2008). We, therefore, asked whether stable expression of WT and mutant LAP*/C/EBPβ1 isoforms in NIH 3T3 L1 cells alters adipogenesis. As shown in Figure 7E (expression controls Figure 7F), fat cell differentiation was strongly enhanced by the LAP*/C/EBPβ1 R3A mutant, as indicated by upregulation of the adipogenic genes PPARγ, aP2, and adipsin (Figure 7D), in the absence of the usually required hormone cocktail. In contrast, expression of the LAP/C/EBPβ2 isoform or the LAP*/C/EBPβ1 R3L mutant in NIH 3T3 L1 cells did not induce spontaneous differentiation. These results suggest that enhanced SWI/SNF recruitment by unmethylated R3 in LAP*/C/EBPβ1 CR1 predisposes mesenchymal precursor cells to adipogenic differentiation.

Discussion

The transcription factor C/EBPβ functions at the interface of cell metabolism, stress, proliferation, and differentiation. Data presented here show that ras/MAPkinase signalling is coupled to R3 methylation and that the R3 methylation state specifies interactions between C/EBPβ, SWI/SNF and Mediator. Our data show how extracellular signals initially converted to kinase signalling connect to arginine methylation of a transcription factor that in turn determines interaction with the epigenetic and gene regulatory machinery in the nucleus to regulate gene transcription and differentiation. The data are summarized in the conceptual model shown in Figure 8.

A distinctive feature of the intronless single C/EBPβ transcript is that alternative translation initiation at consecutive in-frame start sites may generate three protein isoforms with different N-termini that display activator and repressor functions (Descombes and Schibler, 1991; Calkhoven et al., 2000). The CR1 comprises 21–23 amino acids in different species and is contained in the largest LAP*/C/EBPβ1 isoform. LAP*/C/EBPβ1 regulates a subset of C/EBPβ target genes that are involved in mammary epithelial integrity, proliferation and cell differentiation, innate immunity, and potentially also fat metabolism (Eaton et al., 2001; Bundy and Sealy, 2008).
2003; Uematsu et al., 2007). Our data show that the conserved arginine at position 3 (R3) can be specifically methylated by PRMT4/CARM1 and that the methylation status of R3 determines the interaction between CR1 and other proteins. Proteins that interact with the unmethylated CR1 include components of the SWI/SNF complex and Mediator complex. Both multi-subunit protein complexes had been described earlier to mediate important functions of LAP*/C/EBP expression and gene regulation.

Mass spectrometry combined with mutational analysis, biochemical, molecular genetic, and cell biological data establishes that PRMT4/CARM1 interacts with the TAD of C/EBPβ and dimethylates CR1 specifically at the evolutionary conserved R3 residue. Methylation of R3 impairs the interaction with SWI/SNF and Mediator complexes and, therefore, attenuates activation of myeloid and adipogenic genes. It has recently been shown that adipogenic induction requires activation of C/EBPβ expression and that the LAP* isof orm was able to bypass an early regulatory network of proadipogenic transcription factors (Birsoy et al., 2008). At the same time, genes that are not affected by the presence of CR1 are exempt from R3 regulation. This interpretation is in agreement with a modular and appendicular function of N-terminal CR1 (Pedersen et al., 2001) and is supported by a recent knock in study that shows that LAP*/C/EBPβ may largely replace LAP/C/EBPβ2 (Uematsu et al., 2007).

Of particular attention is the finding of a functional crosstalk between ras/MAPkinase signalling, arginine methylation of C/EBPβ and recruitment of an epigenetic complex. Our data show that phosphorylation of a distal site and methylation of the N-terminal R3 has opposing functions on C/EBPβ-mediated gene activation. The interaction between PRMT4/CARM1 and C/EBPβ and their co-association on the hELA2 promoter is abolished when LAP*/C/EBPβ1 is phosphorylated. Concomitantly, the silent myeloid hELA2 C/EBPβ target gene was activated in fibroblasts, reflecting epigenetic activation of myeloid gene expression by LAP*/C/EBPβ1. The R3A mutation in C/EBPβ that removes the methylation target displayed enhanced co-factor interactions, myeloid gene activation, induction of adipogenesis, and, importantly, resists the repressive effect of PRMT4/CARM1. We conclude that in the absence of signals, association of PRMT4/CARM1 specifically restrains LAP*/C/EBPβ1 functions by R3 methylation, without repressing the overall C/EBPβ activity.

Earlier, we had shown that C/EBPβ is also methylated on a highly conserved lysine residue by G9a and that methylation at K39 compromises its transactivation (Pless et al., 2008). Here, we show that MAPkinase-phosphorylated C/EBPβ is undermethylated. Accordingly, we data support the idea of a negative crosstalk between C/EBPβ phosphorylation and methylation and raise the possibility that in the absence of an activating signal, C/EBPβ becomes methylated and remains transcriptional inactive or displays repressor functions, whereas MAPkinase signalling transiently abrogates C/EBPβ methylation and permits co-activator recruitment and gene activating functions.

Phosphorylation of the RD following receptor tyrosine kinase-ras/MAPK signalling has been shown to activate LAP*/C/EBPβ1 by inducing a structural change that affects interaction and composition of associated Mediator complex (Mo et al., 2004). Data presented here suggest that phosphorylation of the C/EBPβ RD also curtails interaction with PRMT4/CARM1 that is associated with inactive C/EBPβ.
this context, it is interesting to note that in studies of promoter-specific PRMT4/CARM1 functions, enhanced expression of the C/EBPβ target genes Il6 and COX2 (Akira et al., 1990; Poli, 1998; Gorgoni et al., 2001) was observed in CARM1-deficient cells (Covic et al., 2005), supporting an inhibitory function of CARM1 for distinct C/EBPβ functions.

PRMT4/CARM1 is primarily considered in gene transactivation; however, a repressive function on gene transcription has also been described for the histone acetyltransferase CREB-binding protein that when methylated at the R17 domain failed to interact with the transcription factor CREB. In addition, methylation of the steroid receptor co-activator 3 complex leads to co-activator disassembly and decreased steroid receptor-mediated transcription (Xu et al., 2001; Feng et al., 2006). Co-activator functions of PRMT4/CARM1 were described for nuclear hormone receptor-mediated target gene activation, including PPARγ-mediated adipogenesis, which is reduced by 40% in adipose tissue in PRMT4/CARM1 KOs (Yadav et al., 2008). It, therefore, seems that the activity of PRMT4/CARM1 is dispensable for the early activation of adipogenic co-factors (supported by data shown in Supplementary Figure 8), whereas PRMT4/CARM1 is required as a co-activator for PPARγ functions in terminal differentiation. In support of this notion is the observation that MAPkinase signalling and IBMX (enhances protein differentiation. In support of this notion is the observation that the activity of PRMT4/CARM1 is dispensable for the

Materials and methods

Peptides

C/EBPβ peptides covering the TAD were synthesized and are derived from the mouse sequence (Swiss-Prot P28033)—peptide 1: aa 1–21; peptide 2: aa 22–55; peptide 3: aa 49–81; peptide 4: aa 80–113. For protein macroarray screening and peptide pull-down analysis, two peptides were synthesized and HPLC purified covering aa 1–41 in a non-methylated and R3 asymmetrically dimethylated form (PSL).

Protein macroarray screening (UNIPEX)

Two identical UNIPEX protein macroarrays (containing approximately 20,000 sequenced, annotated and verified cDNA clones; Imagenes, Berlin) were overlayed with non-modified and R3 asymmetrically dimethylated N-terminal C/EBPβ peptides covering aa 1–41. In brief, protein macroarrays were rinsed in water, washed with TBS-T-T (500 mM NaCl, 20 mM Tris–HCl (pH 7.5), 0.5% Tween 20, 0.5% Triton X-100) and excess bacterial colony material was scraped off the macroarray. Subsequently, membranes were blocked with 3% skim milk in TBS-T (500 mM NaCl, 20 mM Tris–HCl (pH 7.5), 0.05% Tween 20) for 2 h. The libraries were incubated with 1 μM for both, unmodified and methylated peptides, in blocking solution over night. Interaction partners were detected by using streptavidin-HEP and ECL. Positive scoring clones were localized, according to the manufacturer’s instructions.

Peptide pull down

Streptavidin dynabeads were saturated with non-modified or R3 asymmetrically dimethylated N-terminal C/EBPβ-peptides for 30 min in PBS (pH 7.4). Excess peptides were washed off with wash buffer (PBS (pH 7.4), 0.01% Tween, 0.1% BSA). Beads were then incubated with cell extracts prepared from Raji or HEK-293 cells for 1 h (lysis buffer: 20 mM Heps (pH 7.6), 350 mM NaCl, 30 mM MgCl2, 1 mM EDTA (pH 8), 0.1 mM EGTA (pH 8), 20% glycerol, 0.5% NP40). After washing, bound proteins were eluted with SDS–PAGE loading buffer. Western blots were incubated with appropriate antibodies and revealed by ECL.

In vitro methylation

PCDNA3.1-HA PRMT3, 4, or 5 were transfected into HEK-293 cells; 48 h post transfection, cells were lysed (50 mM Tris pH 8, 150 mM
NaCl, 0.1% NP40, 1 mM EDTA, 5 mM MgCl2 plus protease inhibitors) and immunoprecipitations were performed from lysates with anti-HA for 3 h. Immunocomplexes were coupled to protein G-coated Dynabeads (Invitrogen) for 1 h, washed with lysis buffer and used directly in in vitro methylation studies. Reactions were carried out in PBS, supplemented with 1 μl of S-adenosyl-L-([3H]-methyl)-H)methionine as methyl donor and incubated with either 1 μM of corresponding peptides or 1–2 μg of C/EBPβ (GST-fusion proteins for 3 h at 30°C. Three parts of the reaction was submitted to SDS-PAGE, methylated proteins were detected by fluorography, and one part of the reaction was spotted on phosphocellulose membrane to determine ([3H]methyl incorporation by scintillation counting.

Mass spectrometry
pcDNA3-NFM-8xHis constructs were transiently transfected, expressed in QT6 quail fibroblasts, and extracted under denaturing conditions in lysis buffer (100 mM NaH2PO4 (pH 8.0); 10 mM Tris (pH 8.0); 1 mM Pefablock, and protease inhibitor cocktail (Roche)). Fragment ion spectra were obtained by fragmenting an arginine (pH 8.0), 1 mM Pefablock, and protease inhibitor cocktail (Roche)). Affinity purification was carried out on an AKTA purifier (GE Healthcare) equipped with a HisTrap HP Nickel column (GE Healthcare). Elution was performed with an Imidazole gradient (20–250 mM). For the identification of arginine methylation on C/EBPβ, peptides were generated using Lysyl-endopeptidase (LysC) as described (de Godoy et al., 2008). Peptides were separated by reverse phase chromatography on a nano-Acquity UPLC (Waters) and analysed by electrospray ionization on a Q-TOF premier mass spectrometer (Waters). Peptide spectra were assigned using the MASCOT software (Matrix Science) and refined by hand interpretation. For quantification of endogenous C/EBPβ R3 modification by MRM of untreated or PMA-treated K562 or U937, cell lysates were prepared in lysis buffer (50 mM Tris (pH 8), 150 mM NaCl, 0.5% NP40, supplemented with protease inhibitor cocktail (Roche Applied Biosciences)). For IP, lysates were diluted with Buffer B (20 mM Heps (pH 7.9), 30 mM MgCl2, 1 mM EDTA pH 8, 1 mM EGTA pH 8, 20% glycerol, 0.2% NP40). Immunoprecipitations of CARM1 and hBmR were performed in lysis buffer 50 mM Tris (pH 8), 150 mM NaCl, 0.5% NP40, 1 mM EDTA (pH 8) supplemented with protease inhibitor cocktail. Samples were immunoprecipitated with appropriate antibodies for 2–3 h at 4°C as indicated and immunoprecipitates were collected on protein A- or G-Sepharose beads. Immunoprecipitations of C/EBPβ constructs and PRMT4/CARM1 were performed after transient transfection of QT6 fibroblasts, HEK-293 cells, or K562 cells for endogenous protein interaction studies. Briefly, cells were lysed in buffer (50 mM Tris (pH 8), 150 mM NaCl, 0.5% NP40, 1 mM EDTA, and protease inhibitors). Samples were immunoprecipitated with appropriate antibodies for 2 h at 4°C as indicated and immunoprecipitates were collected on protein A- or G-Sepharose beads. Antibodies: anti-C/EBPβ (Leitz lab), anti-C/EBPβ (Santa Cruz; C-19, H-7), anti-phospho-Thr235 C/EBPβ (Cell Signaling Technology; #3084), anti-FLAG (Sigma), anti-HA.11 (Covance), anti-ASYM24 (Upstate; #125), anti-PRMT4/CARM1 (Cell Signaling Technology; #4438), anti-Bmi (Bioul; A301-014A), anti-BAF155 (Bioul; A301-019A), anti-BAF47/In1 (Sigma; H9912), anti-MED23 (Santa Cruz; SC-12454), anti-MED26 (Santa Cruz; SC-9425), and anti-CDK8 (Santa Cruz; SC-1521). Immunoblots were visualized by ECL (GE Healthcare).

Chromatin immunoprecipitation
Chromatin immunoprecipitations with subsequent quantitative PCR of the hELA2 gene promoter and IgG control were performed as described (Pless et al., 2008). Re-ChIP analysis was performed using the ‘Re-ChIP-IT Magnetic Chromatin Re-immunoprecipitation Kit’ (Active Motif, #53016) according to the manufacturer’s protocol.

Adipogenesis and RT–PCR analysis
Vector construct, LAP/C/EBPβ1, LAP/C/EBPβ2, LAP/C/EBPβ3, or LAP/C/EBPβ R3L were transfected into MetaIeucete according to the manufacturer’s protocol (Invitrogen) in NIH 3T3 L1 fibroblasts and selected by 1.5 μg/ml puromycin. Stable transfectants were seeded in triplicates on tissue culture dishes and grown to confluence. Ten days past confluence, total RNA was
isolated from one dish with High Pure RNA isolation Kit (Roche) and cDNA was prepared using SuperScript cDNA Kit (Invitrogen). Real-time PCR was performed using SYBR Green (Invitrogen) and a Light Cycler (Roche) according to the manufacturer’s instructions. Δp2 forward primer: CAAAAGTGTTGATCCCTTGGT; reverse primer: GATGCTCCCTGTTGAGCC; Δp2 forward primer: GATGCTCCCTCTGTTGAG; reverse primer: TACGTTTATCTGGTGTTGT CAT; Adipsin forward primer: CCTATCTTCCTGGCTCATGCC; reverse primer: GATGCTCCCTGTTGAGCC. Alternatively, spontaneous NIH 3T3 L1 adipogenesis was visualized after fixation with 4% paraformaldehyde by oil red O staining. Protein expression of stable transfectants was determined by western blot.

**Supplementary data**

Supplementary data are available at The EMBO Journal Online (http://www.embojournal.org).

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**Conflict of interest**

The authors declare that they have no conflict of interest.

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**Author contributions**

EKL, OP, and AL designed, and EKL and OP performed experiments and analysed data. MR expressed and purified proteins, and CD performed mass spectrometric analysis. EKL, OP, and AL supervised the work.

**Supplementary data**

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